



NORTH-WEST UNIVERSITY
YUNIBESITHI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

The antioxidant properties of 4-quinolones compared to structurally related flavonoids

Jane Greeff

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Supervisor: Prof. S. van Dyk

Co-Supervisor: Prof. S.F. Malan

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ABSTRACT

Oxidative stress is a common occurrence in neurodegenerative disorders such as Alzheimer's and Parkinson's disease and is suggested to take place before the onset of neurodegenerative disease, leading to, or exacerbating deterioration. The oxidative status of the cells is regulated by antioxidant enzymes responsible for neutralising free radicals. With increasing age the enzymes are overwhelmed by the amount of free radicals requiring deactivation, leading to decreased protection by the body's antioxidant systems. The mitochondria also produce more reactive oxygen species at the cost of producing less ATP. These natural by-products of cellular respiration in the mitochondria act to injure the mitochondria themselves and cell structures containing lipids, proteins and DNA and serve to decrease the lifespan of the cell. During oxygen radical damage of the brain, an area containing high concentrations of oxidisable substrate and oxidative catalysts as well as low concentrations of antioxidant enzymes, apoptosis of the brain cells takes place, contributing to irreversible neurodegeneration. This deterioration is only diagnosed when damage to the brain is sufficient to induce disability and it is too late to be restored. The area of interest in this study was therefore to discover compounds useful in decreasing brain damage caused by reactive oxygen species, thereby curbing the progression of neurodegenerative disease and prolonging the lifespan and quality of life of the patient.

Flavonoids are naturally occurring compounds, abundant in plants with established antioxidant activity. Hydrogen donating substituents on the natural flavonoids are responsible for elevated antioxidant activity and it was therefore hypothesised that synthesising structurally similar compounds containing hydrogen donating functional groups might improve the antioxidant activity observed for the flavonoids. As a result, the flavone moiety was selected as the lead compound, substituted with hydroxyl groups on different positions and were compared to the correlating hydroxyl substituted 2-phenylquinolin-4(1*H*)-ones, which were prepared by the Conrad-Limpach method and characterised by NMR, IR and MS techniques.

Biological activity was evaluated using a range of antioxidant assays to evaluate the potential value of the flavones and synthesised 2-phenylquinolin-4(1*H*)-ones. The oxygen radical absorbance capacity (ORAC) assay was performed to establish the ability of the test series to scavenge peroxy radicals leading to lipid peroxidation. The 2-phenylquinolin-4(1*H*)-ones demonstrated moderate activity, with 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**) observed to be the best of the group. 6-Hydroxyflavone (**5**) however, performed the best of the test series. In the ferric reduction/antioxidant power (FRAP) assay the chemical ability to reduce ferric iron was

evaluated in order to assess the theoretical inhibition of the Haber-Weis reaction, leading to reduced hydroxyl radical production. In this case the 2-phenylquinolin-4(1*H*)-ones showed the best activity of the compounds, with the performance of 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) comparable to that of Trolox, followed by 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**). The superoxide anion (NBT) assay demonstrated the ability of the compounds to scavenge superoxide anions, the first oxygen radical produced by the mitochondria, which is responsible for most of further oxygen radical production in the cell. The 2-phenylquinolin-4(1*H*)-ones showed moderate activity, 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**) demonstrating the best activity in the group while 8-hydroxyquinoline (**3**) was the best superoxide anion scavenger. In the lipid peroxidation (TBARS) assay the ability of the compounds to scavenge the hydroxyl radical was assessed, to ascertain the ability to inhibit the initiation of lipid peroxidation. In this assay 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**) performed the best of the 2-phenylquinolin-4(1*H*)-ones, its 1 mM concentration performing better than the 0.01 mM Trolox concentration, while the compound displaying the best hydroxyl radical scavenging activity in the assay was 4-hydroxyquinoline (**2**).

From the above-mentioned evaluations it was possible to establish that 2-phenylquinolin-4(1*H*)-ones acted as chain-breaking antioxidants, with a postulated hydrogen donor mechanism of action. The slightly acidic amine present in the synthesised series of 2-phenylquinolin-4(1*H*)-ones did not however prove advantageous compared to the basic amine of the quinolines, except in the FRAP assay. It was however clear that hydroxyl substitution lead to an increase in antioxidant activity, with the 8- and 6-hydroxyl substitution of the 2-phenylquinolin-4(1*H*)-ones (**10** and **8**) able to enhance antioxidant activity in the FRAP and TBARS assays and the 7-substitution (**9**) in the ORAC and NBT assays. The hydroxyl substituted 2-phenylquinolin-4(1*H*)-ones outperformed the flavones in the FRAP, NBT and TBARS assays., indicating that under certain conditions the hydroxyl substituted synthesised series may inhibit radical mediated damage better than the flavones and thus show promise as possible neuroprotective agents. It however remains to establish the ability of the test compounds to permeate the blood brain barrier, to determine the antioxidant effect that may be obtained in a living brain.

UITTREKSEL

Oksidatiewe stres is 'n algemene verskynsel in neurologiese agteruitgang soos in Alzheimer en Parkinson se siekte, wat waarskynlik plaasvind voor die aanvang van die neurodegeneratiewe siekte en lei tot, of vererger bestaande agteruitgang. Die oksidatiewe status van die selle word gereguleer deur anti-oksidatiewe ensieme, wat verantwoordelik is vir die neutralisering van vry radikale. Met verhoogde ouderdom word die ensieme oorweldig deur die hoeveelheid vry radikale wat gedeaktiveer moet word en dit lei tot verminderde beskerming deur die liggaam se antioksidatiewe stelsels. Die mitochondria produseer ook meer reaktiewe suurstofspesies ten koste van verlaagde ATP-produksie. Hierdie natuurlike byprodukte van selrespirasie in die mitochondria beskadig die mitochondria self, asook die selstrukture wat lipiede, proteïene en DNA bevat, en verkort die lewe van 'n sel. Gedurende suurstof-gemedieerde beskadiging van die brein, 'n area wat hoë konsentrasies oksideerbare substrate en oksidatiewe kataliste bevat, asook lae konsentrasies anti-oksidatiewe ensieme, vind apoptose van die breinselle plaas, wat bydra tot onomkeerbare neurodegenerasie. Hierdie agteruitgang word eers gediagnoseer wanneer die skade aan die brein genoegsaam is om gebreke te veroorsaak en dit reeds te laat is om die skade om te keer. Die area van belangstelling in hierdie studie was daarom om verbindings te vind wat nuttig is in die vermindering van neuronale degenerasie veroorsaak deur reaktiewe suurstofspesies en daardeur die verloop van die neurodegeneratiewe siekte in te perk en die lewensduur en lewenskwaliteit van die pasiënt te verbeter.

Die flavonoïede is verbindings van natuurlike oorsprong, wat algemeen in plante voorkom en bewese anti-oksidatiewe werking het. Protonskenkende substituentte op die natuurlike verbindings is verantwoordelik vir verhoogde anti-oksidatiewe werking. Op grond hiervan is gepostuleer dat die sintese van struktureel ooreenstemmende verbindings wat protonskenkende funksionele groepe bevat, moontlik die anti-oksidant aktiwiteit van die flavonoïede kon verbeter. Die flavoonstruktuur is daarom gekies as die leidraadverbinding en met hidroksielgroepe in verskillende posisies gesubstitueer om met die ooreenstemmende hidroksielgesubstitueerde 2-fenielkinolien-4(1*H*)-one te vergelyk, wat deur die Conrad-Limpach metode berei en deur KMR, IR en MS gekarakteriseer is.

Die biologiese aktiwiteit is geëvalueer deur die flavone en 2-fenielkinolien-4(1*H*)-one aan 'n reeks anti-oksidanttoetse te onderwerp. Die suurstofradikaal-absorbansiekapasiteit (ORAC) toets is gedoen om vas te stel wat die vermoë van die toetsverbinding is om peroksielradikale op te ruim wat lei tot lipiedperoksidasie. Die 2-fenielkinolien-4(1*H*)-one het matige aktiwiteit getoon, met 7-hidroksie-2-fenielkinolien-4(1*H*)-oon (**9**) die beste van die groep en 6-hidroksieflavoon (**5**) die beste van die toetsreeks. In die ferri-ioonreduksie/anti-

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Vanuit die bogenoemde resultate was dit moontlik om vas te stel dat die 2-fenielkinolien-4(1*H*)-one as kettingbrekende anti-oksidente optree, met 'n gepostuleerde protonskenkende werkingsmeganisme. Die effense suur amien teenwoordig in die 2-fenielkinolien-4(1*H*)-one het egter nie gunstige aktiwiteit getoon relatief tot die basiese amien van die kinoliene nie, behalwe in die FRAP toets. Dit is egter duidelik dat hidroksielsubstitusie tot 'n toename in anti-oksidentaktiwiteit gelei het, met die 8- en 6-hidroksielsubstitusie van 2-fenielkinolien-4(1*H*)-one (**10** and **8**) wat die anti-oksidentaktiwiteit in die FRAP en TBARS toetse verhoog het terwyl die 7-substitusie (**9**) voordelig was in die ORAC en NBT toetse. Die hidroksielgesubstitueerde 2-fenielkinolien-4(1*H*)-one het beter aktiwiteit getoon in die FRAP, NBT en TBARS toetse, wat 'n aanduiding was dat die hidroksielgesubstitueerde reeks onder sekere toestande, radikaalgemedieerde skade beter kan inhibeer as die flavone en dus potensiaal toon as neurobeskermende geneesmiddels. Dit is egter nodig om die bloed-breinskansdeurlaatbaarheid van die toetsverbindings te evalueer om die anti-oksidenteffek vas te stel wat verkry sal word in 'n lewende brein.

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LIST OF ABBREVIATIONS

$^{\circ}\text{C}$	Degrees Celsius
$\cdot\text{L}$	Carbon-based Radical
$\cdot\text{OH}$	Hydroxyl Radical
$\cdot\text{OL}$	Lipid Alkoxy Radical
$^{13}\text{C NMR}$	Carbon-thirteen Nuclear Magnetic Resonance Spectroscopy
$^1\text{H NMR}$	Proton/Hydrogen-one Nuclear Magnetic Resonance Spectroscopy
A^{\cdot}	Stable Antioxidant Radical
AAPH	2,2'-Azobis(2-amidinopropane)-dihydrochloride
ACE	Angiotensin Converting Enzyme
A-H	Antioxidant
AIDS	Acquired Immune Deficiency Syndrome
APCI-MS	Atmospheric Pressure Chemical Ionisation Mass Spectrometry
ArO^{\cdot}	Oxidised Antioxidant / Stable Phenoxide Radical
ArOH	Radical Acceptor / Phenolic Antioxidant
$\text{ArOH}^{+\cdot}$	Antioxidant / Phenol Cation Intermediate
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BHT	2,6-Di-tert-butyl-4-methylphenol
BSA	Bovine Serum Albumin
C	Carbon atom
Ca^{2+}	Calcium

CAT	Catalase
COMT	Catechol-O-Methyl Transferase
COOH	Carboxylic acid
CoQ	Coenzyme Q
Cu,Zn-SOD	Copper, Zinc-Superoxide Dismutase
DCM	Dichloromethane
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DPE	Diphenyl Ether
DQF-COSY NMR	Double Quantum Filtered Correlated Spectroscopy
EI-MS	Electron Impact Mass Spectrometry
Eq	Equation
EtOH	Ethanol
f_0	Initial Fluorescence
Fe	Iron atom
Fe²⁺	Ferrous Ion
Fe²⁺-TPTZ	Ferrous-Tripyridyltriazine Complex
Fe³⁺	Ferric Ion
Fe³⁺-TPTZ	Ferric-Tripyridyltriazine Complex
FeCl₃	Iron(III)chloride
FeCl₃.6H₂O	Iron(III)chloride Hexahydrate
Fig	Figure

FL	Fluorescein
FRAP	Ferric Reducing/Antioxidant Power
g	Gravity force
GAA	Glacial Acetic Acid
GABA	Gamma-amino-butyric acid
GP	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H⁺	Proton/Hydrogen atom
H₂O	Water molecule
H₂O₂	Hydrogen Peroxide
HCl	Hydrochloric Acid
HHE	4-Hydroxy-2-hexenal
HREI-MS	High Resolution Mass Spectrometry
HSQC NMR	Heteronuclear Single Quantum Correlation Nuclear Magnetic Resonance Spectroscopy
<i>In vitro</i>	Biological method performed outside a living organism
<i>In vivo</i>	Biological method performed inside a living organism
IR	Infrared Spectroscopy
K₂HPO₄	Dipotassium Hydrogen Phosphate
KCl	Potassium Chloride

KCN	Potassium Cyanide
KH₂PO₄	Potassium Dihydrogen Orthophosphate
L	Litre
L·	Carbon-based Radical
LH	Fatty Acyl Chain
LOO·	Hydroperoxyl Radical / Lipid Peroxyl Radical
LOOH	Lipid Hydroperoxide
MAO	Monoamine oxidase
MAO-B	Monoamine oxidase-B
MDA	Malondialdehyde
MeOH	Methanol
mg	Milligram
mM	Millimolar
mmHg	Millimetre Mercury
Mn-SOD	Manganese Superoxide Dismutase
MPDP⁺	2,3-Dihydropyridinium Intermediate
MPP⁺	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Mass spectrometry
N	Nitrogen atom
Na₂HPO₄	Di-sodium hydrogen orthophosphate anhydrous
NaAc.3H₂O	Sodium Acetate Trihydrate

NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBD	Nitro-blue Diformazan
NBT	Nitro-blue Tetrazolium
NH	Amine group
nm	Nanometres
NMDA	N-methyl-D-aspartate
nmol/L	Nanomoles per Litre
NMR	Nuclear Magnetic Resonance Spectrometry
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
O	Oxygen atom
O₂	Molecular Oxygen
O₂⁻	Superoxide Anion
OH	Hydroxyl group
OH⁻	Hydroxyl anion
OH·	Hydroxyl radical
ONOO⁻	Peroxynitrite
PBS	Phosphate Buffer Solution
ppm	Parts per Million
PQS	<i>Pseudomonas</i> Quinolone Signal
p-TosOH	<i>para</i> -Toluene sulfonic acid

P-value	Indicator of Significant Difference
R.S.D.	Relative Standard Deviation
RFU	Relative Fluorescence Units
RNS	Reactive Nitrogen Species
ROO⁻	Fe(TPTZ) ₂ (II) / Peroxyl Anion
ROO[•]	Peroxyl Radical
ROOH	Hydroperoxide
ROS	Reactive Oxygen Species
S.D.	Standard Deviation
S.E.M.	Standard Error of the Mean
SOD	Superoxide Dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric Acid-Reactive Substances
TCA	Trichloroacetic Acid
TMP	1,1,3,3-tetramethoxypropane
TPTZ	2,4,6-Tripyridyl-s-triazine
Trolox	(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
UCP	Uncoupling Protein
Vitamin C	Ascorbate/Ascorbic Acid
Vitamin E	α-Tocopherol
w/v	Weight per volume (grams per 100 millilitres)
v/v	Volume per volume (millilitres per 100 millilitres)

μl	Microlitres
μM	Micromolar

CHAPTER 1: INTRODUCTION

Aging is a natural occurrence in all living organisms that cannot be avoided. As a consequence of highly developed medical services and research, improved health and quality of life leads us to new untreatable diseases related to the increased age of the population. One of the main afflictions concerning this growing population of elderly is neurodegenerative disorders. In an attempt to understand and curb these diseases multiple areas of interest are focussed on, one of which is free radical overproduction in the brain and the toxic effects thereof.

Free radicals are the products of cellular respiration and energy production. Oxygen is essential for the survival of the cell and is responsible for the production of ATP in the mitochondria. This process however leads to toxic by-product formation (Balaban *et al.*, 2005), for which there are antioxidant systems in place, mostly in the form of enzymes. These antioxidant systems are able to attend to the free radicals present in the cell, but as the organism grows older, free radical production is increased and the antioxidant systems fail to regulate the oxidative status effectively. The resulting oxidative damage caused to the cell and its structures lead to cell death (Märkesbery *et al.*, 2001). Brain cells are unable to reproduce or regenerate, causing an assault on neurons of this kind to be fatal to human cognisance, memory and movement. Alzheimer's disease, Parkinson's disease and ischemia reperfusion injury are a few examples of the result of damage to areas in the brain, which is largely irreversible. It is therefore essential to lessen the oxidative burden on neurons before brain damage occurs. Antioxidants are able to lessen the overload of the endogenous antioxidant systems in the brain and prevent the damage caused by free radicals.

Flavones are a class of flavonoids (Robak and Glyglewski, 1988; Bors *et al.*, 1990) and *Pseudomonas* quinolone signal (Déziel *et al.*, 2004), are structurally similar compounds displaying antioxidant activity. The 4-quinolones, a well-known class of antimicrobial agents are also structurally similar to these mentioned antioxidants and are postulated to display increased antioxidant activity when substituted and compared to flavones, due to the increased number of hydrogen donating functional groups.

1.1 Research Objective

1.1.1 Objectives for this Study:

- To identify a natural antioxidant flavonoid structure that may be modified to assess the effect of key functional groups on antioxidant activity, and to establish a series of structurally similar compounds relevant in proving structure-activity relationships

- To synthesise the 2-phenylquinolin-4(1*H*)-one series, based on the lead compound flavone and to confirm the identity of the compounds with NMR, IR and MS techniques
- To establish the extent of antioxidant activity of the synthesised 2-phenylquinolin-4(1*H*)-one series when compared to a correlating flavone series, supplemented with relevant quinolines, by employing the chemical and biological evaluations, oxygen radical absorbance capacity (ORAC), ferric reduction/antioxidant power (FRAP), superoxide anion (NBT) and lipid peroxidation (TBA)
- To establish antioxidant structure-activity relationships of 4-quinolones

1.1.2 Proposed Series

Flavonoids are a class of antioxidants found in nature that consist of many types of chemical structures (Cotelle *et al.*, 1996). Antioxidant activity may be attained with various substitutions to the basic flavonoid structure (Fig 1.1), one of which is the hydroxyl substitution. In this study flavone was used as a lead compound (Fig 1.2) to synthesise a series of hydroxyl substituted 2-phenylquinolin-4(1*H*)-ones.

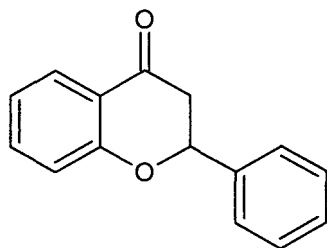
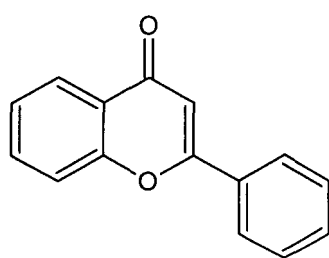
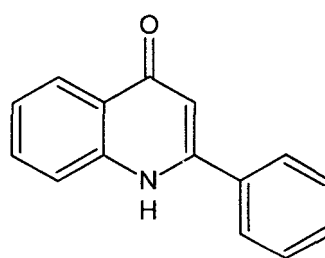


Figure 1.1 The Basic Flavonoid Structure



Flavone

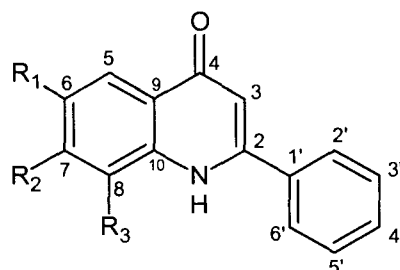


2-Phenylquinolin-4(1*H*)-one

Figure 1.2 Structure similarity between Flavone and 2-Phenylquinolin-4(1*H*)-one

2-Phenyl-4-quinolones have structures resembling that of flavones, a sub-type of flavonoids, but contain an additional hydrogen donating group, the amine. Due to this extra hydrogen

donor, it is postulated that the 4-quinolone may show the same or even more potent antioxidant activity when compared to flavone. 2-Phenylquinolin-4(1*H*)-ones (**7** to **10**) were proposed to establish structure-activity relationships. The effect of hydroxyl groups on antioxidant activity on positions 6, 7 and 8 was assessed, while the aromatic substitution on C-2 was a constant, used to facilitate comparison between the structures of 4-quinolones and flavones.



(**7**) $R_{1,2,3} = H$

(**8**) $R_{2,3} = H, R_1 = OH$

(**9**) $R_{1,3} = H, R_2 = OH$

(**10**) $R_{1,2} = H, R_3 = OH$

The complete series of compounds (Fig 1.3) in this study consisted of the quinolines (**1** to **3**), flavones (**4** to **6**) and synthesised 2-phenylquinolin-4(1*H*)-ones (**7** to **10**). Including quinoline (**1**) in the series determined the effect of the basic quinoline structure present in all 2-phenylquinolin-4(1*H*)-ones while 4-hydroxyquinoline (**2**) is structurally similar to the unsubstituted 2-phenylquinolin-4(1*H*)-one (**7**), which enabled determination of the effect of the protonated amine group. Substitution of both flavone (**4**) and 2-phenylquinolin-4(1*H*)-one (**7**) with 6- and 7-hydroxyl groups were compared to determine the effect of the presence and position of the hydroxyl substitution. As 8-hydroxyflavone was not commercially available, 8-hydroxyquinoline (**3**) was included in the series to compare structurally to 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**). The synthesised 2-phenylquinolin-4(1*H*)-ones (**7** to **10**) were compared to the appropriately substituted flavones (**4** to **6**) and certain quinolines (**1** to **3**) in chemical and biological evaluations.

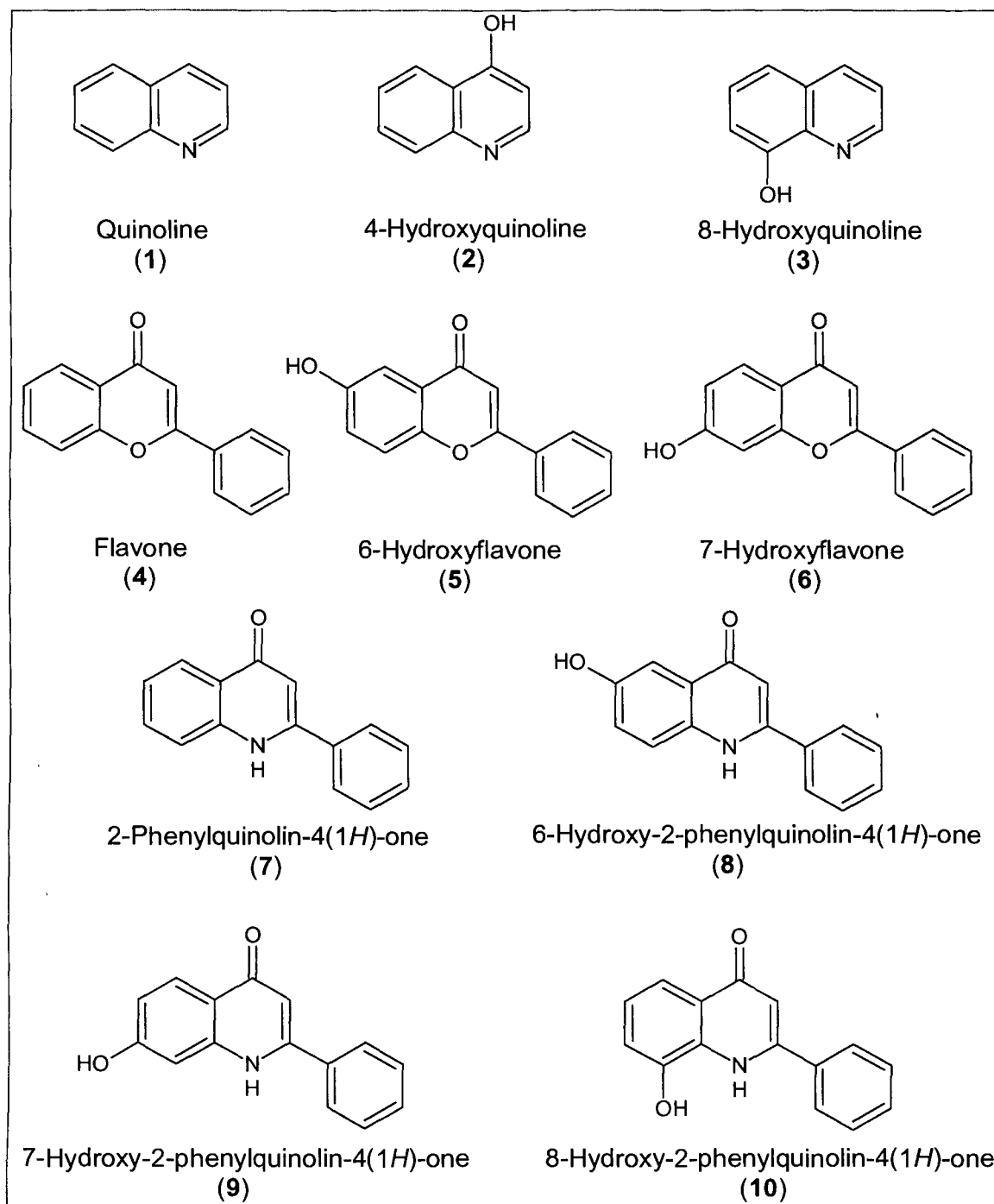


Figure 1.3 Complete Series of Test Compounds (1 to 10)

1.1.3 Rationale for Antioxidant Evaluations

A well-known contraindication of fluoroquinolones (Fig 1.4) is the simultaneous intake of divalent cations, as metal ions present in biological fluids or produced by other drugs, such as iron, aluminium, magnesium, calcium and copper have the ability to chelate or complex the fluoroquinolone structure (Turel, 2002). This might be useful in antioxidant activity since

the Fenton reaction is catalysed by ferrous (Fe^{2+}) and the Haber-Weiss reaction by ferric (Fe^{3+}) ions. Chelation of iron(III) by fluoroquinolones (Turel, 2002), will effectively remove the catalyst of the Haber-Weiss reaction, thereby producing less hydroxyl radicals. Iron-complexation involves both the carbonyl oxygen and the C3-carboxylic acid oxygen in fluoroquinolones (Turel, 2002), however a 3-hydroxy-4-quinolone, the *Pseudomonas* Quinolone Signal (PQS) (Fig 1.5), is an iron(III)-chelator found in nature without the characteristic C3-carboxylic acid group. Its iron chelating ability was linked by Déziel *et al.* (2004), to the 3-hydroxyl group. Since none of the test compounds, was substituted at C-3 (Déziel *et al.*, 2004), it was assumed that iron chelating activity was not an attribute of any of the test compounds in this series. Therefore the ability of the synthesised 2-phenylquinolin-4(1*H*)-ones (7 to 10), to reduce ferric iron was assessed in the ferric reducing/antioxidant (FRAP) assay to indicate a measure of hydroxyl radical production inhibition through the Haber-Weiss reaction, thereby reducing oxidative stress. The estimation of the iron chelating ability of the test compounds would however assist in the FRAP assay, since chelation of the ferric ion utilised in the assay would give falsely high ferric reducing activity results.

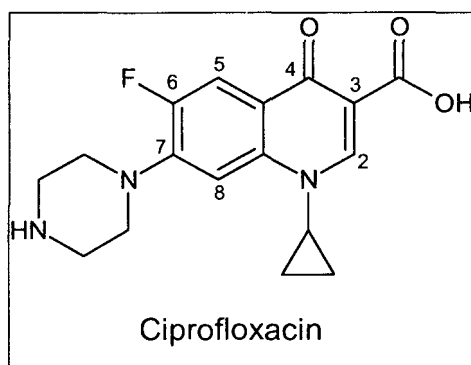


Figure 1.4 Fluoroquinolone Antibiotic (Park *et al.*, 2007)

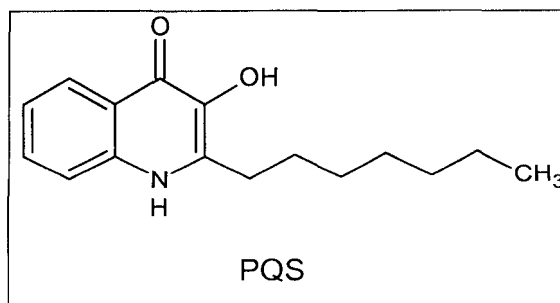


Figure 1.5 *Pseudomonas* Quinolone Signal (Diggle *et al.*, 2007)

Since polyphenols are reported to act via the hydrogen donor mechanism of action (Ou *et al.*, 2001), the ability of the synthesised series to scavenge the peroxy radical in the oxygen absorbance capacity (ORAC) assay, the hydroxyl radical in the lipid peroxidation thiobarbituric acid (TBA) assay and the superoxide anion in the nitro-blue tetrazolium (NBT) assay, was experimentally assessed together with the ferric reducing ability. These chemical and biological evaluations would therefore give a wide range of data for the performance of the synthesised 2-phenylquinolin-4(1*H*)-one series, (**7** to **10**) together with their structure related compounds (Fig 1.3), enabling determination of structure-activity relationships for some of the key oxidative reactions. Compounds displaying favourable activity and the structure-activity relationships concluded will assist in determining the antioxidant effect of the test compounds, enabling antioxidant drug design suitable for the prevention of antioxidant damage contributing to neurodegenerative disease (Fu *et al.*, 1998).

CHAPTER 2: LITERATURE REVIEW

2.1 The Free Radical Theory of Aging

All atoms have orbitals surrounding their nuclei that contain electrons. Some elements have outer orbitals that are not filled, but have single, unpaired electrons that do not bond with other atoms within a molecule. These unbalanced electrons are reactive and unstable and seek to be bonded to another atom, thus rendering them free radicals. These destructive free radicals can be either reactive oxygen species (ROS) or reactive nitrogen species (RNS). Figure 2.1 illustrates the outer orbital of oxygen, nitrogen and hydrogen atoms, the most important elements in the free radical theory, as well as the bonds formed between them:

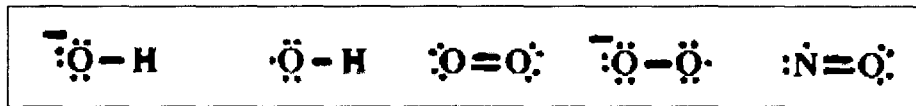
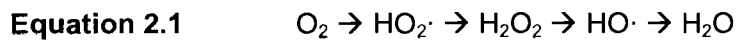


Figure 2.1 Lewis structures, indicating all outer shell electrons of respectively the hydroxide ion, hydroxyl radical, molecular oxygen, superoxide anion and nitric oxide (Best, 1990)

2.1.1 Reactive Oxygen Species and Free Radicals

Reactive oxygen species (ROS) are formed when oxygen molecules (O_2) are converted to water molecules (Markesbery *et al.*, 2001) in the aerobic organism. The univalent reductions yield the superoxide anion (O_2^-), then hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO\cdot$), before producing the safe water molecule (Imlay *et al.*, 1988):

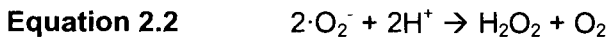


Molecular oxygen

From Figure 2.1 it is possible to see that molecular oxygen, also called triplet oxygen, has two uncoupled electrons in its outer shell, an aspect that gives it different properties from other free radicals. Normally oxygen forms two pi-bonds with other atoms, but in the event of excitation, or addition of energy, these two uncoupled electrons are placed in one outer p-orbital, leaving the other p-orbital empty. In this state, oxygen is called singlet oxygen and is a destructive reactive oxygen species with high affinity for the multiple unsaturated double bonds in DNA, proteins and polyunsaturated fatty acids (Best, 1990).

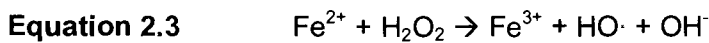
Molecular oxygen may receive one electron to bond one of its uncoupled electrons from the electron transport chain in the inner mitochondrial membrane. This generates a superoxide anion (O_2^-) that reacts with oxidised cytochrome c, a carrier (Joubert *et al.*, 2004), or with cytochrome oxidase, also known as complex IV (Orii, 1982) in the electron transport chain

(Section 3.1.1). Superoxide anions produced in the mitochondria are reduced by the enzyme superoxide dismutase (SOD) to hydrogen peroxide (H₂O₂) (Markesbery *et al.*, 2001):

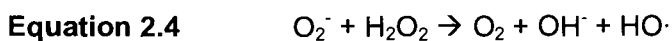


Hydrogen peroxide

Hydrogen peroxide is in itself not a free radical but is able to oxidise unsaturated double bonds nonetheless and furthermore leads to free radical production through the Fenton reaction (Markesbery *et al.*, 2001). This reaction between hydrogen peroxide and the reduced, ferrous ions (Fe²⁺) produces the hydroxyl radical (HO·), one of the strongest and most destructive free radicals present in the body. This hydroxyl radical is responsible for oxidative damage to DNA, proteins and lipid membranes and is a contributing factor in neurodegenerative disease (Fu *et al.*, 1998):



Accordingly, the damage caused is proportional to the concentration of reduced ferrous ions (Fe²⁺) available. Iron ions are generally protein bound, however in the cerebrospinal fluid and during ischemic reperfusion injury, as in stroke (Section 6.2), ferrous ions are unbound, catalysing the Fenton reaction to convert hydrogen peroxide to hydroxyl radicals (HO·), hydroxyl anions (OH⁻) and oxidised, ferric ions (Fe³⁺) (Markesbery *et al.*, 2001). It is therefore necessary to either chelate, or reduce ferric ions in order to prevent the Fenton reaction from generating hydroxyl radicals (Imlay *et al.*, 1988). In addition, the ferric iron (Fe³⁺) produced by the Fenton reaction, acts as a catalyst in the Haber-Weiss reaction, further oxidising hydrogen peroxide to hydroxyl radicals:



Hydrogen peroxide and ferrous ions cause oxidative stress through these two reactions. The body may prevent this detrimental chain of events by reducing hydrogen peroxide to water in the mitochondria, with the endogenous antioxidants (Radi *et al.*, 1991) catalase and glutathione peroxidase (Section 5.1.2-5.1.3).

2.1.2 Reactive Nitrogen Species

Figure 2.1 illustrates nitric oxide (NO), one of the reactive nitrogen species (RNS). In this study the focus will remain on the reactive oxygen species (ROS) except for nitric oxide, since it is a precursor in hydroxyl radical generation (Markesbery *et al.*, 2001). Nitric oxide is a free radical generated by the enzyme nitric oxide synthase (NOS) and is an important

endogenous vasodilator. However, in the case of ischemia reperfusion injury, excitotoxicity or inflammation, nitric oxide together with the superoxide anion (O_2^-), produces peroxynitrite ($ONOO^-$), a very destructive free radical (Markesbery *et al.*, 2001):



Peroxynitrite, as well as the hydroxyl radicals further generated by it, interferes with DNA, proteins and lipids by causing DNA cross-linking and lipid peroxidation through the formation of aldehydes and ketones (Roussyn, 1996). Superoxide anion scavengers and the enzyme superoxide dismutase will therefore clear up excessive superoxide anions produced in the mitochondria, before free radicals are further produced.

2.2 The Mitochondria

Mitochondria are organelles present in the cell's cytoplasm and are invaluable, given that they are the energy producers of the cell. Most of the reactive oxygen species (ROS) however, are formed inside the mitochondrial electron transport chain, or the cytochrome chain (Balaban *et al.*, 2005). This chain of proteins and enzymes are attached to the inner membrane and are responsible for oxidative phosphorylation, a process that consists of oxidative respiratory reactions and ATP-generation. The mitochondrial cytoplasm can resist the damaging effect of free radicals to some extent (Balaban *et al.*, 2005), but too great a burden on the antioxidant mechanisms leads to the destruction of the mitochondria and cell death. The oxidative stress caused by free radicals, gives rise to the 'swirl phenomenon', a process of rearranging the cristae of the mitochondrial membrane, which causes a breakdown of mitochondrial function, decreased energy production and a shorter lifespan of the cell (Balaban *et al.*, 2005). Therefore aging of the organism assists in the termination of mitochondrial function and energy production. An improvement of oxidative phosphorylation will result in reduced reactive oxygen species formation (Loschen *et al.*, 1971), and a longer lifespan of the cell as well as the organism.

Reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide are generated by the cytochrome chain's membrane proteins (Lambeth, 2004), the enzymes NADPH oxidase and cyclooxygenase in the cytoplasm, as well as a result of lipid metabolism within peroxisomes. According to Silva and Schapira (2001), 90% of reactive oxygen species present in the cell, are produced by oxidative phosphorylation, and as a consequence of aging, oxidative phosphorylation becomes less effective, producing less energy and more superoxide anions. It is therefore important to take note of the reactions that take place in the respiratory/cytochrome chain.

The Cytochrome Chain

The cytochrome chain or respiratory chain is attached to the inner mitochondrial membrane and is responsible for oxidative phosphorylation and ATP-production. The chain consists of four protein complexes, which transfer electrons and so produces superoxide anions and hydrogen peroxide, as illustrated by figure 2.2 and equation 2.2. The decreasing shades of pink of the four complexes indicate their decreasing ability to reduce molecular oxygen to superoxide anions, with complex I having the highest and complex IV the lowest ability (Balaban *et al.*, 2005). The decrease in voltage caused by the transfer of electrons from complex I to III is the greatest, and therefore produces the most superoxide anions. In Parkinson's disease (Section 6.3) the substantia nigra tends to have defective protein complex I (Smigrodzki, 2004) causing enhanced free radical production responsible for oxidative damage (Tretter, 2004).

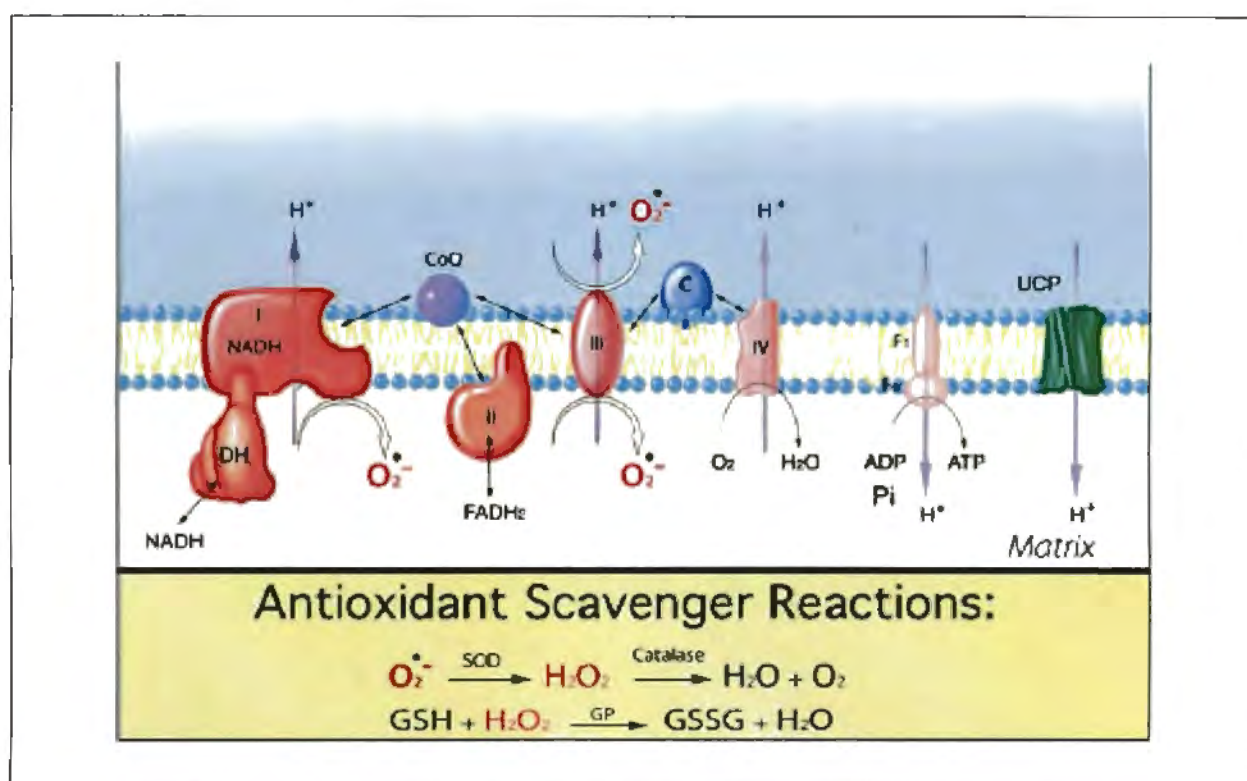


Figure 2.2 Schematic model of reactive oxygen species generation in the mitochondria (Balaban *et al.*, 2005)

Complexes I and II transfer electrons to complex III *via* the carrier, coenzyme Q (CoQ), which is itself reduced, (CoQH₂), then oxidised (•CoQ[•]). This state of coenzyme Q (•CoQ[•]) is responsible for most of the superoxide anions produced by complex III. Some of these anions move to the matrix, where it is converted to hydrogen peroxide by enzymes, such as manganese superoxide dismutase (Mn-SOD), Eq 2.2, while some move to the cytoplasm

(Muller, 2004). The produced hydrogen peroxide is then converted by either catalase (CAT) or glutathione peroxidase (GP) to harmless water molecules. Cytochrome c reductase (complex III), transfers the received electrons to the carrier, cytochrome c, which in turn transfers the electrons to cytochrome c oxidase (complex IV), which is responsible for converting the protons to water. Increased age of the organism causes a decrease in the activity of complex IV, resulting in increased superoxide anions and hydrogen peroxide and decreased metabolism of the oxidants to water.

Free radical production and cytochrome c-release during programmed cell death (Silva and Schapira, 2001), cause oxidation of lipids in the mitochondrial membrane (Boveris and Chance, 1973). This leads to enhanced proton leak, reactive oxygen species generation and further damage to protein, lipids and DNA (Best, 1990). As a consequence of ageing, cardiolipin levels decrease, which is responsible for holding cytochrome c to the inner mitochondrial membrane (Lutter, 2001). The release of cytochrome c into the cytoplasm takes place by either a Ca^{2+} -dependent or -independent mechanism and initiates necrosis or apoptosis, respectively (Best, 1990). In the Ca^{2+} -dependent mechanism, Ca^{2+} -overload in the mitochondria causes the opening of mitochondrial permeability transition pores between the matrix and the cytoplasm (Li, 2004), through which solutes and water enter, causing the mitochondria to burst and leading to necrosis (Best, 1990). The Ca^{2+} -independent mechanism, leading to apoptosis, requires Bax/Bax proteins to form pores in the outer mitochondrial membrane (Ott, 2002). Although programmed cell death is a necessary process, increased antioxidant action might reduce the oxidative stress caused to the surrounding cells.

There is however the uncoupling protein (UCP) in the respiratory chain that acts as an antioxidant by alleviating the oxidative burden created during electron transport. According to Speakman *et al.* (2004), the uncoupling protein decreases the membrane potential, contrary to the protein complexes, subsequently reducing superoxide anion production (Echtay *et al.*, 2002) and increasing the lifespan of the cell. The body generally makes use of proteins and enzyme antioxidant systems to correct the equilibrium between the pro-oxidants and antioxidants, thereby alleviating oxidative stress.

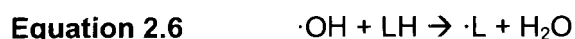
2.3 Oxidative Stress

Reactive oxygen species cause oxidative stress within the cell that ultimately leads to oxidative damage of DNA and proteins and peroxidation of the lipid membrane (McCord, 1985). This compromises the cell's ability to function and ultimately leads to cell death, which is devastating in the case of the brain, where neurons cannot regenerate. According to

McCord (1985), the brain is all the more susceptible to oxidative stress since it consists mainly of polyunsaturated fat, has high aerobic activity and levels of iron and fairly low levels of antioxidants. It is therefore important to know how radicals interact with biological matter if we are to decrease the damage caused by reactive oxygen species.

2.3.1 Free Radical damage to Lipids

Lipid peroxidation occurs when the cell experiences oxidative stress. It is the collective name for different reactions taking place during the initiation, propagation and termination stages. In the initiation stage, Eq 2.6, a fatty acyl chain (LH) donates one hydrogen atom, leaving a carbon-based radical (\cdot L). In the following propagation stage, Eq 2.7 and 2.8, the radical on the fatty acyl chain (\cdot L) obtains an oxygen molecule, forming a hydroperoxyl radical (LOO \cdot), which abstracts a hydrogen atom from another fatty acyl chain (LH), as in the initiation stage, to generate a lipid hydroperoxide (LOOH) and a new carbon-based radical. In the termination of lipid peroxidation, two radical species neutralise each other (Markesbery *et al.*, 2001), at the cost of a cross-link or covalent bond being formed between the two lipids (Best, 1990). Lipid peroxidation then leaves behind lipid hydroperoxides (LOOH), which may be disposed of in the Fenton reaction, Eq 2.9, to yield the very reactive and damaging lipid alkoxy radical (\cdot OL).



The membrane damage caused by this ongoing, self-propagating process is perceptible as split fatty acyl chains, aldehydes and cross-links between lipids and between lipids and proteins (Faber, 1995). According to Esterbauer *et al.* (1991), aldehydes such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) and 4-hydroxy-2-hexenal (HHE), can react with protein, nucleic acids and lipids, hence the use of malondialdehyde (MDA) as a standard of lipid peroxidation in biological assays. It has been shown that lipid peroxidation is enhanced in neurodegenerative diseases such as Alzheimer's (Markesbery *et al.*, 2001) and Parkinson's disease (Coyle and Puttfarcken, 1993), causing drug design to turn to lipid peroxidation inhibitors for preventative action. Amyloid β -peptide and iron are important initiators of lipid peroxidation (Mattson, 1998), facilitating neuronal degeneration through

apoptosis and excitotoxicity (Mark *et al.*, 1995; Keller *et al.*, 1997). Certain antioxidants may interfere with lipid peroxidation at the different stages of oxidation. Superoxide dismutase (SOD), catalase (CAT) and iron chelators interfere with the initiation stage, whereas reduced glutathione (GSH), ascorbate and α -tocopherol are responsible for limiting the propagation of lipid peroxidation (Markesbery *et al.*, 2001).

2.3.2 Free Radical damage to DNA and Protein

Oxidative damage to DNA is one of the most deleterious prospects of reactive oxygen species, since the consequences are far-reaching. It leads directly to the miscoding of proteins and, as result of this a detrimental cycle of events ensues, leading to the failure in regulation and function of the cell, and finally cell death. This is of course devastating when applied to the brain and its vital functions. Given that mitochondrial function declines with age (Shigenaga *et al.*, 1994) and nearly all oxidative steps take place within the mitochondria, it comes as no surprise that mitochondrial DNA is more prone to age-related oxidative damage than nuclear DNA (Richter *et al.*, 1988). Balaban *et al.* (2005) stated that the concept of the 'vicious cycle' includes the initial damage to mitochondria caused by reactive oxygen species, as well as the resulting mitochondrial dysfunction, which in turn produce more reactive oxygen species. Hirai *et al.* (1998), concluded that oxidative modification of mitochondrial DNA is an early event in Alzheimer's disease and accumulation of deleted mitochondrial DNA may further oxidative damage in neurons. The majority of the damage to DNA is caused by the hydroxyl radical, which together with peroxynitrite (ONOO⁻) and singlet oxygen cause direct oxidation of DNA. The superoxide anion and hydrogen peroxide cause DNA damage indirectly *via* the Fenton reaction, by producing hydroxyl radicals (Markesbery *et al.*, 2001). DNA oxidation is visible in the form of strand breaks, sister chromatid exchange, DNA- and DNA-protein cross-linking and base modification (Markesbery *et al.*, 2001). Single strand breaks are predominant, when singlet oxygen is the cause of oxidation and when specific repair enzymes restore areas of damaged purine bases (Viola *et al.*, 2004). There may be a double insult of increased oxidative damage and a deficiency of repair mechanisms in Alzheimer's disease (Markesbery *et al.*, 2001).

2.3.3 Oxidative Stress and the Brain

It is plain that oxidative stress plays a role in multifactorial diseases associated with age, cognitive decline and dementia, since neurons are especially susceptible to oxygen radical damage and lipid peroxidation of the brain is evident (Pratico, 2002). Pratico and Delanty, (2000) indicated that oxidative damage was an early factor in neuronal death, leading to various degrees of neurodegeneration and making this area ideal for drug intervention. They also pointed out that isoprostanes, lipid isomers of prostaglandins, are produced by lipid

peroxidation and may be used as non-invasive, biomarkers for measuring lipid peroxidation *in vivo*.

2.4 Antioxidants

Halliwell and Gutteridge (1989) defined an antioxidant as a substance that, when present at low concentration, significantly delays or inhibits oxidation of an oxidative substrate, while Møller and Loft (2006) described it as any substance that directly scavenges reactive oxygen species or indirectly acts to up-regulate antioxidant defences or inhibit reactive oxygen species production (Møller and Loft, 2006). It is therefore important to know what the functions of endogenous and exogenous antioxidants are, in order to treat oxidative stress effectively.

2.4.1 Antioxidant Enzymes

The main antioxidant systems in the body concerned in curbing oxygen radical production are the enzymes catalase (CAT), glutathione peroxidase (GP) and glutathione reductase (GR), which convert hydrogen peroxide to water molecules (Fig. 3) (Markesbery *et al.*, 2001). The hydrogen peroxide is produced when superoxide dismutase (SOD) converts superoxide anions to hydrogen peroxide (Eq 2.2, Section 2.1). Antioxidants may act by one of two mechanisms: by preventing initiation of oxidation or by acting as a chain breaking antioxidant. Prevention of initiation of oxidation occurs through inhibition of superoxide anion production, hydrogen peroxide degradation and metal ion chelation or reduction (Fig 3.), while chain-breakers act by scavenging radicals already produced, mostly hydroxyl radicals and thereby inhibiting the chain of oxidative events leading to damage of lipid membranes, proteins and DNA (Halliwell and Gutteridge, 1989). In order to scavenge radicals it is postulated that the antioxidant must act as a hydrogen-donor in order to reduce the oxidising free radical and so quench the ability of the radical to oxidise biological matter. The antioxidant should form a stable radical in order not to harm biological matter itself. Therefore, it is postulated that antioxidants containing H-donating groups, will show enhanced antioxidant activity through the scavenging of radicals.

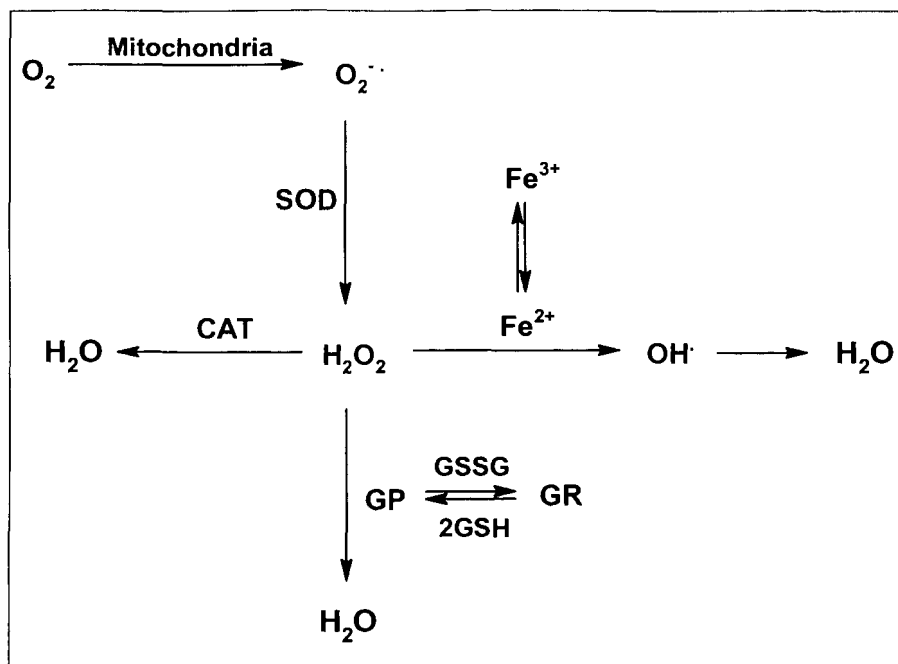


Figure 2.3 Pathways of neutralising hydrogen peroxide

2.4.1.1 Superoxide Dismutase

The first antioxidant enzyme in the cascade of oxidative events is superoxide dismutase (SOD), which is responsible for scavenging superoxide anions produced by the mitochondrial respiratory chain (Fig. 3). This enzyme facilitates the dismutase of superoxide anions to oxygen and hydrogen peroxide. Although hydrogen peroxide is one of the reactive oxygen species, it is not a radical, and therefore less harmful, and has many antioxidant enzymes ready to deactivate it.

Two types of the enzyme are present intracellularly, namely copper-zinc superoxide dismutase, Cu,Zn-SOD (SOD1), in the cytoplasm and manganese superoxide dismutase, Mn-SOD (SOD2), inside the mitochondria (Weisiger and Fridovich, 1973). An extracellular form of superoxide dismutase, Cu,Zn-SOD (SOD3), is needed to inhibit the reduction of nitric oxide by superoxide anions (Eq 2.5, Section 2.2) in blood vessels (Fukai, 2009). According to Serra *et al.* (2003), an increase in superoxide dismutase decreases the rate of telomere shortening, indicating that this enzyme actively prevents ageing.

2.4.1.2 Catalase

The antioxidant enzyme catalase is located in the peroxisomes and decreases the hydrogen peroxide levels by converting hydrogen peroxide to water and oxygen (Fig. 3). According to Putnam *et al.* (2000), the enzyme contains a heme group, which is responsible for reduction of two molecules hydrogen peroxide to one oxygen and two water molecules in two steps.

Firstly, the heme group reduces a hydrogen peroxide molecule to water and forms a covalent bond with the remaining oxygen, this newly formed compound then oxidises a second hydrogen peroxide molecule to oxygen after which it releases the remaining heme-bound oxygen in the form of water. This is a very efficient antioxidant mechanism.

2.4.1.3 Glutathione Peroxidase and Glutathione Reductase

Glutathione peroxidase is also responsible for deactivating hydrogen peroxide (Fig. 3). The enzyme catalyses the reaction in which two reduced glutathione (GSH) molecules donate two hydrogen atoms to hydrogen peroxide, to form two water molecules and one oxidised glutathione (GSSG):



The oxidised glutathione (GSSG) is then converted back to reduced glutathione (GSH) by glutathione reductase (GR), which is described by Silva and Schapira (2001) as the engine responsible for driving this antioxidant system. As the organism ages the expression of antioxidant enzymes decrease (Packer *et al.*, 1997), causing decreased antioxidant activity and increased neuronal damage.

2.4.2 Other Antioxidants

2.4.2.1 Vitamins

The most well-known exogenous antioxidants are vitamins C and E. Vitamin C, or ascorbate, is a hydrophilic substance (Markesbery *et al.*, 2001) actively transported into the brain and present in relatively high concentrations (Harrison and May, 2009). It is functional against an array of neurodegenerative diseases, such as ischemic stroke, Alzheimer's disease, Parkinson's disease, and Huntington's disease, due to its glutamate, GABA and dopamine effects (Harrison and May, 2009).

Vitamin E, or α -tocopherol, is a chain-breaking, lipophilic antioxidant active in lipid membranes (Packer, 1991). It is responsible for radical scavenging and inhibition of lipid peroxidation (Burton & Ingold, 1989); however these positive effects diminish at high concentrations, resulting in a pro-oxidative effect (Cillard *et al.*, 1980). It is nevertheless a very potent inhibitor of radical mediated injury and is utilised as an adjunct in various diseases. Kashif *et al.* (2004) found that vitamin E surpassed the ability of vitamin C in increasing glutathione levels, improving the performance of superoxide dismutase, reduced glutathione and catalase and inhibiting lipid peroxidation. Both these vitamins though

contribute to the oxidative health of the brain and are well-known strategies against neurodegeneration.

2.4.2.2 Melatonin

Melatonin, an indoleamine neurohormone, is produced by the pineal gland and is effective in ischemia/reperfusion of the brain (Cheung, 2003), Alzheimer's (Pappolla *et al.*, 2000) and Parkinson's disease (Antolin *et al.*, 2002). It is a more efficient radical scavenger than vitamin E and glutathione according to Reiter *et al.* (1997) and Hara *et al.* (1996); one melatonin molecule is able to scavenge two hydroxyl radicals, indicating the effectiveness of this hormone in inhibiting lipid peroxidation. It is found that even the degradation products of melatonin show some antioxidant activity (Reiter *et al.*, 2003). Melatonin neutralises singlet oxygen (Poeggeler *et al.*, 1996) and stimulates the antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase and α -reductase, while inhibiting the pro-oxidative enzyme nitric oxide synthase (NOS) (Reiter, 1998). Altogether this is a remarkable antioxidant hormone, stimulating development of similar structures in the search for antioxidant drugs.

2.4.2.3 Uric acid

Uric acid is a substance produced by the body that protects against vitamin C degradation and may inhibit the Fenton reaction through the complexation of iron (Best, 1990). Hensley *et al.* (1998) found decreased uric acid in Alzheimer's disease patients, indicating the importance of this substance in scavenging peroxynitrite in the brain (Whiteman and Halliwell, 1996).

2.4.2.4 Natural Plant Antioxidants: Flavonoids

Antioxidants have attracted a great deal of attention as potential agents for preventing age-related oxidative damage (Reiter *et al.*, 2003). Flavonoids are a class of well-known natural compounds that possess antioxidant and radical scavenging properties (Robak and Gryglewski, 1988; Bors *et al.*, 1990). The term flavonoid is used for a great variety of chemical structures, including the chalcones, flavanols, flavanones, flavones, flavonols, flavylum salts or anthocyanidins, isoflavonoids, neo-, and biflavanoids, which together with the ample possibilities for substitution, makes for an interesting class of compounds with varying activities (Bors *et al.*, 1990). For the purpose of this study we will focus on the flavones, especially polyhydroxyflavones, which are known antioxidants. Robak and Gryglewski (1988) have noted that quercetin, myricetin and rutin (Fig 2.4) showed especial superoxide anion scavenging activity, indicating the importance of a catechol moiety on the B ring and C-7 hydroxyl substitution. Qin *et al.* (2008), found that flavonoids with three

hydroxyl substitutions presented increased antioxidant activity, confirming our hypothesis that an increased number of hydrogen-donating groups on the structure, increase antioxidant capacity.

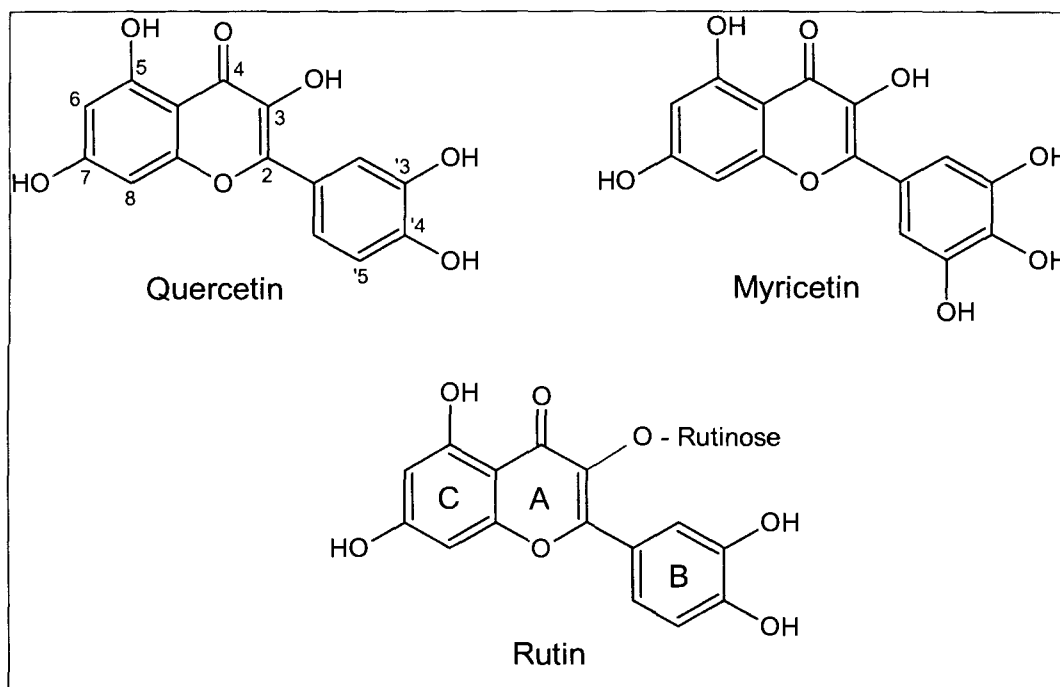


Figure 2.4 Flavone structures with proven antioxidant activity

According to Cotelle *et al.* (1996), a catechol moiety on the B ring and hydroxyl substitution on C-6 and C-7 enhances enzymatic antioxidant and radical scavenging activity, which is especially useful in preventing oxidant damage to ischemic tissue. Flavones with both C-7 and B ring substitution showed complementary action and proved to be very good antioxidants (Cotelle *et al.*, 1996). Khlebnikov *et al.* (2006) indicated that hydroxyl groups on either positions 3, 7 and 8 enhance reactive oxygen species scavenging. It is however uncertain whether flavones act directly or indirectly against superoxide anions (Cotelle, 2001). This effect on superoxide anions will however prove advantageous to the preservation of nitric oxide in the blood vessels, preventing vascular dysfunction (Chan *et al.*, 2003). Duarte *et al.* (1993) stated that the C3-hydroxyl group is essential for endothelium-dependent relaxation, as is the case with quercetin (Fitzpatrick *et al.*, 1993). The scavenging ability is however associated with the ability of flavones to form stable radicals (Cotelle *et al.*, 1996), an important factor in antioxidant activity.

2.4.2.5 Quinolones

Fluoroquinolones are well-known antimicrobial drugs that are able to infiltrate the cerebrospinal fluid and are used to treat a multitude of infections, including meningitis. Ronald and Low (2004) expressed the antibacterial structure-activity relationship for fluoroquinolones as: N1, C2-H, C3-COOH, C4-O, C6-F and C7-piperazine. Park *et al.* (2007), found that ciprofloxacin reduced ischemia and increased cell survival in a rat focal cerebral ischemic animal model and that a modified structure of ciprofloxacin, devoid of the C-6 fluorine and C-3 carboxylic acid, but containing a C-2 methyl, lessened antibacterial activity while improving anti-ischemic activity and neuroprotection.

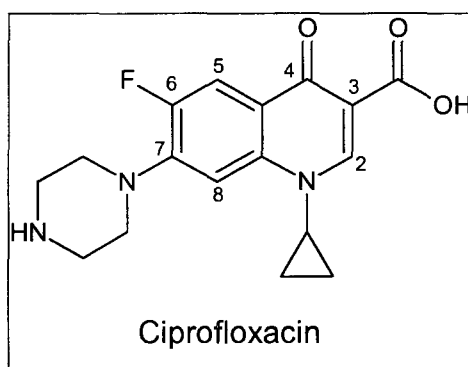


Figure 2.5 Fluoroquinolone Antibiotic (Park *et al.*, 2007)

Quinolone structures that have shown antioxidant activity include the plant quinolone alkaloid, 4-carboethoxy-6-hydroxy-2-quinolone, found in a rice species (Chung and Shin, 2007). It is reported to have radical scavenging activity and prevents neurodegeneration and cytotoxicity. This further indicates the effect of hydroxyl substitution on these polycyclic compounds in antioxidant activity.

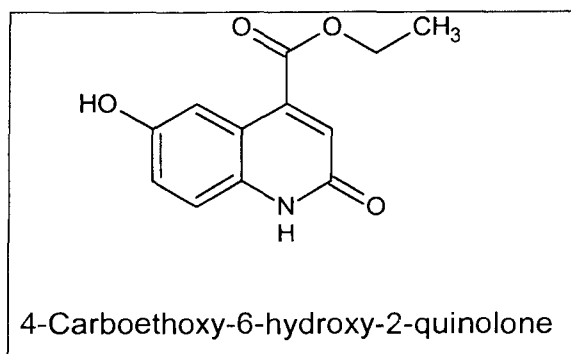


Figure 2.6 Antioxidant alkaloid (Chung and Shin, 2007)

It is postulated that substituting a 2-phenyl-4-quinolone structure with hydroxyl groups as is found on the natural antioxidant flavones, might retain flavone antioxidant activity, or even increase it, since the quinolone contains an extra hydrogen-donating group, the amine.

2.5 Ageing and Age-Related Neurodegenerative Diseases

Ageing is a natural process that occurs in all aerobic organisms due to oxidation that effect changes in the integrity of the cell leading to dysfunction of mitochondria and vascular systems, inflammation and cellular senescence (Fukai, 2009). Ineffective respiration in the mitochondrial respiratory chain, along with contributing factors such as ion catalysts and insufficient antioxidant concentrations, lead to oxygen radical production, which cause oxidative stress and radical-mediated damage to biological structures. Neurodegeneration is only visible when enough cells in the brain and spinal cord have died or ceased to function, to cause disability, visible as impaired memory, movement or speech. By the time symptoms of disability present themselves it is too late to reverse the radical-induced damage, but the ongoing effect thereof might be slowed or even stopped if the oxidative burden is lightened. Biomarkers might also indicate oxidative damage earlier in the disease, leading to treatment, before symptoms of disability develop.

2.5.1 Alzheimer's Disease

Alzheimer's disease is still not fully understood and is an increasing burden on our society given that we do not have an effective preventative drug or cure. Dementia afflicted 30 million people worldwide in 2008 and is it is postulated by Alzheimer's disease International (2009) that the number of dementia cases will increase to over 100 million by 2050. Currently more than 50% of dementia cases in Caucasian populations can be attributed to Alzheimer's disease. According to Pratico (2002), Alzheimer's disease is characterised by progressive loss of memory, speech, and cognisance, proportional to increased age. Genetic mutations cause early-onset familial Alzheimer's disease, but in late-onset Alzheimer's disease, which amounts to 90% of patients, genetic risk factors are only part of the cause. Neurofibrillary tangles, senile plaques, oxidative stress, gliosis, microglia activation, and inflammation are common occurrences in this disease, however Pratico (2002) has proved that oxidative stress is an early event in Alzheimer's disease, turning our focus to antioxidants to curb the disease at (one of) its origin(s).

The distinctive free radical assault on DNA, protein and lipid membranes is seen in the form of lesions in the brains of Alzheimer's patients. This occurs as a result of increased iron concentrations in Alzheimer's disease patients' grey matter, cytochrome-c oxidase irregularities and amyloid β -peptide aggregates (Christen, 2000). Hensley *et al.* (1994)

demonstrated that amyloid β -aggregates generate free radicals, which lead to oxidative stress and cell death in the brain that may be reversed by radical scavengers, such as vitamin E. Other drugs that may alleviate oxidant damage are *Ginkgo biloba* extracts, desferrioxamine, an iron chelator, nonsteroidal anti-inflammatory drugs and oestrogen therapy, though only in women (Christen, 2000 and Huang, 2008). Cholinesterase inhibitors decrease acetylcholine degradation and temporarily improve cognisance, though memantine has shown ability to slow the progression of Alzheimer's disease (Huang, 2008).

The difficulty in treating this disease lies in the fact that it is not possible to diagnose prodromally and that it is multifactorial in origin. Pratico *et al.* (1998) however showed that isoprostane levels, a toxic product produced during peroxidation of arachidonic acid, may be used as a biomarker indicative of central nervous system oxidative damage, since levels are elevated only in areas affected by the disease. Bateman *et al.* (2007) also stated that angiotensin converting enzyme (ACE)-levels may be used as a biomarker of amyloid- β peptide levels in the cerebrospinal fluid. The choroidal epithelium, a regulator of cerebrospinal fluid secretion and homeostasis, degenerates with age causing inflammation, production of ACE and intracellular adhesion molecule and decreased clearance of amyloid β -protein. Vascular dysfunction results from the amyloid β -deposits, since vasodilator response to endothelium-dependant vasodilators is inhibited (Kalaria, 2007). The supply of oxygenated blood to the brain plays a key role in neuronal homeostasis. One of the central factors protecting the brain is blood circulation. As a result of age the terminal arterioles of the deep white matter lose the ability to control blood flow effectively (Kalaria, 1996), leading to chronic hypoxia (Fernando *et al.*, 2006) and ischemic injury. This will ultimately damage cerebral endothelium, violate the blood brain barrier and cause oedema (Farral and Wardlaw, 2009). In the elderly, the blood brain barrier is less capable of regenerating itself assisting vascular anomalies and inflammation (Finch, 2005). Autoregulation of cerebral blood flow ensures that pressure remains between 50 and 160 mmHg and controls the amount of nutrients, oxygen and glucose taken to the brain. This is hindered when autonomic nuclei degenerate, leading to altered control of vascular wall smooth muscle (Kalaria, 2007). When amyloid precursor protein is over-expressed, causing accumulation of amyloid- β deposits (Iadecola, 2004) and radical generation, it further hinders autoregulation. This indicates that blood flow and pressure play an important role in neurodegeneration.

2.5.2 Ischemic Stroke

In normal blood vessels nitric oxide is released from the endothelium and diffuses into smooth muscle to produce vasorelaxation. Superoxide anion reduction of nitric oxide contributes to vasoconstriction and inflammation (Fukai, 2009), while producing peroxynitrite,

a very destructive reactive nitrogen species. When these vascular events occur, the chances of developing ischemia and ischemic stroke are increased, linking neurodegeneration and vascular degeneration to a common cause, namely reactive oxygen species. Vascular degeneration then further aggravates neurodegeneration, as is seen in Alzheimer's disease. In areas of high neuronal activity increased blood flow (Kalaria, 2007) is essential and reduced perfusion will therefore inhibit neuronal processes. Vasorelaxation is therefore important and may be induced by drugs that act on vascular smooth muscle (Herrera *et al.*, 1996) or inhibit calcium influx (Chan *et al.*, 2000). Both acute and transient ischemias occur as a result of reactive oxygen species, and cause further production of radicals. After an acute ischemic episode increased malondialdehyde levels are visible (Sinha *et al.*, 2001), while increased calcium influx is seen after transient episodes of ischemia. Both cases lead to lipid peroxidation, oxidative stress and neurotoxicity (Rios and Santamaria, 1991). Qin *et al.* (2008), conclude that reperfusion of ischemic tissue, although essential, should be attenuated to reduce the occurrence of excitotoxicity, intracellular calcium overload, protein inhibition and oxygen radical production. This damage profile might be mediated by flavonols, natural products with both antioxidant and vasorelaxation activities which exhibit the ability to protect against ischemic damage (Wang *et al.*, 2004).

2.5.3 Parkinson's Disease

In Parkinson's disease an imbalance between acetylcholine and dopamine exists, giving rise to the characteristic shuffle, tremors and rigidity, therefore increasing dopamine while decreasing acetylcholine levels will effectively treat Parkinson's disease. The brain stem cells also contain the characteristic Lewy bodies, or intracytoplasmic inclusions (Silva and Schapira, 2001). The well-known drug L-dopa is a precursor to dopamine in the periphery and the brain. According to Eidelberg and Pourfar (2007), addition of carbidopa assists in the selective conversion to dopamine in the brain, and enhances the efficacy of the drug. The effect of L-dopa is however short lived and may even contribute to deterioration of the disease. This on-off phenomenon may be treated with apomorphine, a dopamine agonist. Other drugs include the dopamine agonists and the antiviral drug amantadine (Eidelberg and Pourfar, 2007). Parkinson's disease may also be treated by inhibiting the degradation of dopamine by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT), and by anticholinergics. Symptomatic relief of tremors may be attained with the use of beta-blockers while antihistamines and tricyclic antidepressants supplement levodopa in the brain (Eidelberg and Pourfar, 2007).

This disease is also related to neurodegeneration and vascular dysfunction by free radical damage, due to amassing iron ions, malfunctioning mitochondrial complex I (Silva and

Schapira, 2001) and insufficient concentrations of glutathione in the substantia nigra (Perry *et al.*, 1982). The defective complex I is responsible for superoxide anion production, which in turn exaggerates the complex I defect (Silva and Schapira, 2001). This increase in production of superoxide anions warrants an increase in superoxide dismutase levels; however nitric oxide production is also increased, inhibiting superoxide dismutase and complexes II, III and IV, causing cell death through oxidative damage. As in Alzheimer's disease, malondialdehyde levels are increased (Jenner, 1991), indicating that lipid peroxidation of neuronal membranes takes place. Melatonin, a neurohormone antioxidant, may reverse mitochondrial respiratory chain inhibition of 1-methyl-4-phenylpyridinium (MPTP) (Absi *et al.*, 2000), enhancing the respiratory chain activity, while decreasing radical production.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was originally synthesised as a meperidine analogue designer drug in northern California. With the use of this drug individuals developed an akinetic rigid syndrome and severe loss of neurons in the substantia nigra, without Lewy bodies (Langston *et al.*, 1983). MPTP, a protoxin, causes inhibition of oxidative phosphorylation and generation of free radicals. It is mainly converted to the active 1-methyl-4-phenylpyridinium (MPP^+) form by MAO-B, *via* the 2,3-dihydropyridinium (MPDP) intermediate (Silva and Schapira, 2001). Consequently MAO-B antagonists will prevent MPTP toxicity through prevention of degradation to MPP^+ . The radicals produced by MPP^+ as a result of its permanent inhibition of mitochondrial complex I, may be scavenged by radical scavenging antioxidants (Cleeter *et al.*, 1994), decreasing the rate of destruction of this toxic degradation product.

It is possible to limit the oxidative damage caused by Parkinson's disease and MPTP and its metabolites, as well as that of Alzheimer's disease and ischemic stroke, by employing antioxidant scavenger drugs, reducing the oxidative damage leading to disability and neuron-loss in the elderly and improving these patients' prognosis. This study will consequently focus on the natural antioxidant group flavones and the antioxidant activity observed for this group in selected antioxidant assays, which will be compared to structurally similar synthesised 2-phenylquinolin-4(1*H*)-ones, with relevant hydroxyl substitutions to establish antioxidant structure-activity relationships as well as the antioxidant mechanism of action of the 2-phenylquinolin-4(1*H*)-ones. The conclusions drawn from the assessments will therefore assist in the design of new antioxidant therapeutic approaches in the search for an effective neuroprotective drug.

CHAPTER 3: 2-PHENYLQUINOLIN-4(1H)-ONES

3.1 Introduction

Oxygen free radical production in the cell is a typical occurrence and is regulated by the body's antioxidant enzymes and other antioxidant molecules. However as the body ages it becomes less equipped to control increasing amounts of reactive oxygen species, resulting from ageing mitochondria (Balaban *et al.*, 2005; Markesbery *et al.*, 2001), which effectively produce more radical by-products and less ATP. Consequently the necessity to support and supplement the endogenous antioxidant systems arises. The plant kingdom has provided us with a multitude of naturally occurring polyphenolic compounds able to mediate antioxidant activity, of which the flavonoids are a prominent class of compounds (Laguerre *et al.*, 2007). Study of the structure-activity relationships of flavonoids have lead researchers to confirm the hypothesis that polyphenolic compounds, containing hydrogen donating groups, are the source of good antioxidant activity in plant extracts (Robak and Glyglewski, 1988; Bors *et al.*, 1990). Certain positions of substitution on the flavonoid structure, as well as the type and number of hydrogen donors are factors responsible for altering the observed antioxidant activity (Laguerre *et al.*, 2007).

3.2 Chemical properties of Polyphenols

Phenolic structures as chemical entities are well suited to attenuate oxidative attack. A phenol is an aromatic ring substituted with a hydroxyl group; this moiety is responsible for antioxidant activity since the hydroxyl group may donate its hydrogen atom to a hydrogen acceptor, thereby forming a stable phenoxide radical (Silva, 2009). The newly formed oxygen radical is stabilised by the aromatic ring, allowing the hydrogen atom transfer reaction to continue unimpeded, producing phenoxide radicals at the cost of reducing hydrogen acceptors, or in this case reactive oxygen species (Silva, 2009).

Antioxidants may be categorised as either radical production inhibitors or as radical scavengers. Polyphenols fall into the second category, acting by scavenging radicals that have already been produced, thereby interrupting the chain of ongoing oxidative reactions. Chain-breakers may act by one of two different mechanisms that result in the same redox state: single electron transport where two one-electron reactions result in a phenoxide radical (Section 5.1), or hydrogen atom transfer where the oxidant abstracts a hydrogen atom from the antioxidant, also leaving a phenoxide radical (Section 4.1), (Ou *et al.*, 2002). In both these mechanisms the antioxidant is oxidised to a stable radical and the oxidant is reduced, leaving it unable to oxidise biological matter. Polyphenolic compounds may act by either of these mechanisms, since the end result is the same: production of a stable phenoxide radical

(Ou *et al.*, 2002). It is therefore no surprise that the flavonoids are good antioxidants (Laguerre *et al.*, 2007), since the polycyclic phenolic structure is able to form a stable radical once the hydrogen-donor group has been oxidised to deactivate a free radical.

3.3 Flavones as Lead Compounds for 2-Phenylquinolin-4(1H)-ones

Flavones are a sub-type of flavonoids that have displayed antioxidant activity when appropriately substituted with hydrogen donor groups (Laguerre *et al.*, 2007). The similarity between the flavone and a 2-phenyl substituted 4-quinolone is clearly visible in figure 3.1:

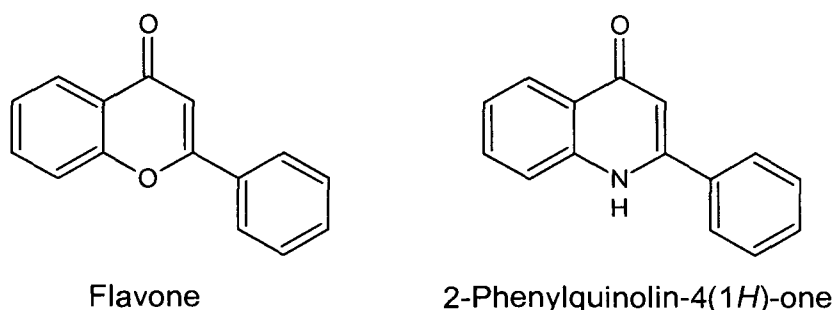
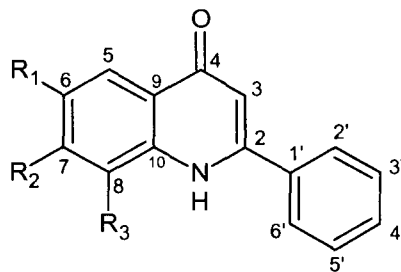


Figure 3.1 Structure similarity between Flavone and 2-Phenylquinolin-4(1H)-one

Although 4-quinolones are not particularly known for antioxidant activity, it is hypothesised that the 2-phenylquinolin-4(1H)-one structure might retain the flavone's antioxidant activity, since an extra hydrogen-donating group, the amine, is added to the basic skeleton. It was found by Park *et al.* (2007), that the antibacterial activity of fluoroquinolones, as described by Ronald and Low (2004), could be altered to yield neuroprotection through prevention of apoptosis, by excluding the characteristic fluorine and carboxylic acid groups and substituting a methyl group on position 2 (Fig 2.5). Fluoroquinolones are used in the treatment of bacterial meningitis and are therefore distributed to the cerebrospinal fluid (Destache *et al.*, 2001). As a result we suggested that enlarging this C-2 methyl group to an aromatic ring, might result in neuroprotection through enhanced lipophilicity and ability to cross the blood brain barrier, while retaining the basic skeleton of the flavone. This might prove enlightening with regards to the biologic activity of the amine versus the oxo group, as well as antioxidant structure-activity relationships of hydroxyl-substitution on the 2-phenylquinolin-4(1H)-one structure. The positions on the 2-phenylquinolin-4(1H)-one skeleton investigated in this study were the 6, 7 and 8-hydroxyl substitutions:



(7) R_{1,2,3} = H

(8) R_{2,3} = H, R₁ = OH

(9) R_{1,3} = H, R₂ = OH

(10) R_{1,2} = H, R₃ = OH

3.4 Synthesis of 2-Phenylquinolin-4(1H)-ones

The compounds (7 to 10) were synthesised by the Conrad-Limpach synthetic route described by Somanathan and Smith (1981), where aniline reacts with a β -keto-ester to form the 2-phenylquinolin-4(1H)-one (Fig 3.2). Identification of the compounds were done by physical means such as NMR, MS and IR (Appendix A).

3.4.1 Standard Experimental Techniques

3.4.1.1 Instrumentation

3.4.1.1.1 Melting Point Determination

The melting points for the synthesised compounds were established using a Stuart melting point SMP10 apparatus with glass capillary tubes holding the sample in the heating chamber. A plateau temperature was quickly reached after which the exact melting point was observed by slowly ramping to the melting point of the compound.

3.4.1.1.2 Mass Spectrometry

A Thermo Electron LXQ ion trap mass spectrometer with atmospheric pressure chemical ionisation (APCI) source set at 300 °C was used to obtain low resolution APCI, with capillary voltage at 7.0 V and Corona discharge at 10 μ A while a Thermo Electron DFS magnetic sector mass spectrometer at 70 eV and 250 °C was used to obtain high resolution electron impact (EI) spectra. Samples were introduced by a heated probe and perfluorokerosene was used as reference compound.

3.4.1.1.3 Infrared Absorption Spectra

Infrared spectra (IR) were obtained at 4000-40 cm^{-1} from a Nicolet Nexus 470-FT-IR Spectrometer, using KBr pellets and the diffuse reflectance method.

3.4.1.1.4 Nuclear Magnetic Resonance Spectroscopy

^1H , ^{13}C , HSQC and COSY NMR were obtained from a Bruker Advance 600 Spectrometer, in a 14.09 Tesla magnetic field, using ultra shield plus magnet. ^1H NMR and ^{13}C NMR were recorded at frequencies of 600 MHz and 300 MHz, respectively. Tetramethylsilane was used as a point of reference at $\delta = 0$, against which all chemical shifts were measured in parts per million (ppm). ^1H NMR signal multiplicity was denoted as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet) or m (multiplet).

3.4.1.2 Chromatography

3.4.1.2.1 Thin Layer Chromatography

TLC Silicagel 60 F_{254} aluminium sheets from Merck, Darmstadt, Germany, were used for thin layer chromatography and compounds eluted with the appropriate mobile phase, constituted immediately before use. The R_f -value was observed under UV-light at 254 nm and 366 nm and calculated by dividing the distance of sample elution by the fixed distance of the mobile phase.

3.4.1.2.2 Column Chromatography

Glass columns with the appropriate length and thickness were used in column chromatography. Merck[®] Silica 60 (0,063 - 0,200 mm) was used as the stationary phase, with the appropriate mobile phase constituted immediately before use.

3.4.2 Traditional Synthesis of 2-Phenylquinolin-4(1*H*)-ones

All 2-phenylquinolin-4(1*H*)-ones (**7** to **10**) were prepared in a one pot synthesis according to the general Conrad-Limpach method described by Somanathan and Smith (1981), (Fig 3.2). The 4-quinolone structure was prepared by cyclisation of aniline, (**7**), (279.39 mg, 0.003 moles) or the appropriately substituted aminophenol, (**8** to **10**), (327.39 mg, 0.003 moles) with ethyl benzoylacetate (576.66 mg, 0.003 moles). The reaction was stirred in 100 ml benzene at reflux temperature, with *para*-toluene sulfonic acid (*p*-TosOH), (50 mg, 2.629 mole) as catalyst and was dehydrated with the Dean-Stark apparatus to yield the intermediate compound (Fig 3.3). The reaction was halted in the presence ethanol, a by-product produced by the reaction and was therefore dehydrated in order to maintain conversion of reactants to product (Lange *et al.*, 2001). Diphenyl ether (15 ml) was added

after an extended reaction time of 19 hours and subsequent removal of benzene, to enable reflux at a temperature of 265°C for 30 minutes, in which time cyclisation of the intermediate generated the product.

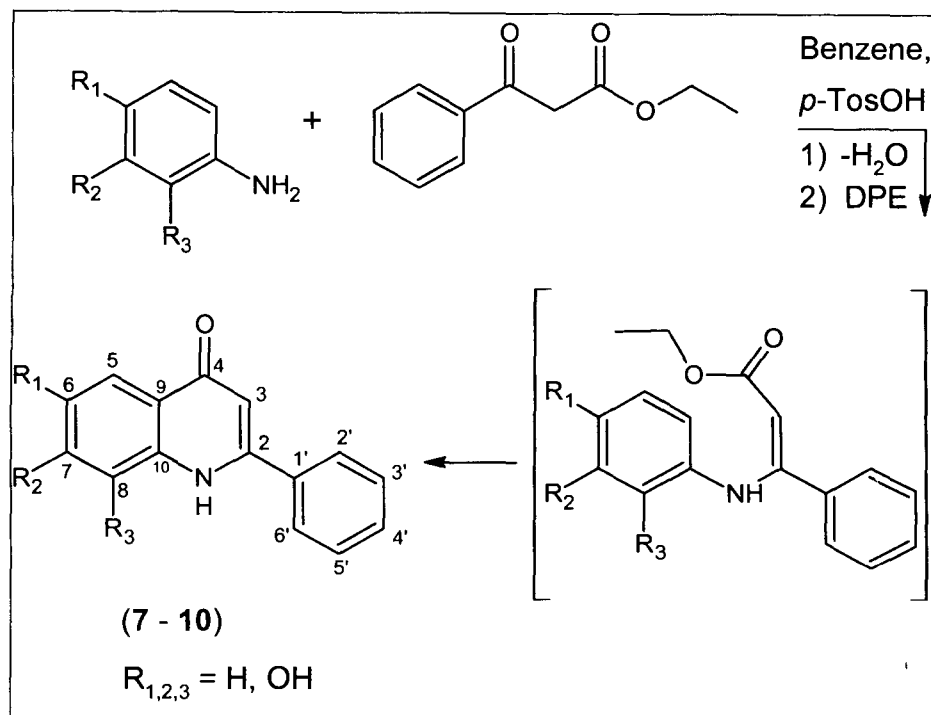


Figure 3.2 General scheme for Conrad-Limpach Synthetic Method with *p*-TosOH (*para*-Toluene Sulfonic Acid) and DPE (Diphenyl Ether)

An extensive work-up procedure was needed for some of the synthesised compounds. The removal of diphenyl ether was however a constant and was achieved with a short silica column, eluting over several hours with petroleum ether until all diphenyl ether was eluted from the product mixture. Petroleum ether effectively removed diphenyl ether while leaving the product mixture at the base line as a muddy, grainy solid. This procedure was a time-consuming step, but proved to be the most economic and successful removal of diphenyl ether. All spectra obtained to verify the identity of the compounds are presented in Appendix A.

3.4.3 Optimisation of Conrad-Limpach Method

Optimisation of these chemical syntheses was attempted by employing solvent-free microwave irradiated synthesis as described by Lange *et al.* (2001). The open-vessel setup of Razzaq and Kappe (2007), was employed, but with a 20 cm Vigreux column, to allow for the by-product ethanol to be removed from the reaction. Aniline and ethyl benzoylacetate was cyclised in a 1:2 ratio in a CEM Discover System microwave reactor at 300 W at

290°C, this process was repeated 2 to 3 times and the reaction followed by thin layer chromatography (TLC). Enhanced reaction was seen when the microwave reactor chamber was cooled with positive air flow. Further optimisation of the method is needed, however 2-phenylquinolin-4(1*H*)-one (**7**) was successfully synthesised and confirmed by TLC in approximately one hour, with the workup sped up considerably due to the absence of diphenyl ether. Precipitation with diethyl ether yielded almost pure compound, but further column chromatography was necessary, eluting with DCM 97%, MeOH 3%, (as in Section 3.4.4.1). Further study into this method will be done to facilitate speedy, optimised and eco-friendly synthesis of 2-phenylquinolin-4(1*H*)-ones. The traditional Conrad-Limpach method (Section 3.4.2) was used as a general route as higher yields were obtained by this method.

3.4.4 Test Compounds

3.4.4.1 2-Phenylquinolin-4(1*H*)-one (**7**)

Aniline (279.39 mg, 0.003 moles) was cyclised with ethyl benzoylacetate (576.66 mg, 0.003 moles). *In vacuo* removal of benzene yielded a yellowish oil, which was refluxed with diphenyl ether. The product was purified by column chromatography, initially eluting with petroleum ether, to remove diphenyl ether, then with 97% DCM, 3% MeOH (*R_f* = 0.115) to obtain the crude product, which was suspended in toluene and filtered to obtain the pure product, as an off-white amorphous solid (Yield: 203 mg, 0.917 mmole, 30.58%).

Physical data

R_f = 0.115 (97% DCM, 3% MeOH); C₁₅H₁₁NO; mp = 259°C; APCI-MS (300 °C, 7.0 V, 10 uA) *m/z*: (spectrum 1): 221.64 (M⁺); HREI-MS (spectrum 2): calc. 221.25394, exp. 221.08371; *v_{max}* (spectrum 3, KBr, cm⁻¹): 3200.0, 3150.0, 3120.0, 3100.0, 3080.0, 3052.7, 1733.8, 1683.6, 1635.6, 1582.2, 1547.7, 1501.3, 1473.5, 1449.9, 1432.8, 2971.4, 800.5; δ_H (spectrum 4, 600 MHz, DMSO-d₆): 6.34 (s; 1H; H-3), 7.331 (m; 1H; J=12.22 Hz; H-6), 7.576 (s; 3H; H-8; H3'; H5'), 7.664 (m; 1H; H-7), 7.761 (d; 1H; J=7.96Hz; H-4'), 7.83 (d; 2H; J=2.9Hz; H-12, H-6'), 8.094 (d; 1H; J=7.47Hz; H-5), 11.732 (s; 1H; H-1); δ_C (spectrum 5, 150 MHz, DMSO-d₆): 107.24 (C-3), 118.80 (C-4'), 123.25 (C-6), 124.65 (C-5, C-1'), 127.37 (C-2', C-6'), 128.96 (C-3', C-5'), 130.39 (C-8), 131.74 (C-7), 134.25 (C-2), 140.54 (C-9), 150.05 (C-10), 176.90 (C-4)

3.4.4.2 6-Hydroxyl-2-phenylquinolin-4(1*H*)-one (**8**)

Ethyl benzoylacetate (576.66 mg, 0.003 mole) was refluxed with 4-aminophenol (327.39 mg, 0.003 mole). Removal of benzene yielded a dark yellow oil, which was refluxed with diphenyl ether. The petroleum ether column was flushed with ethyl acetate after removal of diphenyl

ether and the crude product precipitated from toluene. After an acid base extraction the deep brown solid was taken to dryness and resuspended in toluene. Further purification was however necessary. A Discovery Solid Phase Extraction Tube, from SUPELCO, Sigma Aldrich, was utilised with positive pressure to retain the product (97% DCM, 3% MeOH), which was flushed from the tube with methanol to yield a still dark brown amorphous solid (Yield: 83 mg, 0.35 mmole, 11.66%). Further purification was unsuccessful.

Physical data

R_f = 0.71 (EtOH); C₁₅H₁₁NO₂; mp = 151°C; APCI-MS (300 °C, 7.0 V, 10 uA) *m/z*: (spectrum 8) 237.67 (M⁺); HR-MS (spectrum 9): calc. 237.25334, exp. 237.07833; *v*_{max} (spectrum 10, KBr, cm⁻¹): 3300.0, 3230.0, 3200.0, 3130.0, 3100.0, 3070.0, 2972.1, 2950.0, 1704.6, 1592.6, 1580.0, 1560.0, 1510.6, 1490.0, 1449.7, 832.8; δ_H (spectrum 11, 600 MHz, DMSO-d₆): 6.22 (s; 1H; H-3), 6.40 (d; 1H; J=8.03Hz; H-7), 6.45 (d; 1H; J=8.03Hz; H-8), 6.66 (d; 2H; J=8.08Hz; H-2', H-6'), 7.17 (m; 2H; J=8.08Hz; H3'; H5'), 7.41 (m; 1H; H-4'), 7.55 (s; 1H; H-5), 9.71 (s; 1H; OH-6); 11.61 (s; 1H; NH-1); δ_C (spectrum 12, 150 MHz, DMSO-d₆): 105.77 (C-3), 107.23 (C-4'), 115.14 (C-2', C-6'), 115.22 (C-7), 115.52 (C-8), 120.17 (C-3'), 120.60 (C-6), 121.98 (C-5'), 128.21 (C-1'), 128.98 (C-5), 131.47 (C-2), 140.67 (C-9), 148.21 (C-10), 186.23 (C-4)

3.4.4.3 7-Hydroxyl-2-phenylquinolin-4(1H)-one (9)

Ethyl benzoylacetate (576.66 mg, 0.003 mole) was refluxed with 3-aminophenol (327.39 mg, 0.003 mole). Evaporation of benzene yielded an amber coloured oil, which was refluxed with diphenyl ether. The diphenyl ether was removed from the mixture by employing column chromatography, eluting with petroleum ether. The product was further purified by acid-base extraction and silica column chromatography (97% DCM, 3% MeOH; R_f = 0.61, EtOH). Suspending the product firstly in toluene, then in ethanol yielded the pure product by filtration as a sand-coloured crystalline solid (Yield: 178 mg, 0.75 mmol, 25.0%).

Physical data

R_f = 0.61 (97% DCM, 3% MeOH); C₁₅H₁₁NO₂; mp = 267°C; APCI-MS (300 °C, 7.0 V, 10 uA) *m/z*: (spectrum 15): 237.62 (M⁺); HR-MS (spectrum 16): calc. 237.25334, exp 237.07854; *v*_{max} (spectrum 17, KBr, cm⁻¹): 3200.0, 3100.0, 3086.0, 3050.0, 3010.0, 2960.0, 2925.8, 2900.0, 1598.6, 1578.5, 1529.7, 1510.0, 1500.0, 1480.0, 1449.9, 1436.1, 1417.2, 852.6; δ_H (spectrum 18, 600 MHz, DMSO-d₆): 6.38 (s; 1H; H-3), 6.57 (d; 1H; J=5.01Hz; H-6), 7.151 (d; 1H; J=5.01Hz; H-5), 7.50 (s; 1H; H-8), 7.58 (m; 3H; H-2', H-4', H-6'), 7.83 (m; 2H; H3'; H5'), 12.16 (s; 1H; OH-7), 14.52 (s; 1H; NH-1); δ_C (spectrum 19, 150 MHz, DMSO-d₆): 105.86 (C-3), 107.50 (C-5), 108.02 (C-6), 127.34 (C-1'), 127.83 (C-3', C-5'), 129.17 (C-2',

C-6'), 131.02 (C-14), 133.58 (C-2), 133.99 (C-8), 141.39 (C-9), 151.76 (C-10), 161.0 (C-7), 182.15 (C-4)

3.4.4.4 8-Hydroxy-2-phenylquinolin-4(1H)-one (10)

Ethyl benzoylacetate (576.66 mg, 0.003 mole) was refluxed with 2-aminophenol (327.39 mg, 0.003 mole), after which the solvent was evaporated and the reaction heated at reflux with diphenyl ether. Removal of diphenyl ether from the reaction mixture was done as described above and the brown crude eluted from the column with ethanol. After acid-base extraction, precipitation of the pure product in ethanol yielded the pure product as a brown amorphous solid (Yield: 87 mg, 0.37 mmol, 12.2%).

Physical data

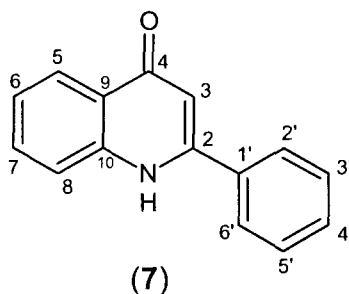
R_f = 0.767 (EtOH); C₁₅H₁₁NO₂; mp = 272°C; APCI-MS (300 °C, 7.0 V, 10 uA) *m/z*: (spectrum 22): 237.64 (M⁺), HR-MS (spectrum 23): calc. 237.25334, exp. 237.07957; ν_{max} (spectrum 24, KBr, cm⁻¹): 3293.7, 3110.0, 3100.0, 3050.0, 2970.0, 2900.0, 2550, 1781.6, 1620.0, 1573.4, 1514.4, 1482.7, 1447.8, 1409.3, 1350, 849.9; δ_H (spectrum 25, 600.17 MHz, DMSO-d₆): 6.54 (s; 1H; H-3), 7.10 (dd; 1H; J=7.25Hz; H-7), 7.19 (t; 1H; J=7.25Hz; H-6), 7.51 (m; 3H; H-2', H-4', H-6'), 7.56 (dd; 1H; J=7.25Hz; H-5), 7.88 (m; 2H; H3'; H5'), 10.57 (s; 1H; OH-8); δ_C (spectrum 26, 150.91 MHz, DMSO-d₆): 106.54 (C-3), 114.02 (C-5), 114.24 (C-7), 123.94 (C-6), 125.06 (C-1'), 127.54 (C-3', C-5'), 128.18 (C-8), 128.78 (C-2', C-6'), 130.06 (C-4'), 135.36 (C-2), 148.43 (C-9), 150.87 (C-10), 181.08 (C-4)

3.5 Results and Discussion

The chemical shifts reported by Gottlieb *et al.* (1997), for 300.1 MHz ¹H and 75.5 MHz ¹³C NMR was used for guidance in the interpretation of the ¹H and ¹³C NMR at 600.17 MHz and 150.91 MHz respectively. All spectra are presented in Appendix A.

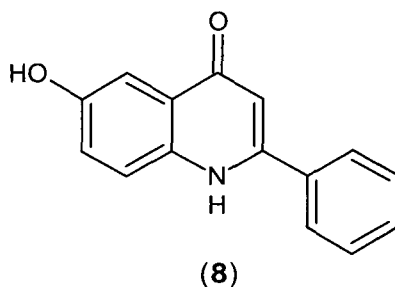
The low resolution atmospheric pressure chemical ionisation (APCI-MS) technique was used as the electron impact (EI-MS) technique appeared to be too harsh. As structure characterisation was done using more than one reliable method, namely NMR and IR the mother ion was distinguished without difficulty, to further confirm the identity of the compound.

3.5.1 2-Phenylquinolin-4(1H)-one (7)



2-Phenylquinolin-4(1H)-one (7) was successfully synthesised and confirmed by NMR (Spectra 4 to 7). The calculated mass, 221.25 and experimental mass, 221.08, measured by HR-MS was constant and confirmed the molecular mass of the compound. Infrared indicated the amine group at 3052.7 cm^{-1} and 1582.2 cm^{-1} and the carbonyl at 1733.8 cm^{-1} . The ^1H and ^{13}C NMR showed all the characteristic signals of the synthesised compound: a singlet at δ_{H} 11.73 ppm (Spectrum 4) was observed for the amine group NH-1 and a singlet at δ_{H} 6.34 ppm for H-3. The carbonyl carbon on C-4 was indicated at δ_{C} 176.9 ppm (Spectrum 5) and C-3 at δ_{C} 107.24 ppm. The aromatic region of the ^1H NMR could only be characterised by employing COSY and HSQC spectra (Spectra 6 and 7), since overlapping signals on the ^1H and ^{13}C NMR made characterisation uncertain. These NMR spectra are indicative of the 2-phenylquinolin-4(1H)-one (7) structure and gave confirmation of the compound. The signal at δ_{H} 2.5 ppm (Spectrum 4) was caused by DMSO- d_6 solvent, at δ_{H} 3.33 ppm by water and at δ_{H} 2.09 ppm by acetone. Their correlating signals at δ_{C} 30.5 ppm for acetone and δ_{C} 39.5 ppm for solvent (Spectrum 5) was also noted.

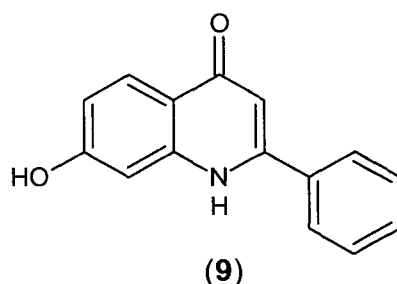
3.5.2 6-Hydroxy-2-phenylquinolin-4(1H)-one (8)



6-Hydroxy-2-phenylquinolin-4(1H)-one (8) was successfully synthesised and confirmed by NMR (Spectra 11 to 14). The calculated mass, 237.25 was comparable to that measured by HR-MS, 237.07833. The infrared spectrum indicated the amine group at 2972.1 cm^{-1} and 1510.6 cm^{-1} , the hydroxyl group at 3070.0 cm^{-1} and the carbonyl at 1704.6 cm^{-1} . The ^1H and ^{13}C NMR showed distinguishing signals at δ_{H} 11.61 ppm, for the NH-1 (spectrum 11), the

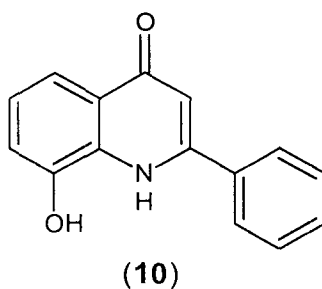
6-hydroxyl at δ_{H} 9.71 ppm and an H-3 singlet at δ_{H} 6.22 ppm. The carbonyl carbon was signified at δ_{C} 186.23 ppm (spectrum 12) and C-3 at δ_{C} 105.77 ppm. The aromatic region of the ^1H NMR was clarified by making use of COSY and HSQC NMR (spectra 13 and 14). These chemical shifts confirmed the presence of 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**). The signal at δ_{H} 2.5 ppm (spectrum 11) was caused by DMSO- d_6 solvent, at δ_{H} 3.33 ppm by water and at δ_{C} 39.52 ppm (spectrum 12) by DMSO- d_6 . DCM was responsible for the signals at δ_{H} 5.76 ppm and at δ_{C} 54.84 ppm, while ethanol was identified as δ_{H} 1.06 (t), 3.44 (q) and 4.63 (s) ppm and δ_{C} 56.07 ppm.

3.5.3 7-Hydroxy-2-phenylquinolin-4(1*H*)-one (**9**)



7-Hydroxy-2-phenylquinolin-4(1*H*)-one (**9**) was successfully synthesised and confirmed by NMR (Spectra 18 to 21). The experimental mass (237.07854), measured by HR-MS was comparable to the theoretical mass. Infrared showed the signals for the amine group at 3086.0 cm^{-1} and 1598.6 cm^{-1} , the hydroxyl at 2925.8 cm^{-1} , and the carbonyl at 1578.5 cm^{-1} . A singlet at δ_{H} 14.52 ppm (spectrum 18) was observed for the amine group NH-1, at δ_{H} 12.16 ppm for the hydroxyl OH-7 and a singlet at δ_{H} 6.38 ppm for H-3. The C-4 carbonyl was clearly visible at δ_{C} 182.15 ppm (spectrum 19) and C-3 at δ_{C} 105.86 ppm. The aromatic region of the ^1H NMR was characterised with the use of COSY and HSQC NMR spectra (spectra 20 and 21). These NMR spectra are indicative of the compound (**9**). The signals below δ_{H} 4.4 ppm (spectrum 18) and δ_{C} 57 ppm (spectrum 19) was the result of ethanol, water and DMSO- d_6 solvent.

3.5.4 8-Hydroxy-2-phenylquinolin-4(1*H*)-one (**10**)



8-Hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) was successfully synthesised and confirmed by NMR (Spectra 25 and 28). Infrared spectrometry indicated the amine group at 3293.7 cm⁻¹ and 1573.4 cm⁻¹, the hydroxyl at 3050.0 cm⁻¹ and the carbonyl at 1781.6 cm⁻¹. The calculated mass, 237.25 was confirmed by the experimental mass 237.07957. A singlet at δ_{H} 10.57 ppm (spectrum 25) was observed for the hydroxyl group on OH-8 and a singlet at δ_{H} 6.54 ppm for H-3. The carbonyl carbon on C-4 was indicated at δ_{C} 181.08 ppm (spectrum 26) and C-3 at δ_{C} 106.54 ppm. The ¹H NMR aromatic region was effectively interpreted by employing COSY and HSQC NMR (spectra 27 and 28). These NMR spectra gave confirmation of compound 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**). The signals obtained at δ_{H} 1.06 (t), 3.44 (q) and 4.63 (s) ppm and δ_{H} 3.33 ppm (spectrum 25) were produced by ethanol and water respectively, while the signal at δ_{H} 2.5 ppm was caused by the DMSO-d₆ solvent. The correlating signals were observed for ethanol at δ_{C} 18.51 and 56.07 ppm and for the solvent at δ_{C} 39.52 ppm (spectrum 26).

CHAPTER 4: OXYGEN RADICAL ABSORBANCE CAPACITY

4.1 Introduction

As a result of normal aerobic respiration, the body produces damaging free radicals, that if left unchecked lead to destruction of the lipid membrane, DNA and protein structures. This alters the functionality of the cell, ultimately leading to cell-death, which is one of the contributing factors in neurodegenerative disorders (Fu *et al.*, 1998). The oxygen radical absorbance capacity (ORAC) assay was developed to assess the ability of compounds to scavenge peroxy radicals, one of the main contributors to lipid peroxidation (Ou *et al.*, 2001). The assay utilises AAPH (2,2'-azobi(2-amidinopropane) dihydrochloride) to thermally induce peroxy radical generation at 37 °C (Ou *et al.*, 2001, Perez-Jimenez and Saura-Calixto, 2006). The fluorescent probe, fluorescein ($pK_a = 6.4$) is oxidised by the peroxy radicals present in the assay, causing its fluorescent intensity to diminish (Ou *et al.*, 2001). Inhibition of fluorescent decay therefore indicates the ability of the tested antioxidant to scavenge peroxy radicals, protecting fluorescein. A Trolox standard curve was generated, allowing for the measurement of the area under the curve (AUC) and the expression of the values obtained as Trolox equivalents. A blank solvent sample was performed in parallel to allow for the effect of the solvent and all other reagents in this assay, to be subtracted from the measurements obtained for the test compounds (Ou *et al.*, 2002; Perez-Jimenez and Saura-Calixto, 2006). Since ORAC measures both reaction time and the extent of the reaction in hydrophilic conditions (Ou *et al.*, 2001), the kinetic observation was expressed as the area under the fluorescence decay curve (AUC). The AUC of the test compound indicates the ability of the antioxidant to scavenge peroxy radicals when compared to the AUC of the blank, by lengthening the plateau phase observed in figure 4.1.

In this experiment fluorescein was measured at 485/530 nm excitation and emission. The pH-sensitive fluorescent probe undergoes a significant decrease in fluorescence in acidic media, therefore the use of phosphate buffer, pH 7.4 was compulsory to maintain fluorescence, as the assay was performed in DMSO with slightly acidic test compounds. DMSO was used successfully by Kurilich *et al.* (2002) as a solvent, to assist in the aqueous dissolution of samples, though more lipophilic compounds warrant the use of randomly methylated β -cyclodextrin (RMCD) to enable aqueous dissolution and accurate testing in this hydrophilic assay (Huang *et al.*, 2002).

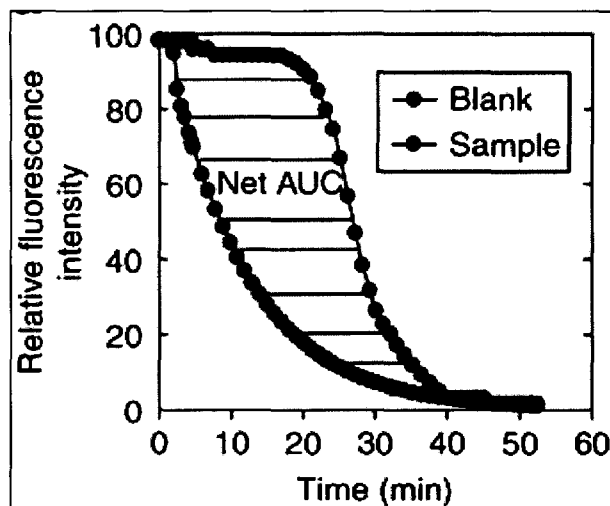


Figure 4.1 Schematic representation of the Net Area Under the Curve

The underlying reaction in the ORAC assay is a hydrogen transfer reaction (Ou *et al.*, 2001) and according to Prior *et al.* (2005), should therefore not be affected by organic solvent; it has however been reported that organic solvents give falsely high ORAC-values when this effect is not incorporated into the standard curve (Fernández-Pachón *et al.*, 2004; Villaño *et al.*, 2005 and Zhou & Yu, 2004). Perez-Jimenez and Saura-Calixto (2006), found a direct relationship between high ORAC-values and the apolarity of the solvent and Pedrielli *et al.* (2001) and Pinelo *et al.* (2004) stated that polar solvents may in fact hinder hydrogen donation, due to hydrogen bonds formed in the polar medium. Nonetheless phenolic antioxidants exhibit good activity in this assay as the phenol donates a hydrogen atom to quench the peroxy radical, producing a stable phenoxyl radical (Huang *et al.*, 2002). Ou *et al.* (2001) demonstrated the oxidation of fluorescein (FL) in equation 4.1, thereby illustrating the hydrogen donor mechanism of action. The ORAC assay generates peroxy (ROO^{\bullet}) radicals with AAPH, which abstract a hydrogen atom from the phenolic antioxidant (ArOH) (Eq 4.2), more rapidly than from the normal substrate fluorescein, resulting in a stable phenoxyl radical (ArO^{\bullet}) and unoxidised fluorescein: The stable phenoxyl radical (Eq 4.2) does not further partake in oxidative reactions, thereby terminating the lipid peroxidation chain reaction (Ou *et al.*, 2002).



As the oxidation of fluorescein is visible as fluorescent decay over time, the reduction in decay, or length of the plateau phase (Fig 4.1) can be measured to estimate the ability of the antioxidant to scavenge peroxy radicals.

4.2 Experimental

The ORAC assay was developed by Ou *et al.* (2001), using fluorescein as the fluorescent probe to measure fluorescent decay caused by AAPH-induced peroxy radicals. A Trolox standard was used to calibrate the assay, allowing for expression of results in Trolox equivalents (μM) per litre sample.

4.2.1 Materials and Methods

4.2.1.1 Chemicals

Dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), fluorescein (3,6'-dihydroxy-spiro(isobenzofuran-1(3*H*),9'(9*H*)-xanthen)-3-one) disodium salt, flavone, 6-hydroxyflavone and 7-hydroxyflavone were purchased from Sigma Aldrich, St. Louis, MO and 4-hydroxyquinoline from Aldrich Chemical Company, Milwaukee, WIS, USA. Dimethyl sulphoxide (DMSO) was purchased from Saarchem (PTY) Ltd., Wadeville, and quinoline from Saarchem (PTY) Ltd., Muldersdrift, South Africa. 8-Hydroxyquinoline was obtained from Merck, Darmstadt and 2,2'-azobis(2-amidinopropane)-dihydrochloride (AAPH) and (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from Sigma Aldrich, Steinham, Germany. All the above chemicals, save DMSO and the test compounds, were generously donated by the Department of Biochemistry, NWU, Potchefstroom Campus.

4.2.1.2 Reagents

Phosphate buffer, 75 mM, was prepared by diluting 1 M K_2HPO_4 with 1 M KH_2PO_4 aqueous solution in a 61.6:38.9 v/v ratio and further diluting it with Milli-Q water to 75 mM. The pH was adjusted to 7.4 and the buffer was stored in the fridge. A 265 mM fluorescein stock solution was prepared, which was further diluted with buffer before each assay to yield a 112 nM working solution. The 72 mM AAPH solution was prepared with buffer immediately before use and kept on ice, while a sufficient quantity of the 250 μM Trolox standard solution was prepared before each assay, dissolving Trolox in DMSO by tip-sonication for two minutes.

4.2.2 Sample Preparation

The water insoluble test compounds were dissolved in DMSO to yield final concentrations of 0.1 mM, 0.01 mM and 0.001 mM in the well-plate in 10% DMSO. According to Prior *et al.* (2003), DMSO acts as an antioxidant and was included in the blank to correct this effect.

4.2.3 Instrumentation

The ORAC assay was performed at 485 nm excitation and 530 nm emission wavelengths in static mode on a BioTek FL600 plate reader, using KC4 software. The sensitivity of the well plate was determined before initiation of the reaction, in order to ascertain that emission values fell in the range the plate reader was able to measure.

4.2.4 ORAC assay

A Trolox standard curve (Fig 4.2) was generated by further diluting the Trolox working solution with DMSO to yield 20 μ l standard solutions in a final 10% DMSO concentration in the first row of an opaque 96-well plate. Concentrations of 0, 2.5, 5, 10, 15 and 20 μ M were tested in duplicate, in wells next to each other, since it was found that the Biotek plate reader gave unreliable measurements in the outer columns and bottom row of the well plate. Therefore, applying the 'forward-then-reverse' method of Ou *et al.* (2001), would result in two unreliable values for the same sample, or in the case of the standard, two unreliable blank values, jeopardising the data of the entire plate. A multi-channel pipette was used to add 80 μ l of the 112 nM fluorescein solution to each well. The computer protocol was started with the sensitivity set at 146. The first measurement of the wells, containing both standard and test compounds, was taken and the relative fluorescence units (RFU) established to be approximately 65 000. The sensitivity was therefore not adjusted and the reaction started by adding 100 μ l of 72 mM AAPH solution, yielding a final 36 mM concentration in the well. The computer protocol was started anew and the fluorescent decay measured at 5 minute intervals over a period of 3 hours.

A second order polynomial slope was obtained for the standard curve, enabling us to use the area under the curve (AUC) to determine the Trolox equivalents of the assayed compounds. The standard curve was forced through zero to simplify calculations. The test compounds were diluted to give 20 μ l volumes with final concentrations of 0.1 mM, 0.01 mM and 0.001 mM in 10% DMSO, and were placed in the opaque well plate in triplicate, next to each other. The assay was further performed as for the standard.

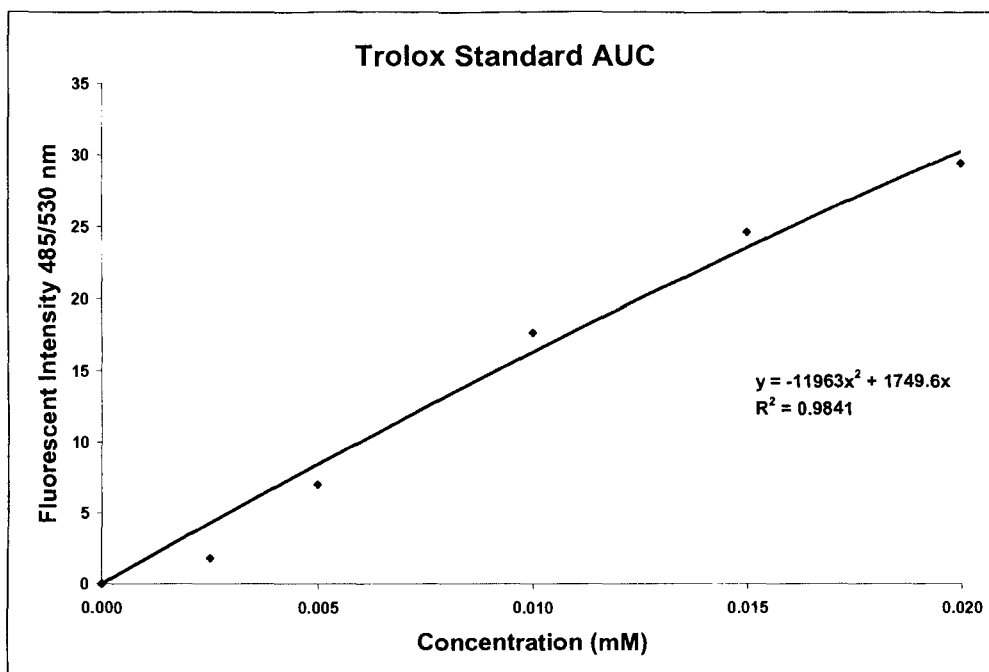


Figure 4.2 Regression displaying Fluorescent Decay in the presence of Trolox Standard Concentrations

4.2.5 Data Collection

The formulae utilised by Cao *et al.* (1999), were applied to obtain the ORAC-values as micromoles Trolox equivalents per litre sample. The fluorescent decay measurements obtained for the test compounds over 3 hours were interpolated into the AUC formula (Eq 4.3), to calculate the area under the fluorescent decay curve (Ou *et al.*, 2001), where f_0 is the initial fluorescence at 0 minutes, f_n the fluorescence value at time n and CT the cycle time in minutes, (in this case 5 minutes), thereby expressing all measurements relative to the initial measurement:

Equation 4.3
$$\text{AUC} = (0.5 + f_5 / f_0 + f_{10} / f_0 + f_{15} / f_0 + \dots + f_{65} / f_0 + f_{70} / f_0) \times \text{CT}$$

Equation 4.4
$$y = ax^2 + bx + c$$

Equation 4.5
$$x = -b + \sqrt{[b^2 - 4a(c-y)] / 2a}$$

The net area under the fluorescent decay curve (y-value) was obtained by subtracting the blank AUC-value from the test compound AUC-values, and used in the second order polynomial regression equation, where $c = 0$ when the equation is forced through

zero (Eq 4.4, Fig 4.2). The relative ORAC-value (x-value, Eq 4.5) was then calculated in Trolox equivalents per litre sample ($\mu\text{M/L}$) (Ou *et al.*, 2001).

4.2.6 Statistical Analysis

The data was captured in Microsoft Excel and the mean of three values converted to ORAC-value \pm S.D. Statistical acceptability was determined at a relative standard deviation of less than 5%. Further statistical analysis was performed using a Paired Student-Newman Keuls t-test on GraphPad Prism.

4.3 Results

The ORAC-values indicated the relative protection of fluorescein by the sample antioxidants compared to that afforded by Trolox, by integrating into the Trolox regression equation (Fig 4.2) (Kuti, 2004). Higher ORAC-values indicated prolonged fluorescence and protection of fluorescein against oxidation.

Table 4.1 Relative ORAC-values obtained in the presence of Quinolines, Flavones and 2-Phenylquinolin-4(1*H*)-ones (N=3).

Antioxidant	ORAC-value (μM Trolox Equivalents/L)		
	\pm SEM		
	0.001 mM	0.01 mM	0.1 mM
Quinoline (1)	1.396 \pm 0.091	-20.59 \pm 0.049	-2.761 \pm 1.169
4-Hydroxyquinoline (2)	1.495 \pm 0.256	-0.714 \pm 0.758	-2.229 \pm 0.928
8-Hydroxyquinoline (3)	10.226 \pm 2.443	30.463 \pm 0.027	55.417 \pm 2.165
Flavone (4)	-0.602 \pm 0.459	1.507 \pm 0.386	2.396 \pm 1.333
6-Hydroxyflavone (5)	21.443 \pm 1.042	37.074 \pm 1.068	59.021 \pm 4.140
7-Hydroxyflavone (6)	-10.41 \pm 0.751	-18.23 \pm 0.542	-19.36 \pm 0.306
2-Phenylquinolin-4(1 <i>H</i>)-one (7)	-4.553 \pm 0.890	-1.773 \pm 0.071	1.911 \pm 1.263
6-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (8)	12.276 \pm 0.568	29.011 \pm 1.294	58.288 \pm 1.269
7-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (9)	15.273 \pm 0.911	26.406 \pm 0.832	57.514 \pm 3.343
8-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (10)	9.184 \pm 0.4451	26.069 \pm 0.556	65.394 \pm 0.911

4.4 Discussion

Peroxyl radicals are one of the key radicals in lipid peroxidation responsible for the propagation of oxidation, causing destruction of lipid membranes and compromising the integrity of the cell (Markesbery *et al.*, 2001). Inhibition of this radical would therefore be beneficial in diseases where radical-mediated damage is observed, for example in neurodegenerative disorders (Fu *et al.*, 1998). The oxygen radical absorbance capacity (ORAC) assay determined the ability of test compounds to scavenge AAPH-induced peroxyl radicals, thereby protecting fluorescein from oxidation and prolonging its fluorescence (Kuti, 2004). AAPH is a thermolabile compound used for peroxyl radical production in this assay, therefore the AAPH solution was prepared directly before use and kept on ice. The quinolines, flavones and 2-phenylquinolin-4(1*H*)-ones were evaluated to assess oxygen radical absorbance capacity at 5 minute intervals over a 3 hour period and the respective ORAC-values are displayed in figure 4.3. The fluorescent decay curves (Appendix B) demonstrated the ability of the test compounds to scavenge peroxyl radicals as relative fluorescent units (RFU) over time.

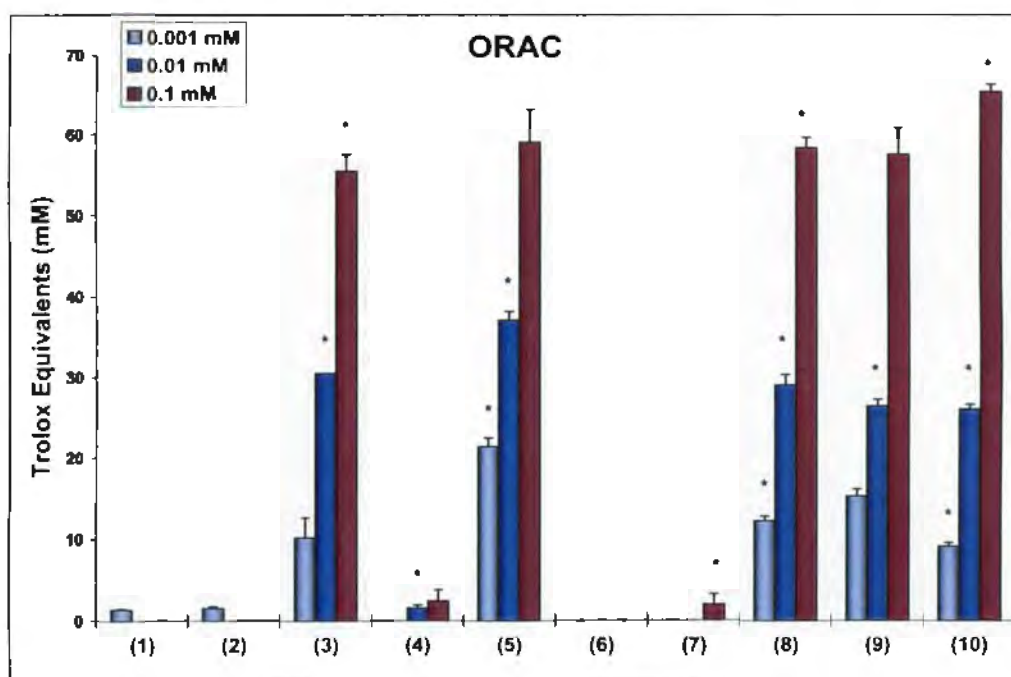


Figure 4.3 ORAC-values obtained for all the test compounds at three concentrations expressed as Trolox Equivalents per litre sample; *RSD<5%

To calculate the ORAC-value, which encompassed both time and the extent of reaction in one single value, the AUC of the blank was subtracted from the calculated AUC-value of the sample and interpolated into the Trolox regression equation, forced through zero (Ou *et al.*, 2001). Negative ORAC-values were obtained in table 4.1 when the blank AUC had a greater value than that of the sample AUC. The compounds and concentrations that produced negative ORAC-values were omitted in the above figure 4.3, as negative values indicated the absence of peroxy scavenging activity. Conversely, the fluorescent decay curves (Appendix B) together with figure 4.3, demonstrated that the 0.01 mM and 0.1 mM concentrations of 8-hydroxyquinoline (3), 6-hydroxyflavone (5) and 6-, 7- and 8-hydroxy-2-phenylquinolin-4(1*H*)-one (8 to 10) did not show the standard decrease in fluorescence to 5% of the initial value (Cao and Prior, 1999), indicating that either the reaction time was too short, or more likely, as there was no such problem observed for the other test compounds, the drug concentrations were too high, overpowering AAPH and causing near continuous protection of fluorescein. This observation led to the conclusion that these compounds were superior to the other compounds, which demonstrated normal decay at the same concentrations. This fact however makes statistical analysis of the different compounds compared to one another a pointless exercise, since all compounds did not reach the same endpoint and could therefore not be compared effectively. It was however possible to compare the 0.001 mM concentration of all the compounds, as they appeared to allow appropriate decay (Fig 4.4) as well as the 0.1 mM concentration of flavone (4) and 2-phenylquinolin-4(1*H*)-one (7) (Fig. 4.5). It appears that in figures 4.4 and 4.5, none of the test compounds came close to the peroxy scavenging activity displayed by Trolox, considering that none of the compounds displayed a plateau region in the fluorescent decay curves as observed for Trolox (Fig 4.6 and Appendix B), where the plateau region lengthened in relation to the increased concentration of Trolox, thereby decreasing the rate of decay (Prior *et al.*, 2003). After the plateau, fluorescein was decayed as was observed for the blank, indicating the time and extent of peroxy radical scavenging activity of Trolox.

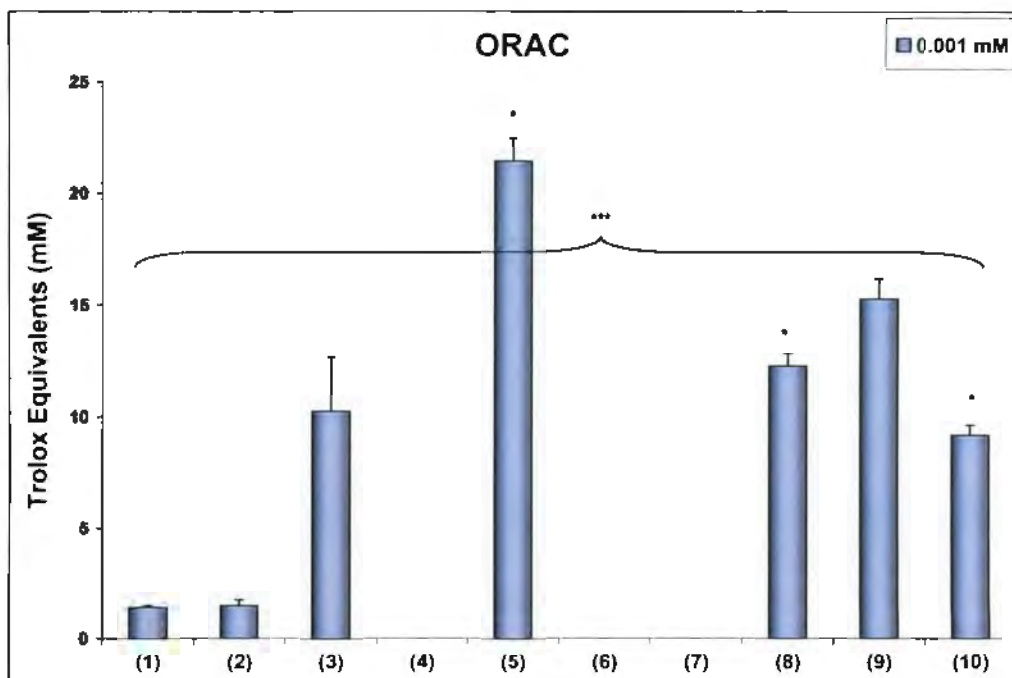


Figure 4.4 The ORAC-values obtained for all test compounds at 0.001 mM concentration, expressed as Trolox Equivalents per litre sample; *RSD<5%; ***p<0.0001 vs. 6-hydroxyflavone (5) (Paired t-test).

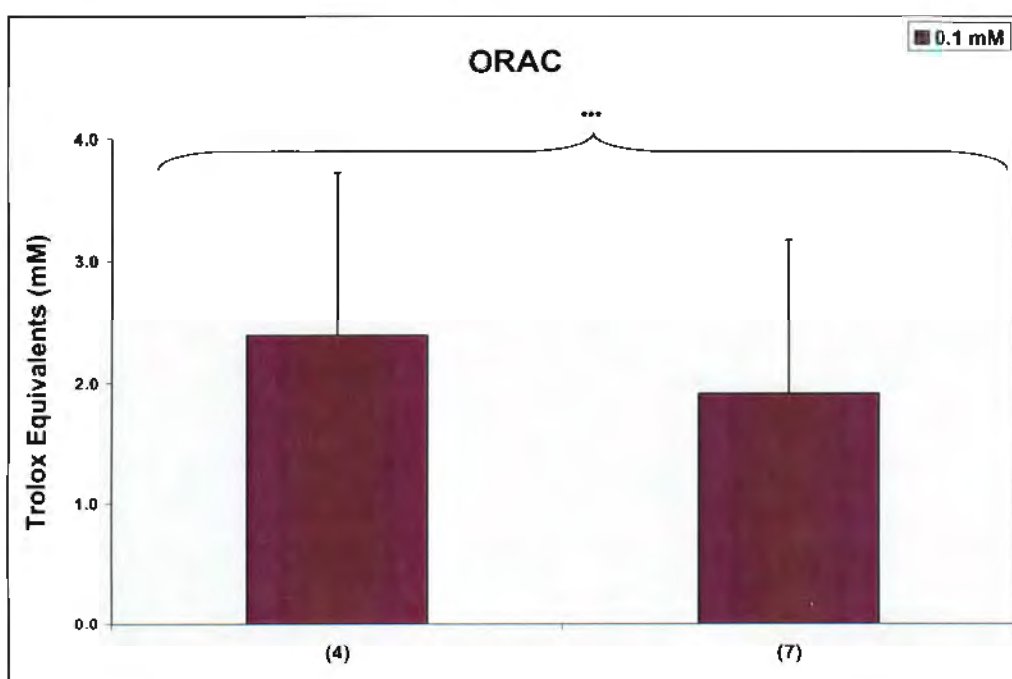


Figure 4.5 The ORAC-values obtained for flavone (4) and 2-phenylquinolin-4(1*H*)-one (7) at 0.1 mM concentration, expressed as Trolox Equivalents per litre sample; ***p<0.0001 vs. 0.001 mM 6-hydroxyflavone (5) (Paired t-test).

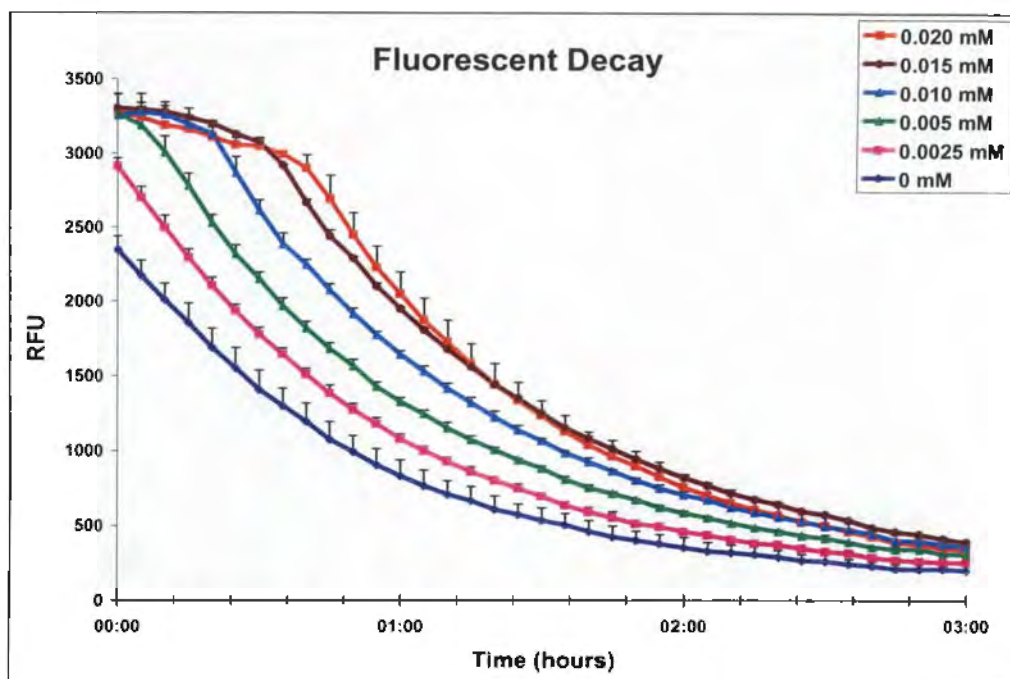


Figure 4.6 Fluorescence Decay observed for Trolox Standard in the presence of AAPH

From figure 4.4 it is clear that flavone (4), 7-hydroxyflavone (6) and 2-phenylquinolin-4(1*H*)-one (7) did not show peroxy scavenging ability at 0.001 mM concentration, while quinoline (1) and 4-hydroxyquinoline (2) demonstrated low activity. This fact was confirmed by the fluorescent decay curves (Appendix B), as it showed these compounds to perform closely to, or worse than the blank used in the Trolox decay curve (Fig 4.5). In figure 4.5 the 0.1 mM concentration of flavone (4) and 2-phenylquinolin-4(1*H*)-one (7) also displayed low activity, with flavone showing increased activity. In general, the hydroxylated compounds had higher values than the unsubstituted compounds, except for 7-hydroxyflavone (6), which performed the worst of all the test compounds (table 4.1). In contrast 6-hydroxyflavone (5) demonstrated the best peroxy scavenging activity at 0.001 mM ($p < 0.0001$). 4-Hydroxyquinoline (2) however showed relatively poor activity, comparable to the unsubstituted quinoline (1). The position of the hydroxyl thus seems to be an important factor. It was further noted that the activity of the 8-hydroxyl substituted 2-phenylquinolin-4(1*H*)-one (10) and quinoline (3) were comparable at 0.001 mM concentration, indicating that the mechanism of action was centred on the 8-hydroxyl substitution and not the amine group. The same can however not be said of the 6- and 7-hydroxyl substitutions, where either the flavones (5 and 6) or the 2-phenylquinolin-4(1*H*)-one (8 and 9) performed better than its counterpart. It is not clear why this inconsistency should be observed between the flavones and 2-phenylquinolin-4(1*H*)-ones. Clearly the position of the hydroxyl together with the type of

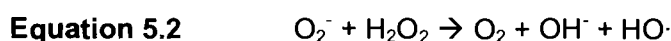
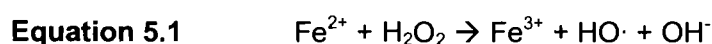
functional group in the ring had an effect on the proton donating ability of the compound. Solubility or stability in solution over the 3 hour period might also have been a contributing factor to the low activity of 7-hydroxyflavone (**6**). In general the hydroxylated 2-phenylquinolin-4(1*H*)-ones (**8** to **10**) as a group showed relatively good activity. The hydroxyl substitution was able to donate a proton in this hydrogen donor transfer mechanism to deactivate the peroxy radical and form a stable phenoxyl radical (Thomas, 1994; Silva, 2009). The unsubstituted compounds all had poor activity, indicating the importance of the hydroxyl substituent. Notably, 2-phenylquinolin-4(1*H*)-one (**7**) performed the worst of the unsubstituted compounds (table 4.1), but also showed the greatest increase in activity with hydroxyl substitution. This low radical absorption capacity of 2-phenylquinolin-4(1*H*)-one (**7**) is a clear indication that the amine functionality does not act as a hydrogen donor according to the suggested mechanism of action. The structure activity relationship for the 2-phenylquinolin-4(1*H*)-ones (**8** to **10**) were however established to be enhanced in the order: 7>6>8-hydroxyl substitution. This might be explained by the fact that the 7-hydroxyl is *para* to the C-4 carbonyl group, which by an –M effect, could be responsible for increasing the compound's ability to stabilise the phenoxyl radical. It is however not clear why the same tendency was not observed for the 7-hydroxyflavone (**6**). As this assay was performed at a pH of 7.4, the amine group might have dissociated as a weak base, causing the conjugated anionic nitrogen not to act as a proton donor. Therefore the amine-containing quinolines and 2-phenylquinolin-4(1*H*)-ones did not show as prominent peroxy scavenging activity in this assay as 6-hydroxyflavone (**5**) and could probably be correlated to the behaviour of the compounds at physiological pH. In this case, the amine and carbonyl groups would have an impact on the ionisability of the phenol in the 6-, 7- and 8-hydroxyl substituted compounds.

The *in vitro* 'total antioxidant capacity' of the compounds was thus confirmed in this experiment. It will however be of great value to establish the effect of these compounds on biological systems *in vivo*, since there are many factors to be taken into account, not all of which can be simulated in an entirely chemical scavenging assay. It is also possible that the phenolic compounds were hindered in this hydrophilic assay from donating protons, yielding lower values than could be expected, since hydrogen bonds formed in the aqueous medium might have interfered with hydrogen atom transfer (Pedrielli *et al.*, 2001; Pinelo *et al.*, 2004).

CHAPTER 5: FERRIC REDUCING/ANTIOXIDANT POWER

5.1 Introduction

Reactive oxygen species overproduction in the elderly as a consequence of respiration is believed to contribute to ageing and age-related disorders (Fu *et al.*, 1998). When hydrogen peroxide (H_2O_2) is enzymatically produced in the cell certain enzymes and elements assist in the breakdown of hydrogen peroxide to either metabolically safe or harmful products (Fig 2.3). Reduced ferrous ions (Fe^{2+}) catalytically oxidise hydrogen peroxide to the destructive hydroxyl radical ($\text{HO}\cdot$) in the Fenton reaction (Eq 5.1). The ferric ions (Fe^{3+}) in turn catalyse further oxidation of hydrogen peroxide in the Haber-Weiss reaction (Eq 5.2) (Markesbery *et al.*, 2001):



The antioxidant effect obtained by limiting these two reactions by removing the catalysts involved would be significant and the ability to reduce ferric ions to its ferrous form will effectively inhibit the Haber-Weiss reaction. However at high concentrations antioxidants may in effect drive the Fenton reaction, producing more hydroxyl radicals (Carr and Frei, 1999). As a result, it is necessary to use various biological evaluations to assess the antioxidant effects of test compounds at certain concentrations.

Non-enzymatic antioxidants are responsible for inhibiting oxidative reactions 5.1 and 5.2 by being oxidised itself, reducing the oxidant and rendering it unable to cause further oxidation (Benzie and Strain, 1996). The oxidation and reduction of the oxidant and antioxidant may be classified as redox reactions (Eq 5.3 and 5.4), in which case the antioxidant acts as a reductant (Benzie and Strain, 1996). To assess the reduction ability, the ferric reducing/antioxidant power (FRAP) assay, a purely chemical evaluation, was done to establish the chemical ability of the test compound to reduce ferric iron. This method however does not necessarily indicate good antioxidant activity, or even inhibition of the Haber-Weiss reaction *in vivo* (Halliwell and Gutteridge, 1995), since there are many factors that may influence antioxidant capacity in biological matter. Benzie and Strain (1996), developed a colorimetric method based on the ability of the test compound to reduce the ferric-tripyridyltriazine complex (Fe^{3+} -TPTZ) to its blue, ferrous form (Fe^{2+} -TPTZ), at 37 °C in a non-physiological environment of pH 3.6. This reaction is a one electron exchange transfer reaction (Ou *et al.*, 2002), Equation 5.3, with the rate-limiting factor the reducing, or electron donating ability of the antioxidant sample (ArOH). Ultimately reaction 5.3 leads to a second

electron transfer reaction, Equation 5.4, producing the stable antioxidant radical (ArO[•]). In the FRAP assay the radical donor (ROO[•]) is Fe³⁺-TPTZ and the anion (ROO⁻) is Fe²⁺-TPTZ (Ou *et al.*, 2002):



Polyphenols are chain-breaking antioxidants (Ou *et al.*, 2002) that function by scavenging radicals. The phenol moieties (Thomas, 1994) of the hydroxyl substituted test compounds, act as electron donors, driving the reduction reaction, while the aromatic ring of the phenolic antioxidant acts to stabilise the oxygen radical produced (ArO[•]), thereby increasing reducing ability of the antioxidant (ArOH) (Silva, 2009). It is hypothesised that the 2-phenylquinolin-4(1*H*)-ones will be superior to flavones due to the extra electron donor group, the amine. However electron donating ability is directly affected by the surrounding pH and solvent (Perez-Jimenez and Saura-Calixto, 2006); DMSO, for instance may hinder electron donation, since it does not contain a hydrogen atom able to stabilise the oxygen ion (ArO[•]) by hydrogen bonding, as is the case in aqueous media (Silva, 2009). The acidic pH of this method also interferes with the single electron transfer reaction (Ou *et al.*, 2002), thereby affecting the antioxidant results obtained for the test compounds. Iron chelation activity of the test compounds may also influence the availability of the ferric radical in this assay, thereby giving falsely high reducing values (Benzie and Strain, 1996). It is therefore necessary to further study this aspect of the test compounds to rule out interference.

The commercially available quinolines (**1** to **3**) and flavones (**4** to **6**) were assayed together with the synthesised 2-phenylquinolin-4(1*H*)-ones (**7** to **10**) to establish the ferric reducing ability of the quinolones compared to that of the natural antioxidant flavones. Quinoline is the skeleton of the 2-phenylquinolin-4(1*H*)-one structures and was assayed to establish ferric reducing structure-activity relationships. The assay was performed over a period of 33 minutes (Pulido *et al.*, 2000), to allow the reaction to reach an endpoint, since it has been shown by Benzie and Strain (1996), that not all antioxidants are equally rapid or effective ferric reductants over time. However, as it was proven in this assay and confirmed by Pulido *et al.* (2000), not all compounds reach an endpoint by this time. Ou *et al.* (2002), is of the opinion that the FRAP reaction 'is too slow to be of any practical use', since it is difficult to compare antioxidants' total ferric reducing abilities when the endpoints of the reactions have not yet been reached. In this experiment the data obtained at 33 minutes was used to compare the final reducing ability of the test compounds to that of Trolox, a well known, water soluble vitamin E derivative. Absorbance values were taken at 6 minute intervals,

starting at 3 minutes after initiating the FRAP reaction, to illustrate the reduction kinetics of the test compounds. The method as described by Benzie and Strain (1996) was used with the exception of a Trolox standard. The reduction power or FRAP-value was obtained by converting the mean 0.1 mM Trolox absorbance value at 33 minutes to the 100% Trolox equivalent, as this concentration yielded the highest value in the experiment at the fixed end time. The results were consequently expressed as percentage 0.1 mM Trolox equivalents. The effect of the organic solvent used for dilution of water insoluble test compounds, was countered by incorporating the same amount of organic solvent in the standard as in the samples (Pulido *et al.*, 2000).

5.2 Experimental

The method described by Benzie and Strain (1996), was used with the adaptation of a Trolox standard employed to enable expression of data as percentage Trolox equivalents. A blank was deducted from all measured absorbance values to correct the effect of the well plate, solvents and other reagents present in the assay.

5.2.1 Materials and Methods

5.2.1.1 Chemicals

Glacial acetic acid, hydrochloric acid and dimethyl sulphoxide (DMSO) was purchased from Saarchem (PTY) Ltd., Wadeville, while quinoline was purchased from Saarchem (PTY) Ltd., Muldersdrift, South Africa. Sodium acetate trihydrate (NaAc.3H₂O) was purchased from BHD, Darmstadt, whereas iron(III)chloride hexahydrate (FeCl₃.6H₂O) and 8-hydroxyquinoline was purchased from Merck, Darmstadt and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from Sigma Aldrich Chemical Corporation, Steinheim, Germany. Flavone, 6-hydroxyflavone, 7-hydroxyflavone and 2,4,6-tripyridyl-s-triazine (TPTZ) were obtained from Sigma Aldrich, St. Louis, MO and 4-hydroxyquinoline from Aldrich Chemical Company, Milwaukee, WIS, USA. All the above chemicals, save DMSO and the test compounds, were generously donated by the Department of Biochemistry, NWU, Potchefstroom Campus.

5.2.1.2 Reagents

Acetate buffer (300 mM) was prepared by dissolving 1.55 g sodium acetate (NaAc.3H₂O) in 8 ml glacial acetic acid and diluting it to 500 ml with double distilled water. The pH was adjusted to 3.6. A 40 mM hydrochloric acid (HCl) solution was prepared by adding 200 µl concentrated HCl to 50 ml double distilled water. A 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) solution was prepared by dissolving 31.24 mg TPTZ in 10 ml of 40 mM HCl solution. A 20 mM iron(III)chloride hexahydrate (FeCl₃.6H₂O) solution was prepared by adding 4.56 mg FeCl₃.6H₂O to 10 ml acetate buffer. The FRAP reagent was constituted of 25 ml acetate

buffer, 2.5 ml TPTZ solution and 2.5 ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution and was mixed directly before addition to the well-plate, given that crystallization takes place rapidly. Double distilled water was used throughout this experiment.

5.2.1.3 Sample Preparation

The Trolox standard, as well as the test compounds was prepared in DMSO to yield final concentrations of 0.1 mM, 0.01 mM and 0.001 mM, in a final 10% DMSO concentration.

5.2.1.4 Instrumentation

Absorbance values were measured at 595 nm with a BioTek Synergy HT Reader, using Gen5 1.05 software.

5.2.2 FRAP Assay

The 0.1 mM, 0.01 mM, 0.001 mM and 0 mM Trolox standards were prepared in a 1:9 ratio with double distilled water to yield a volume of 100 μl in each well of a transparent 96-well plate. The wells containing the same concentration were placed next to each other in triplicate, since it was found that the plate reader gave unreliable measurements of the outer columns and bottom row of the well plate. The use of the 'forward-then-reverse' method (Ou *et al.*, 2001) would therefore not be suitable in this instance. The FRAP reagent was constituted and added with a multi-channel pipette to the wells; 125 μl was added to all wells, followed by a second 125 μl addition, yielding a final 250 μl in each well. Care was taken to start the Gen5 1.05 computer protocol at exactly 1.5 minutes after the first row of the well plate was filled with the first 125 μl of FRAP reagent. This time dependence was implemented to enhance the reproducibility of the assay. The FRAP protocol had a lag time of 3 minutes at 37 °C before the first absorbance measurement was taken at 595 nm, followed by measurements at 6 minute intervals for a period of 33 minutes. The Trolox standards are presented in figure 5.1 and clearly indicate the 3 to 4 fold increase in activity of the 0.1 mM series ($***p < 0.0001$) and yielded a higher FRAP-value than the test compounds; therefore the absorbance mean of 0.1 mM Trolox at 33 minutes was converted to the 100% Trolox equivalent FRAP-value.

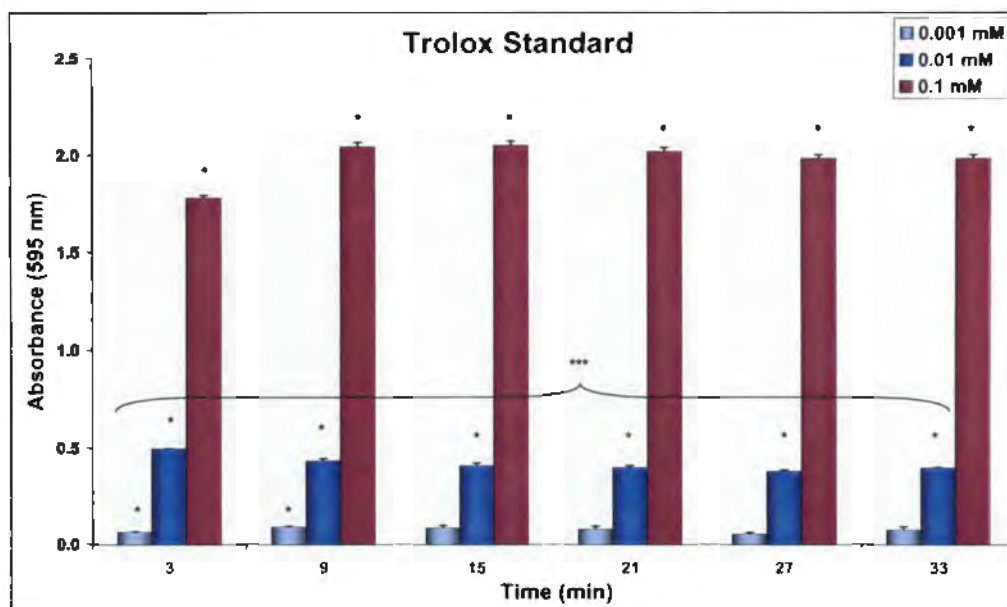


Figure 5.1 The absorbance values at 595 nm of three concentrations of Trolox over a period of 33 minutes. Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox (Paired t-test)

5.2.3 Data Collection

After subtracting the blank-value obtained at 595 nm, the absorbance values for all test compounds were expressed as percentage Trolox equivalents, employing the 0.1 mM Trolox standard value at the fixed time of 33 minutes as a 100% reference (Fig 5.1). The blank corrected for the absorbance of the well plate, solvent and all other reagents used in this assay.

5.2.4 Statistical Analysis

The assay was performed in triplicate over a period of 6 time intervals to yield a total of 18 absorbance measurements per concentration series. The series was analysed statistically against the 0.1 mM Trolox standard series in a Paired Student-Newman Keuls t-test on GraphPad Prism with significant difference indicated at $p < 0.05$. Three series, or three concentrations, per test compound were illustrated together to enable comparison (Appendix C); the data was expressed as the mean of three values \pm S.D. at 6 time intervals. Standard deviation (S.D.) and relative standard deviation (R.S.D.) was calculated in Microsoft Excel and were statistically acceptable at R.S.D. < 5%.

5.3 Results

The test compounds were assayed for the ability to reduce ferric-TPTZ; higher absorption values of the blue ferrous compound therefore indicated stronger ferric reducing ability. The means \pm S.D., in table 5.1, were obtained for all compounds in three concentrations over 33 minutes. The absorbance values were expressed at each time interval as a percentage Trolox equivalent (table 5.1 and Fig 5.2).

Table 5.1 FRAP Values obtained over 33 minutes for Quinolines, Flavones and 2-Phenylquinolin-4(1*H*)-ones (N=3)

Test Compound (mM)	FRAP value (% Trolox Equivalents) \pm S.D.					
	at Time (min)					
	3	9	15	21	27	33
Quinoline (1)						
0.001	0.28 \pm 0.11	0.98 \pm 0.14	1.41 \pm 0.25	1.39 \pm 0.29	2.04 \pm 0.21	1.97 \pm 0.21
0.01	0.48 \pm 0.00	1.21 \pm 0.04	0.98 \pm 0.36	1.92 \pm 0.53	2.19 \pm 0.57	2.42 \pm 0.57
0.1	0.08 \pm 0.00	0.53 \pm 0.21	0.88 \pm 0.14	0.83 \pm 0.21	1.08 \pm 0.14	0.98 \pm 0.11
4-Hydroxyquinoline (2)						
0.001	1.39 \pm 0.21	2.72 \pm 0.32	2.47 \pm 0.18	2.42 \pm 0.11	2.80 \pm 0.07	2.82 \pm 0.36
0.01	1.13 \pm 0.14	2.19 \pm 0.07	1.82 \pm 0.53	2.57 \pm 0.25	2.62 \pm 0.32	1.69 \pm 0.53
0.1	0.03 \pm 0.14	0.15 \pm 0.82	0.38 \pm 1.00	0.66 \pm 1.68	1.44 \pm 0.29	0.23 \pm 0.61
8-Hydroxyquinoline (3)						
0.001	0.66 \pm 0.04	1.49 \pm 0.21	2.42 \pm 0.18	2.72 \pm 0.18	2.55 \pm 0.29	2.65 \pm 0.04
0.01	0.66 \pm 0.25	1.87 \pm 0.18	1.94 \pm 0.14	2.70 \pm 0.57	3.40 \pm 0.78	3.68 \pm 0.78
0.1	4.79 \pm 0.25	7.56 \pm 0.39	9.53 \pm 0.75	11.80 \pm 0.04	13.74 \pm 0.21	15.41 \pm 0.25
Flavone (4)						
0.001	0.20 \pm 0.46	0.53 \pm 0.71	-0.03 \pm 0.36	0.23 \pm 0.14	0.30 \pm 0.18	0.20 \pm 0.14
0.01	1.39 \pm 0.21	2.12 \pm 0.04	3.20 \pm 0.43	3.25 \pm 0.14	3.56 \pm 0.07	3.73 \pm 0.36
0.1	0.33 \pm 0.00	1.69 \pm 0.00	1.20 \pm 0.15	1.49 \pm 0.29	2.70 \pm 0.57	2.72 \pm 0.14
6-Hydroxyflavone (5)						
0.001	0.71 \pm 0.18	1.59 \pm 0.07	1.84 \pm 0.57	3.73 \pm 0.82	1.87 \pm 0.53	1.84 \pm 0.32
0.01	0.66 \pm 0.11	2.07 \pm 0.32	2.24 \pm 0.43	2.27 \pm 0.11	2.67 \pm 0.32	2.47 \pm 0.07
0.1	0.20 \pm 0.04	-0.18 \pm 0.29	-0.63 \pm 0.21	0.35 \pm 0.04	0.55 \pm 0.18	0.61 \pm 0.36

7-Hydroxyflavone (6)						
0.001	0.71 ±0.11	1.77 ±0.32	1.61 ±0.11	2.02 ±0.04	2.22 ±0.53	2.04 ±0.04
0.01	1.64 ±0.14	2.62 ±0.11	2.22 ±0.04	2.04 ±0.14	1.82 ±0.75	2.52 ±0.43
0.1	6.81 ±0.68	7.14 ±1.07	11.50 ±1.11	14.45 ±0.57	18.71 ±0.46	15.13 ±1.14
2-Phenylquinolin-4(1 <i>H</i>)-one (7)						
0.001	0.33 ±0.21	1.59 ±0.50	0.81 ±0.75	1.11 ±0.53	1.92 ±0.61	0.53 ±0.68
0.01	0.40 ±0.11	0.68 ±0.21	1.49 ±0.57	0.78 ±0.07	0.88 ±0.00	1.06 ±0.07
0.1	0.68 ±0.21	2.14 ±0.43	2.19 ±1.00	2.85 ±0.86	2.50 ±0.29	4.79 ±1.00
6-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (8)						
0.001	1.01 ±0.04	2.17 ±0.2	2.34 ±0.07	2.24 ±0.00	2.45 ±0.00	2.14 ±0.32
0.01	4.41 ±0.21	5.72 ±0.07	7.14 ±0.21	8.27 ±0.25	8.95 ±0.14	9.53 ±0.07
0.1	27.86 ±0.78	37.95 ±0.14	44.05 ±0.36	49.90 ±0.36	55.07 ±0.32	58.88 ±0.39
7-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (9)						
0.001	-3.05 ±0.07	-4.29 ±0.32	-4.16 ±0.50	-3.78 ±0.68	-4.72 ±0.46	-3.48 ±0.57
0.01	-2.14 ±0.29	-2.92 ±1.75	-2.19 ±2.57	-1.92 ±2.25	-2.45 ±2.25	-1.26 ±2.28
0.1	0.38 ±0.36	0.01 ±0.33	0.53 ±0.21	1.06 ±0.18	-0.45 ±0.21	1.41 ±0.21
8-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (10)						
0.001	-0.45 ±0.36	0.76 ±0.89	2.09 ±0.71	2.77 ±0.96	2.55 ±1.11	3.56 ±1.11
0.01	8.35 ±0.21	16.19 ±0.04	21.23 ±0.18	22.21 ±1.00	23.63 ±0.82	25.49 ±0.04
0.1	43.57 ±0.96	72.44 ±1.78	86.81 ±1.07	92.79 ±0.61	95.16 ±0.50	97.71 ±0.39

5.4 Discussion

The ability of antioxidants to inhibit the Haber-Weiss reaction is of value in the aerobic organism, in order to modify hydroxyl radical production, leading to lipid peroxidation. The ferric reducing/antioxidant power assay determined the chemical ability of test compounds to reduce ferric ions in an acid medium, measuring the absorption increase at 595 nm. Trolox was used as a reference in order to express results as percentage Trolox equivalents. This assay could be improved by taking measurements more often, for instance every minute, or every few seconds as performed by Benzie and Strain (1996); however the assay was successful in view of the fact that it was able to demonstrate effect on reducing ability over time. At time 33 minutes, the data could be compared (Fig 5.2), illustrating the effect achieved by this time.

Quinoline (1) (Fig 5.2 and Appendix C) showed a rapid increase in reducing ability over time, except for the 0.1 mM concentration, which paradoxically performed the worst of the concentrations, showing an endpoint of the reaction at approximately 15 minutes. The

activity of quinoline measured against that of the Trolox standard was very low ($p < 0.0001$) and showed quinoline to be a poor reductant in this assay. Higher values might however have been obtained for the 0.01 mM series had the reaction continued over a longer time period. 4-Hydroxyquinoline (2) showed a sharp concentration dependant decrease in activity for the 0.1 mM series, however all three series showed some increase in reducing ability over time (Appendix C). Initially the 0.01 mM and 0.001 mM reactions occurred rapidly up to 9 minutes, after which the rate slowed. Only the 0.1 mM series showed reaction increase up to 27 minutes. 8-Hydroxyquinoline (3) demonstrated a significant, concentration dependent increase in activity of the 0.1 mM series ($p = 0.001$) compared to its lower concentrations (Fig 5.2 and Appendix C). This rapid reaction needs to be evaluated over a longer time range, since slowing of the reaction was not visible and further reducing activity might have been obtained over a longer time period.

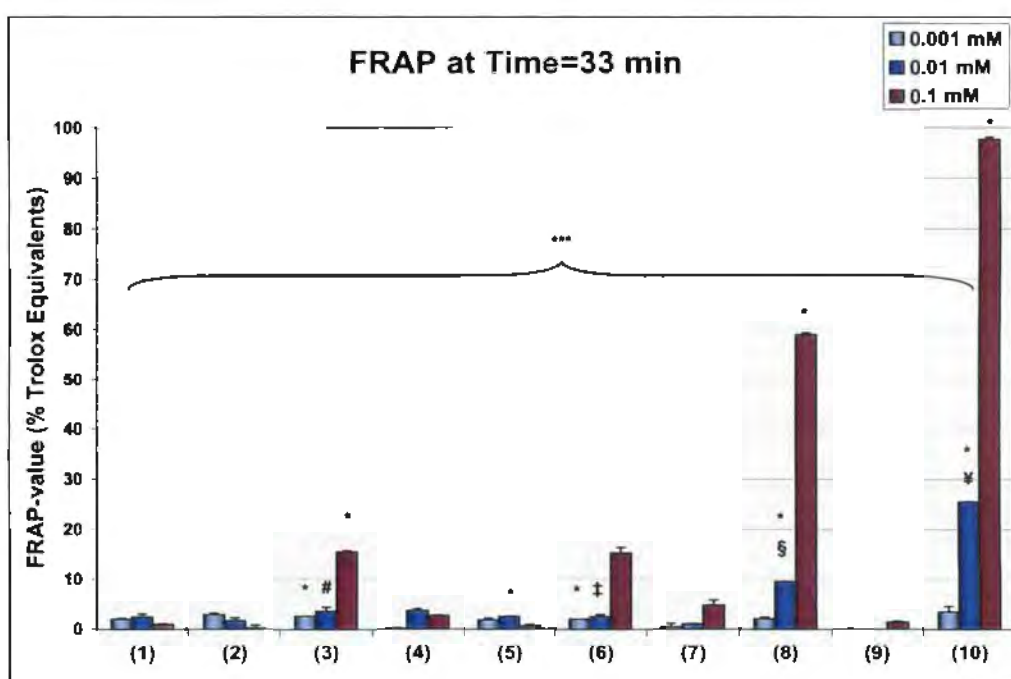


Figure 5.2 FRAP-values obtained for all test compounds in three concentrations at $t = 33$ minutes. Each bar represents the mean \pm S.D.; *R.S.D. < 5%; $N = 3$; *** $p < 0.0001$ vs. 0.1 mM Trolox; # $p = 0.001$ vs. 0.1 mM 8-hydroxyquinoline (3); ‡ $p = 0.0035$ vs. 0.1 mM 7-hydroxyflavone (6); § $p = 0.0002$ vs. 0.1 mM 6-hydroxy-2-phenylquinolin-4(1*H*)-one (8); ¥ $p < 0.0001$ vs. 0.1 mM 8-hydroxy-2-phenylquinolin-4(1*H*)-one (10) (Paired t-test).

Flavone (4) (Fig 5.2 and Appendix C) exhibited its best activity in percentage Trolox equivalents at 0.01 mM concentration. The fast reaction rate slowed after 15 minutes, but a

definitive endpoint for the 0.01 mM reaction was not visible. The 0.001 mM and 0.1 mM flavone series demonstrated more fluctuation, but seemed to reach an endpoint at 21 and 27 minutes, respectively. 6-Hydroxyflavone (**5**) showed an endpoint for the 0.001 mM and 0.01 mM reactions at approximately 9 minutes, except for the 0.001 mM series' value at 21 minutes, which seemed to be an experimental error (Appendix C). It was noted that the 0.1 mM 6-hydroxyflavone (**5**) series' ability to reduce iron was decreased after the first 3 minutes, increasing once more after 21 minutes. Although not significant, a slight decrease in activity over time, as seen in the negative FRAP-values, could indicate the absence of ferrous ions, in which case this compound was a very poor reductant. The 0.1 mM 7-hydroxyflavone (**6**) (Fig 5.2 and Appendix C) showed mild activity as a reductant compared to the Trolox standard (18.71% at 27 minutes), exhibiting approximately 5 times more ferric reducing power than the other flavones and a significant difference compared to its 0.01 mM series ($\dagger p=0.0035$). The 0.01 mM and 0.001 mM 7-hydroxyflavone (**6**) concentrations both reached an endpoint between 3 and 9 minutes, however it is unclear whether the 0.1 mM series would have reached higher values over a longer time.

2-Phenylquinolin-4(1*H*)-one (**7**) (Fig 5.2 and Appendix C) displayed poor reducing ability compared to Trolox (<5% Trolox equivalents), with the two lower concentration series showing considerable fluctuation. 6-Hydroxy-2-phenylquinolin-4(1*H*)-one (**8**) exhibited a concentration dependent increase in activity and a rapid reaction, without seeming to slow (Fig 5.2 and Appendix C). The 0.1 mM series displayed an approximate 5 fold increase in reduction compared to its 0.01 mM series ($\S p=0.0002$), reaching the second highest absorbance value in this evaluation (58.88%). A longer reaction time might have given still better activity. Conversely, it appeared that 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**) (Appendix C) had no reducing activity at 0.001 mM and 0.01 mM concentrations and were indicated as zero in figure 5.2. The negative FRAP-values were obtained when the blank-value was greater than the measured absorbance value of the compound. Only the 0.1 mM concentration series showed positive values for ferrous-TPTZ, displaying a small, but erratic increase in ferric reducing ability over time. Excellent activity, however was seen for 0.1 mM 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) (Fig 5.2 and Appendix C), at 97.71% Trolox equivalents. It appeared that the reaction was nearing its endpoint, comparing favourably to Trolox at the same concentration. The 0.001 mM and 0.01 mM concentrations differed significantly from 0.1 mM Trolox ($***p<0.0001$) and 0.1 mM 8-hydroxy-2-phenylquinolin-4(1*H*)-one ($\yen p<0.0001$). 8-Hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) at 0.1 mM concentration is a good, fast acting ferric reducing agent and is postulated to show favourable antioxidant activity in the lipid peroxidation assay through inhibition of hydroxyl radical production. From this data it is clear that Trolox, 0.1 mM, is a potent ferric

reducing agent compared to the test groups with only one test compound showing comparable activity. A longer time range than 33 minutes might have proved useful as many compounds did not reach a clear endpoint of the reduction reaction. A longer reaction time will however not be viable as crystallisation of the FRAP reagent eventually takes place, interfering with the absorbance values of this colorimetric assay.

Both hydroxyl substituted quinolines (**2**) and (**3**) showed better activity than the unsubstituted quinoline (**1**). However, 8-hydroxyquinoline (**3**) demonstrated significantly better activity than 4-hydroxyquinoline (**2**), indicating that hydroxyl substitution was not the only relevant factor, but also its position on the structure. This might be caused by the location of the hydroxyl group near the amine group in the 8-hydroxyquinoline. Of the flavones, 7-hydroxyflavone (**6**) showed far better activity than either flavone (**4**) or 6-hydroxyflavone (**5**). The negative values obtained for 6-hydroxyflavone (**5**) might be due to solubility problems at high concentrations and indicated the absence of antioxidant activity. 8-Hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) showed unmatched reducing ability in this assay, reaching 97.71% Trolox equivalents, followed by 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**) at 58.88%. This might be the result of the position of the hydroxyl group in close proximity to the amine group in the 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**), as was the case with 8-hydroxyquinoline (**3**), and relative to the carbonyl group in the 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**). Conversely, 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**) performed the worst of all test compounds, only displaying a few positive values at the 0.1 mM concentration and indicating that the hydroxylation *para* to the carbonyl group did not increase the activity of this compound in this experiment. These diverse results (table 5.2) might be accounted for by either poor solubility or iron chelation.

The hydroxyl substitution on the aromatic compound was able to donate electrons to ferric iron, leaving behind an oxygen radical and a reduced ferrous ion. Ionisation was promoted by the stabilising effect of the aromatic ring on the ion. On the other hand, the acidic medium and DMSO present in this assay, inhibited ionisation and reducing ability. Inconsistent reducing ability with regard to concentration was observed for quinoline (**1**), flavone (**4**), 6-hydroxyflavone (**5**) and 2-phenylquinolin-4(1*H*)-one (**7**), where the 0.01 mM concentration performed the best of the three concentrations (Fig 5.2, Appendix C). These compounds, except 6-hydroxyflavone (**5**), were all unsubstituted aromatic compounds with no electron donor group. It was observed that 2-phenylquinolin-4(1*H*)-one (**7**) performed the best of the unsubstituted compounds as a result of the amine group, which was able to donate electrons. The limited activity observed for 6-hydroxyflavone (**5**) could possibly be attributed to precipitation, or the inability to ionise in the acidic medium. The compounds showing

concentration dependant increase in activity, 8-hydroxyquinoline (3), 7-hydroxyflavone (6) and 6-hydroxy-, 7-hydroxy- and 8-hydroxy-2-phenylquinolin-4(1*H*)-one (8 to 10) all had a hydroxyl substitution able to donate electrons. The 7-hydroxyflavone (6) demonstrated better reducing activity than 8-hydroxyquinoline (3), since the compound contained an oxo group that may donate electrons more easily than the deprotonated amine group. Also the 7-hydroxyl is *para* with regards to the carbonyl group, enhancing stabilisation of the antioxidant radical. The 8-hydroxy- and to a lesser degree 6-hydroxy-2-phenylquinolin-4(1*H*)-ones (10 and 8), far surpassed the other test compounds' ability to reduce iron, confirming the electron donor mechanism of action. The ability of the compounds to chelate iron was not estimated and might have contributed to some of the lower values obtained for certain compounds, in particular 7-hydroxy-2-phenylquinolin-4(1*H*)-one (9).

Table 5.2 Hierarchy of the Ferric Reducing/Antioxidant Power of the tested compounds at their highest FRAP-value at the most promising concentration

1.	0.1 mM	Trolox
2.	0.1 mM	8-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (10)
3.	0.1 mM	6-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (8)
4.	0.1 mM	7-Hydroxyflavone (6)
5.	0.1 mM	8-Hydroxyquinoline (3)
6.	0.1 mM	2-Phenylquinolin-4(1 <i>H</i>)-one (7)
7.	0.01 mM	Flavone (4)
8.	0.001 mM	4-Hydroxyquinoline (2)
9.	0.01 mM	6-Hydroxyflavone (5)
10.	0.01 mM	Quinoline (1)
11.	0.1 mM	7-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (9)

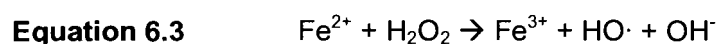
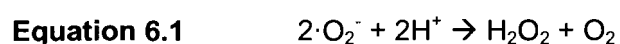
This experiment has indicated the ability of the synthesised series to inhibit hydroxyl radical generation by the Haber-Weiss reaction. The 6- and 8-hydroxy-phenylquinolin-4(1*H*)-ones (8 and 10) showed promise in this acidic, chemical assay. The ability of these compounds to protect against neurodegeneration caused by free radicals must however be further

assessed on biological matter to establish the correlation between chemical and biological antioxidant activity.

CHAPTER 6: SUPEROXIDE ANION SCAVENGING ACTIVITY

6.1 Introduction

Aerobic organisms utilise oxygen for respiration and energy production. The mitochondrial respiratory chain (Fig 2.2) consists of protein complexes that are responsible for converting molecular oxygen (O_2) to superoxide anions (O_2^-). Complex I and II are responsible for the highest production of superoxide (Balaban, 2005), which may be reduced to two products in the mitochondria: to hydrogen peroxide (H_2O_2) by either manganese superoxide dismutase (Mn-SOD) or mitochondrial complex IV (Fig 2.2; Eq 6.1), or to peroxynitrite ($ONOO^-$) by nitric oxide synthase (Eq 6.2). These products lead to hydroxyl radical ($HO\cdot$) production in the Fenton reaction (Eq 6.3) (Lievre *et al.*, 2000).



Build-up of superoxide anions in the mitochondria is countered by the antioxidant enzyme manganese superoxide dismutase. The concentration of this enzyme in the brain is however fairly low and slight increases in free radical production could become a great burden upon cellular antioxidant mechanisms. The oxidative stress caused by these reactive oxygen species sets in motion a cascade of detrimental changes in the molecular structure of lipid membranes, proteins and DNA, causing cell death in the brain and leading to neurodegeneration (McCord, 1985). Smigrodzki (2004) for example, stated that malfunctioning protein complex I in Parkinson's disease, causes enhanced hydrogen peroxide production and oxidative damage to the brain.

The nitro-blue tetrazolium (NBT) assay of Ottino and Duncan (1997), determines the presence of superoxide anions and other free radicals. In this method, radical production is induced in whole rat brain homogenate *in vitro* by potassium cyanide (KCN). Gunasekar *et al.* (1996) concluded that cyanide stimulates N-methyl-D-aspartate receptors to produce nitric oxide and reactive oxygen species, which lead to generation of peroxynitrite and hydroxyl radicals (Eq 6.1 to 6.3). According to Ottino and Duncan (1997) and Waypa *et al.* (2002), cyanide causes cell death by inhibiting the mitochondrial electron transport chain at complex IV, thereby preventing the conversion of superoxide to hydrogen peroxide and producing surplus superoxide anions in the mitochondria. This blockade of complex IV causes inhibition of oxidative phosphorylation and ATP production, causing the cell to die due to a lack of ATP

production. Cyanide is therefore ideal for the generation of superoxide anions in this assay. The radicals produced by cyanide are able to reduce yellow nitro-blue tetrazolium (NBT) to purple, insoluble nitro-blue diformazan (NBD), which has an absorption maximum at 560 nm (Fig 6.1). It is therefore possible to detect the amount of radicals present in the reaction, by quantifying the amount of NBD produced.

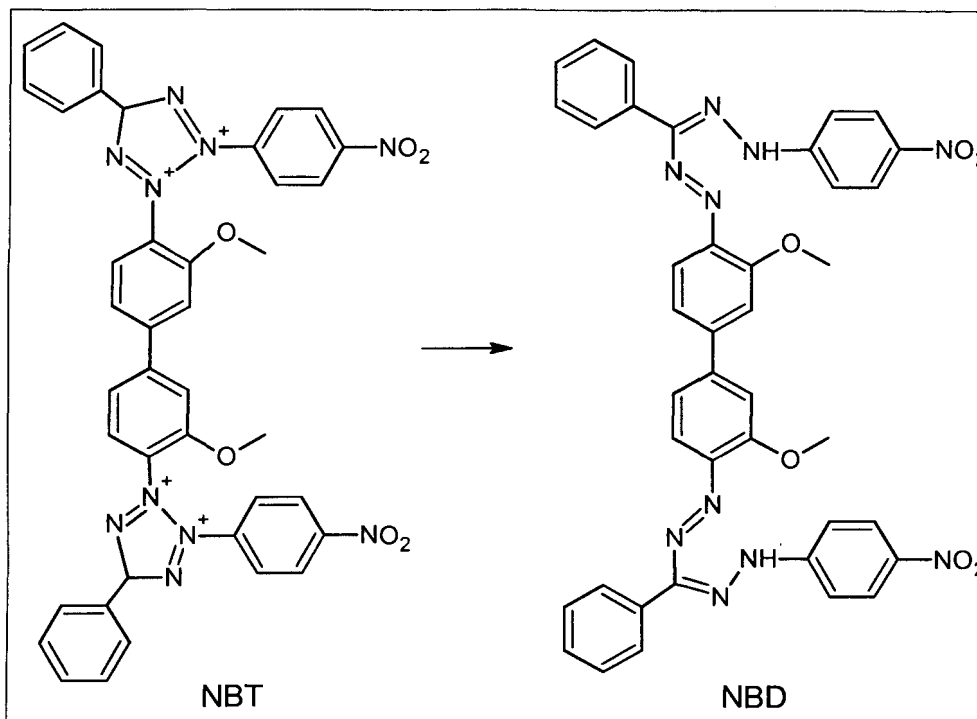


Figure 6.1 Reduction of Nitro-Blue Tetrazolium (NBT) to Nitro-Blue Diformazan (NBD)

The capacity of antioxidants to scavenge radicals may be estimated by determining the amount of NBD produced in the presence of cyanide and the antioxidant. The ability of the test compounds to scavenge cyanide-induced free radicals in rat brain homogenate, thereby preventing reduction of tetrazolium to purple diformazan, could thus be determined. This colorimetric assay quantified scavenging ability according to the intensity of the purple colour generated by free radicals. The quinolines (**1** to **3**), natural antioxidant flavones (**4** to **6**) and the synthesised 2-phenylquinolin-4(1*H*)-ones (**7** to **10**) were compared in this experiment in three increasing concentrations, in order to establish superoxide anion scavenging structure-activity relationships.

The amount of protein present in the assay was determined in order to express the cyanide-induced radical production in equivalents per milligram protein. The protein assay used is a modified method of Bradford (1976), that utilises the dye Coomassie Brilliant Blue G250 as an indicator of protein; it is a red dye that undergoes a change in colour to blue upon binding

protein (Reisner *et al.*, 1975), and causes a shift in absorption maximum, measured at 595 nm. This dye-protein complex is produced within two minutes after initiation of the reaction and remains stable for one hour. The Bradford method is superior to the previously used Lowry protein assay in that it is about four times more sensitive and that cations, such as sodium and potassium, present in the phosphate buffer solution, do not interfere as seen with the Lowry procedure (Bradford, 1976). It is of value to estimate the amount of protein/enzyme present in the whole rat brain homogenate, as enzymatic activity plays a role in the antioxidant activity of the homogenate.

6.2 Experimental

The nitro-blue tetrazolium assay was adapted from Ottino and Duncan (1997). The amount of yellow tetrazolium reduced to purple diformazan by cyanide-induced superoxide anions, was measured. The modified protein assay of Bradford (1976), was used, employing Coomassie Brilliant Blue G250 dye as a colorimetric indicator of protein.

6.2.1 Materials and Methods

6.2.1.1 Chemicals

Potassium cyanide, glacial acetic acid, potassium chloride (KCl), anhydrous di-sodium hydrogen orthophosphate (Na_2HPO_4), potassium dihydrogen orthophosphate (KH_2PO_4), and dimethyl sulphoxide (DMSO) was purchased from Saarchem (PTY) Ltd., Wadeville, quinoline was purchased from Saarchem (PTY) Ltd., Muldersdrift and sodium chloride (NaCl) from BHD, Midrand, South Africa. Nitro-blue diformazan (NBD), nitro-blue tetrazolium (NBT), bovine serum albumin (BSA), Bradford reagent, flavone, 6-hydroxyflavone and 7-hydroxyflavone was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO and 4-hydroxyquinoline was obtained from Aldrich Chemical Company, Milwaukee, WIS, USA. Ethanol and 8-hydroxyquinoline was purchased from Merck, Darmstadt and (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from Sigma Chemical Company, Steinheim, Germany.

6.2.1.2 Animals

The rats used in this experiment were obtained from the North-West University Laboratory Animal Centre and the assay approved by the North-West University Ethics Committee. Adult Sprague Dawley rats were used, weighing between 200 g and 250 g. The decapitation and removal of the brain, was performed by a trained laboratory animal professional.

6.2.1.3 Reagents

A phosphate buffer solution (PBS) was prepared with 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄ and dissolved in 1 L double distilled water. The pH was adjusted to 7.4 and the buffer solution stored in the fridge. A potassium cyanide (KCN) solution was prepared to induce superoxide anion production and stored in the fridge. A stock solution was prepared in double distilled water to yield a final concentration of 1 mM KCN before addition of NBT, since it has been shown by Maharaj (2003), and Ottino and Duncan (1997), that superoxide anion induction by KCN is concentration dependent and that a 1 mM concentration yields appropriate radical production. A 0.1% (w/v) nitro-blue tetrazolium (NBT) solution was prepared fresh every day in an amber bottle to yield a final concentration of 0.028%. The NBT solution contained water and 2% ethanol to yield a final concentration of 0.57% ethanol. Double distilled water was used throughout this experiment.

6.2.1.4 Sample

The studied drugs were dissolved in DMSO to give final concentrations of 1 mM, 0.1 mM and 0.01 mM in 25% DMSO before adding NBT. To estimate the effect of DMSO on the superoxide anion assay, 25% DMSO was tested parallel.

6.2.1.5 Instrumentation

The absorbance was measured with a Labsystems Original Multiscan RC plate-reader and Genesis software.

6.2.2 Preparation of Standards

6.2.2.1 Nitro-Blue Diformazan Calibration Curve

In the nitro-blue tetrazolium (NBT) assay, reduction of tetrazolium to diformazan is measured as a colorimetric indicator of the superoxide anions present. A nitro-blue diformazan (NBD) calibration curve (Fig 6.2) was generated to assist in the calculation of the amount of diformazan produced in the NBT assay. Concentrations of nitro-blue diformazan between 0 and 400 μM, with 100 μM intervals, were prepared in glacial acetic acid to the volume of 255 μl and the absorbance measured at 560 nm.

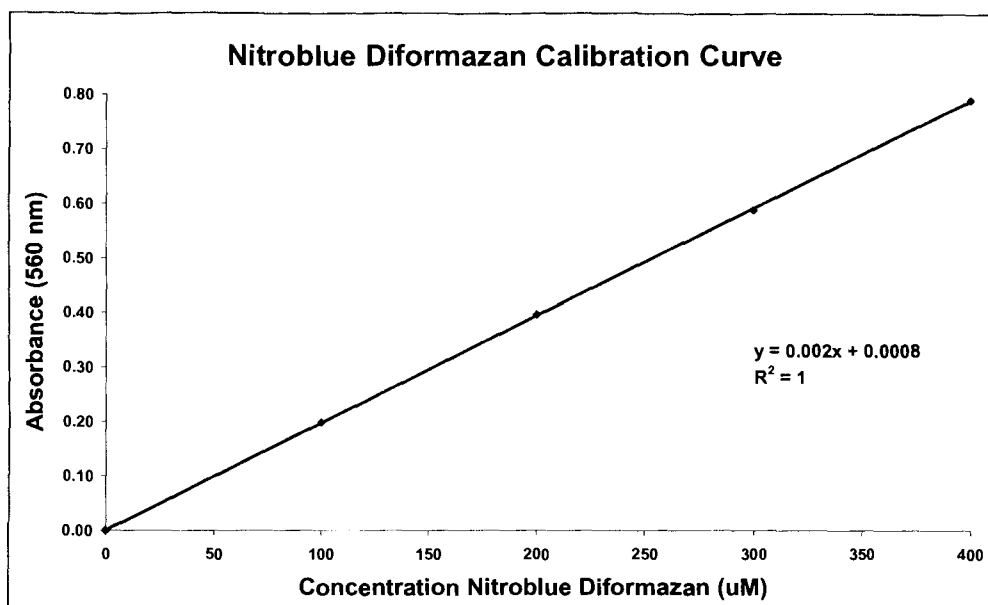


Figure 6.2 Nitro-Blue Diformazan Calibration Curve

6.2.2.2 Bovine Serum Albumin Calibration Curve

The NBT assay is dependent on the amount of protein in the specific rat brain used in the assay; therefore the Bradford protein assay was performed on every rat brain. A bovine serum albumin (BSA) standard was prepared with phosphate buffer solution in concentrations of 0, 0.1, 0.4, 0.7, 1.0 and 1.4 mg/ml, of which 5 μ l was placed in a 96-well plate in triplicate. After adding 250 μ l Bradford Reagent, the well plate was shaken by the plate-reader's mixing function for 30 seconds at room temperature to ensure even mixing. A lag time of 15 minutes ensued, after which the absorbance was measured at 560 nm. This generated a standard curve (Fig 6.3) of known concentration protein as a function of absorbance intensity at 560 nm.

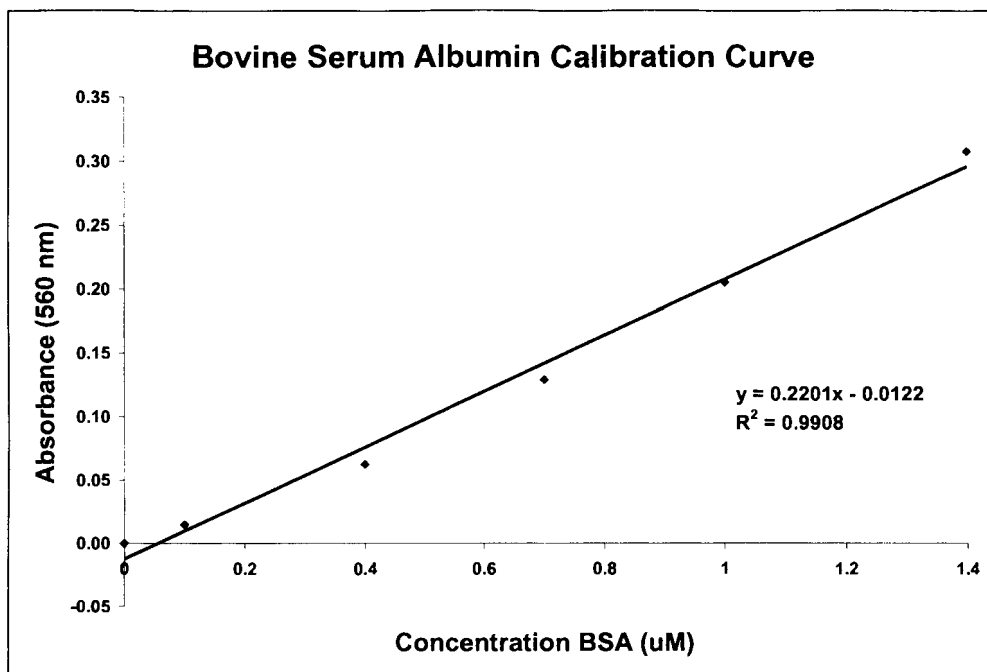


Figure 6.3 Bovine Serum Albumin Calibration Curve

6.2.3 Preparation of Whole Rat Brain Homogenate

The rat was decapitated with a guillotine and the brain removed by cutting laterally through the skull towards the eye on both sides of the head. The top of the skull was removed to reveal the brain, which was easily removed into phosphate buffer solution and held on ice. The 10% (w/v) brain homogenate was formulated with PBS with the aid of a manual glass-Teflon homogeniser.

6.2.4 Nitro-Blue Tetrazolium Assay

6.2.4.1 Exposure of Rat Brain Homogenate to Potassium Cyanide

Potassium cyanide (KCN) was used at a final concentration of 1 mM, before addition of NBT, to generate superoxide anions in 10% (w/v) whole rat brain homogenate. The assay measured the amount of diformazan produced from tetrazolium, thereby establishing the amount of superoxide anions scavenged in the presence of the test compounds when compared to the calibration curve. The positive control, Trolox, was evaluated at the same concentrations as that of the drugs, in the same solvent.

Whole rat brain homogenate, 100 μ l, was incubated at 37 $^{\circ}$ C in an oscillating water bath with 50 μ l KCN and 50 μ l test compound after adding 80 μ l of 0.1% (w/v) NBT solution. The produced free radicals were able to reduce NBT to insoluble, purple NBD, which was extracted from the homogenate pellet after centrifugation, by glacial acetic acid. The top

255 μ l of the glacial acetic acid layer was removed into a 96-well plate and the absorbance measured at 560 nm, using glacial acetic acid as a blank. The absorbance values were converted to micromoles diformazan, by interpolating into the NBD calibration curve and were expressed as micromoles diformazan per milligram protein, by also employing the Bradford Protein Assay to establish the amount of protein present in each run.

6.2.4.2 Exposure of Rat Brain Homogenate to Quinolones, Flavones and 2-Phenylquinolin-4(1*H*)-ones

The studied drugs were tested in 1 mM, 0.1 mM and 0.01 mM concentrations in 25% DMSO solution, before addition of nitro-blue tetrazolium (NBT). 25% DMSO was tested in order to rule out possible scavenging activity of the solvent.

6.2.5 Bradford Protein Assay

The protein assay was performed on every rat brain used in the NBT assay to ascertain the concentration diformazan produced per milligram protein. The unknown protein concentration was determined by making 10, 20 and 50 fold dilutions with PBS of the 10% (w/v) whole rat brain homogenate utilised in the assay, using PBS as a blank. Dilution is necessary since the assay gives unreliable absorbance measurements should the unknown protein concentration be above 1.4 mg/ml. Overall the 10 fold dilution of homogenate was able to yield reliable measurements. The same method was used as in the BSA standard (Section 3.2.1). The absorbance was measured at 560 nm and the values factored into the generated BSA calibration curve and adjusted according to its dilution, to yield the concentration protein (mg/ml) present in the homogenate.

6.2.6 Data Collection

The assay measured the concentration tetrazolium reduced to diformazan as a result of superoxide anion production by cyanide in the presence of the test compound, factoring into the NBD calibration curve (Fig 6.3), while the concentration diformazan produced per milligram protein was determined by interpolating into the BSA calibration curve (Fig 6.4). The final data was expressed as micromoles NBD per milligram protein. Blank homogenate was tested in order to estimate the effect of natural enzymes present in the homogenate on scavenging activity.

6.2.7 Statistical Analysis

The Unpaired Student-Newman Keuls t-test was performed to establish statistical significance of the means obtained from 10 runs per 2 rat brain homogenates, repeating the assay 5 times per brain. Potassium cyanide was assayed with every run and used as a point

of reference. The difference in mean \pm standard error of the mean (S.E.M.) was considered significant when the P-value was less than 0.05, and was calculated in Graphpad Prism.

6.3 Results

In the NBT assay, potassium cyanide increased the NBD absorbance value of the blank homogentate, while the studied drugs reduced the increased absorbance according to their ability to scavenge cyanide-induced superoxide anions (table 6.1 and Fig 6.4). Graphs depicting the inhibition of the different groups of test compounds are included in Appendix D.

Table 6.1 Scavenging of KCN-induced Superoxide Anions by Quinolines, Flavones and 2-Phenylquinolin-4(1*H*)-one (N=10).

Test Compound	Concentration (mM)	Diformazan (μ M/mg protein)	\pm SEM
Blank	-	69.82	\pm 1.94
KCN	1	81.96	\pm 0.65
DMSO	-	78.81	\pm 1.23
Trolox (Positive control)	0.01	73.09	\pm 2.27
	0.1	71.34	\pm 1.70
	1	66.49	\pm 1.28
Quinoline (1)	0.01	72.91	\pm 2.239
	0.1	73.92	\pm 2.574
	1	75.19	\pm 1.138
4-Hydroxyquinoline (2)	0.01	59.8	\pm 1.716
	0.1	74.58	\pm 1.487
	1	75.25	\pm 1.000
8-Hydroxyquinoline (3)	0.01	68.03	\pm 0.7725
	0.1	68.44	\pm 0.8373
	1	69.10	\pm 1.2850
Flavone (4)	0.01	73.61	\pm 0.7274
	0.1	71.3	\pm 0.8867
	1	68.83	\pm 0.9887

6-Hydroxyflavone (5)	0.01	72.37	± 0.7783
	0.1	73.37	± 1.457
	1	77.9	± 77.9
7-Hydroxyflavone (6)	0.01	71.91	± 1.715
	0.1	73.55	± 1.371
	1	77.82	± 0.7191
2-Phenylquinolin-4(1 <i>H</i>)-one (7)	0.01	69.77	± 2.698
	0.1	70.43	± 2.409
	1	71.94	± 2.057
6-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (8)	0.01	73.27	± 2.152
	0.1	73.95	± 1.738
	1	74.73	± 1.484
7-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (9)	0.01	69.29	± 0.6889
	0.1	71.13	± 0.6295
	1	78.95	± 0.951
8-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (10)	0.01	75.12	± 2.533
	0.1	76.62	± 3.500
	1	75.3	± 2.292

6.4 Discussion

The brain is assaulted by free radicals as a consequence of normal mitochondrial respiration. In neurodegenerative disease the brain's antioxidant measures are overwhelmed, causing oxidative stress and cellular damage, ultimately leading to cell death (McCord, 1985). It is therefore of interest to inhibit the overload of free radicals in the brain, by administration of antioxidant drug therapy. Inhibition of radical production, or scavenging of free radicals at the source of production will therefore stop the damaging effects of free radicals before they are able to take place. In this study the known natural antioxidants, flavones, structurally related 2-phenylquinolin-4(1*H*)-ones and certain quinolines were assessed to establish structure-activity relationships in this antioxidant evaluation.

According to Waypa *et al.* (2002), cyanide is a distal inhibitor of the mitochondrial electron transport chain, responsible for blockade of complex IV, oxidative phosphorylation and ATP production (Fig 2.2). The induction of superoxide anion production by 1 mM potassium cyanide (Maharaj, 2003) is evident when the tetrazolium reducing ability of the KCN group is compared to that of the blank homogenate ($p < 0.001$) (Fig 6.4). The addition of the studied drugs was consequently responsible for respective reductions in superoxide and diformazan

production. The Bradford protein assay determined the amount of protein present in each rat brain used in the assay, by factoring into the bovine serum albumin calibration curve (Fig 6.3), enabling us to express the data as diformazan (μM) per milligram protein.

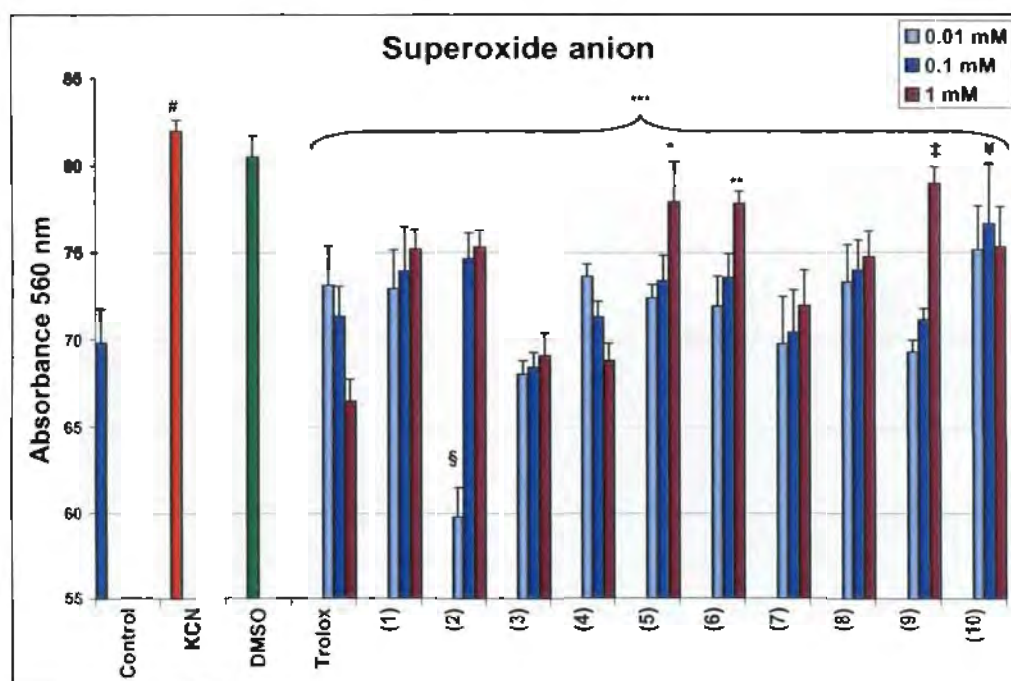


Figure 6.4 The effect of all test compounds on superoxide anion production by KCN in rat brain homogenate. Each bar represents the mean \pm S.E.M.; N=10; # $p < 0.0001$ vs. blank, *** $p < 0.0004$ vs. KCN; § $p = 0.0015$ vs. blank; * $p = 0.0237$ vs. KCN; ** $p = 0.0047$ vs. KCN; ‡ $p = 0.0394$ vs. KCN; ¥ $p = 0.0155$ vs. KCN.

All of the compounds showed significant inhibition of cyanide-induced superoxide production ($p < 0.0394$) in the solvent DMSO, which also caused a slight decrease in superoxide production. Quinoline (1) and 4-hydroxyquinoline (2), at higher concentrations (Fig 6.4, Appendix D) did not show full inhibition of cyanide-induced superoxide anion production, but was comparable to the 0.01 mM and 0.1 mM concentrations of Trolox. The 8-hydroxyquinoline (3) performed better, but 0.01 mM 4-hydroxyquinoline (2) demonstrated the best scavenging activity of all test compounds showing significant activity (§ $p < 0.0015$), completely reversing the effect of cyanide and performing better than Trolox. It is evident that all the quinolines illustrated a concentration dependent decrease in activity, which may be attributed to toxicity on this system.

The unsubstituted flavone (4) performed slightly better than the hydroxyl-substituted flavones, reducing superoxide anion levels to that of the blank at the higher concentrations and scavenging all cyanide-induced superoxide anions at 1 mM. The hydroxyl-substituted

flavones (**5** and **6**) also reduced KCN-induced superoxide production significantly, showing a concentration dependent decrease in activity and less significant difference to KCN at 1 mM concentrations (**p=0.0047 vs. KCN and *p=0.0237 vs. KCN). The activity of the hydroxyl substituted flavones was however less potent than that of flavone (**4**).

The 0.01 mM 7-hydroxy-phenylquinolin-4(1*H*)-one (**9**) showed the best activity of all the 2-phenylquinolin-4(1*H*)-ones, reducing cyanide-induced superoxide production to the blank homogenate level, with the 0.01 mM and 0.1 mM concentrations performing better than Trolox at the same concentrations. Conversely, 1 mM 7-hydroxy-phenylquinolin-4(1*H*)-one (**9**) exhibited the lowest scavenging effect of all the 2-phenylquinolin-4(1*H*)-ones (\ddagger p=0.0394). Of the hydroxyl-substituted 2-phenylquinolin-4(1*H*)-ones the 7-hydroxyl substitution in (**9**) performed the best, followed by the 6-hydroxyl and 8-hydroxyl substitutions in (**8** and **10**). This demonstrated the positive effect of the hydroxyl *para* to the carbonyl group. The unsubstituted 2-phenylquinolin-4(1*H*)-one (**7**) inhibited superoxide production better than the 6- and 8-hydroxy-2-phenylquinolin-4(1*H*)-ones (**8** and **10**), and its activity is comparable to that of the 0.01 mM and 0.1 mM concentrations of 7-hydroxy-phenylquinolin-4(1*H*)-one (**9**).

Both the hydroxylated quinolines (**2** and **3**) presented better scavenging activity than the unsubstituted quinoline (**1**), indicating that the hydroxyl-group increased the activity of quinoline structure. This was however not the case with the flavones or the 2-phenylquinolin-4(1*H*)-ones, where the hydroxyl-substituted compounds (**5**, **6**, **8** and **10**) demonstrated less scavenging activity than the unsubstituted compounds. Of the 2-phenylquinolin-4(1*H*)-ones only 2-phenylquinolin-4(1*H*)-one (**7**) and 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**) reduced superoxide to below the basal level of the blank homogenate, thereby showing enhancement of the natural antioxidant enzymes present in the brain homogenate. The unsubstituted 2-phenylquinolin-4(1*H*)-one (**7**) also performed slightly better than the hydroxyl substituted 2-phenylquinolin-4(1*H*)-ones (**8** and **10**). Unlike the other tested compounds assayed in this experiment, flavone (**4**) was the only compound to show concentration dependent increase in activity. The reason for the decrease in activity with higher concentrations of the other compounds is not clear. It is proposed that higher concentrations of the test compounds may in some way have had a toxic effect on cells, thereby increasing superoxide production, or that solubility was decreased, causing interference. This assay indicates that lower concentrations of the test compounds in the brain will be effective in scavenging radicals and decrease the risk of developing radical-induced neurodegeneration. The effects/toxicity of these compounds at higher concentrations however needs to be investigated further.

CHAPTER 7: LIPID PEROXIDATION

7.1 Introduction

The brain is susceptible to oxidative damage since it consists primarily of fatty acids (McCord, 1985), that are easily oxidised by reactive oxygen species. Peroxidation of polyunsaturated fatty acids takes place at an early stage in neurodegeneration (Pratico and Delanty, 2000) and produces aldehydes and other products of oxidation (Faber, 1995), that lead to the breakdown of the lipid membrane, causing cell death and eventually neurodegeneration. Increased lipid peroxidation, and levels of its bio-marker malondialdehyde (Draper and Hadley, 1990), has been associated with neurodegenerative disorders (Markesbery *et al.*, 2001; Coyle and Puttfarcken, 1993), leading to this study to investigate the possibility of lipid peroxidation inhibition. During oxidation of lipids, carbon-based radicals are generated that produce lipid peroxy radicals, lipid peroxides and, through the Fenton reaction (Eq 7.1), lipid alkoxy radicals ($\cdot\text{OL}$), which are all reactive oxygen species. Lipid peroxidation is initiated by the hydroxyl radical that starts an ongoing chain of events responsible for widespread destruction (Halliwell and Gutteridge, 1989).



Thiobarbituric acid (TBA) reacts with the aldehyde products of lipid peroxidation (Fig 7.1) at high temperatures in a 2:1 ratio, to produce a pink-coloured complex which is spectrophotometrically measured at a wavelength of 532 nm (Ottino and Duncan, 1997).

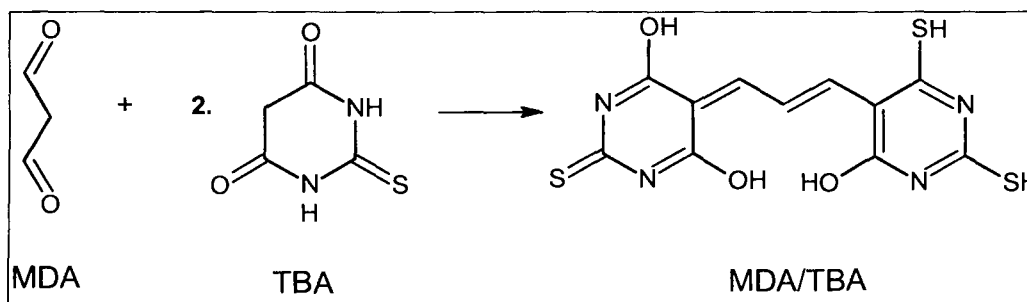


Figure 7.1 The reaction of Malondialdehyde with Thiobarbituric acid to yield a pink TBA₂-MDA Complex.

All aldehydes produced during lipid peroxidation are thiobarbituric acid-reactive substances (TBARS) able to form complexes with TBA and will therefore give unreliable measurements of malondialdehyde (MDA) levels (Esterbauer *et al.*, 1991). TBARS are consequently expressed as malondialdehyde equivalents, however only the amount of free MDA equivalents will be measured in this assay (Draper and Hadley, 1990). Since MDA may

be bound to proteins and other macromolecules (Esterbauer *et al.*, 1991) that interfere with the measurement of free MDA levels, it is necessary to precipitate proteins with trichloroacetic acid (TCA) during the 60 °C incubation, before addition of TBA (Draper and Hadley, 1990). The protein precipitate is then centrifuged off to yield a solution containing free MDA. In addition, the oxidative reaction must be stopped before precipitation of the proteins by adding 2,6-di-tert-butyl-4-methylphenol (BHT). The colorimetric indicator thiobarbituric acid (TBA) is finally added to form a complex with free MDA equivalents produced during toxin-induced lipid peroxidation. The absorbance values obtained were converted to nanomoles MDA equivalents per 1 milligram tissue (Ottino and Duncan, 1997) through the calibration curve generated from 1,1,3,3-tetramethoxypropane (Draper and Hadley, 1990).

The thiobarbituric acid (TBARS) assay was performed on whole rat brain homogenate as a model for lipid peroxidation. Cyanide was shown to be ineffective as a lipid peroxidation inducing agent in Sprague Dawley rats and was substituted with a toxin-solution, consisting of hydrogen peroxide, iron(III)chloride and vitamin C solutions. Although vitamin C is an antioxidant *in vivo*, it interacts with metal ions *in vitro* to produce hydroxyl and lipid alkoxyl radicals (Carr & Frei, 1999). This combination of chemicals induced the Fenton reaction (Eq 7.1), leading to the production of the destructive hydroxyl radical, which is responsible for initiation of the lipid peroxidation reactions.

The toxin was added to the rat brain homogenate first, leaving the drug to scavenge the already produced hydroxyl radicals responsible for aldehyde production, indicating the ability of the compounds to break the chain of oxidation. The hypothesised hydrogen donor mechanism of action is employed here, as radical scavengers donate a proton, thereby deactivating the hydroxyl radical and leaving a stable antioxidant radical (Tan *et al.*, 2002). A range of test compounds with and without hydroxyl substitution was therefore assessed for inhibition of MDA production and consequent lipid peroxidation. The quinoline structurally resembles the core of the 2-phenylquinolin-4(1*H*)-one structure and was included to establish the activity of the basic structure, while the flavones were included as reference antioxidants and to establish the effect of the oxo and the protonated amine groups on hydrogen donating ability. Trolox, a water-soluble derivative of vitamin E and a well known antioxidant, was used as a positive control.

7.2 Experimental

The TBARS method was adapted from Ottino and Duncan (1997) and evaluated the amount of lipid peroxidation that took place by measuring the amount of malondialdehyde equivalents produced after incubation with the toxin.

7.2.1 Materials and Methods

7.2.1.1 Chemicals

1,1,3,3-Tetramethoxypropane (TMP), thiobarbituric acid (TBA), trichloroacetic acid (TCA) and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma Aldrich Chemical Corporation, Steinheim, while iron(III)chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), methanol, ethanol and 8-hydroxyquinoline were purchased from Merck, Darmstadt, Germany. 2,6-Di-tert-butyl-4-methylphenol (BHT), flavone, 6-hydroxyflavone and 7-hydroxyflavone were purchased from Sigma Aldrich, St. Louis, MO and 4-hydroxyquinoline from Aldrich Chemical Company, Milwaukee, WIS, USA. Potassium chloride (KCl), di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4), potassium dihydrogen orthophosphate (KH_2PO_4), hydrochloric acid, butanol, dimethyl sulphoxide (DMSO) and ascorbic acid were purchased from Saarchem (PTY) Ltd., Wadeville, while quinoline was purchased from Saarchem (PTY) Ltd., Muldersdrift, South Africa. Sodium chloride (NaCl) was purchased from BHD, Midrand and hydrogen peroxide (H_2O_2) from Alpha Pharmaceuticals, Durban, South Africa. Double distilled water was used throughout this experiment.

7.2.2 Animals

Ethics approval was obtained by the North-West University Ethics Committee for the use of rats in this assay. Rat brain homogenate was prepared from adult Sprague Dawley rats weighing 200 to 250 g. The rats were bred and maintained in a controlled environment in the North-West University Laboratory Animal Centre. Rats were decapitated and the brains removed by a trained laboratory animal professional.

7.2.2.1 Reagents

The phosphate buffer solution (PBS) was constituted of 8 g NaCl (137 mM), 0.2 g KCl (2.7 mM), 1.44 g Na_2HPO_4 (10 mM) and 0.24 g KH_2PO_4 (2 mM) and dissolved in 1 L double distilled water and the pH adjusted to 7.4. A 0.5% solution of BHT in methanol and 10% solution of trichloroacetic acid (TCA) in double distilled water was made and stored in the refrigerator, together with the phosphate buffer solution. The 0.33% thiobarbituric acid (TBA) solution was made fresh every day, dissolved in double distilled water, in an amber bottle.

7.2.2.2 Sample

The test compounds were dissolved in DMSO to achieve final concentrations of 1 mM, 0.1 mM and 0.01 mM in 10% DMSO. To establish the effect of the solvent 10% DMSO was assessed in parallel.

7.2.2.3 Instrumentation

A Labsystems Original Multiscan RC plate-reader and Genesis software was used to determine absorbances in this experiment at 530 nm.

7.2.3 Malondialdehyde Calibration Curve

A standard solution of 50 nmol/L 1,1,3,3-tetramethoxypropane (TMP) in double distilled water was used in the calibration curve as a standard, and diluted with phosphate buffer solution to achieve TMP concentrations ranging between 0 and 25 nmol/L with 5 nmol/L intervals.

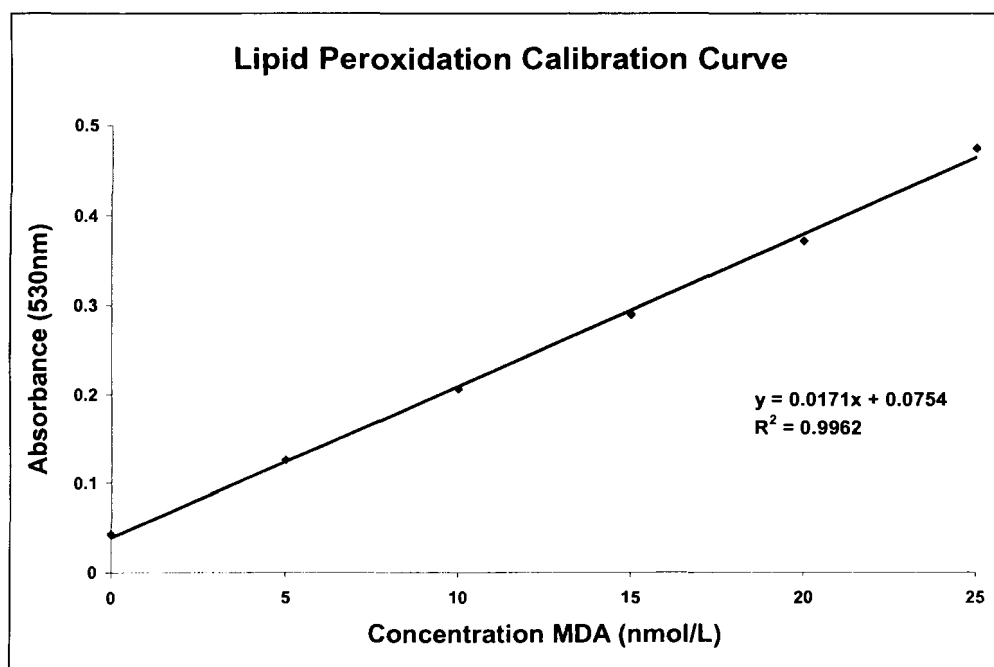


Figure 7.2 MDA Calibration Curve indicating the MDA/TBA-complex formed

Before incubation at 60 °C, BHT, trichloroacetic acid (TCA), and thiobarbituric acid (TBA), solutions were added. After cooling on ice, a butanol extraction was completed by centrifuging at 2000 x g for 5 minutes. From the top butanol layer, 200 µl was transferred into a 96-well plate and the absorbance measured at a wavelength of 530 nm. Butanol was used as a blank to establish the effect of the well plate and butanol on absorption values. A

calibration curve was generated by plotting the absorbance of the TBA/MDA-complex against the concentration MDA (Fig. 7.2).

7.2.4 Preparation of Whole Rat Brain Homogenate

Whole rat brain was homogenised to yield a medium rich in lipids. Decapitation was performed by a trained laboratory animal professional with a guillotine and the skull opened without injuring the brain. The homogenate was prepared with a manual glass–Teflon homogeniser to yield a 10% PBS (v/w) concentration and kept on ice at all times.

7.2.5 Thiobarbituric Acid-Reactive Substances Assay

7.2.5.1 Exposure of Rat Brain Homogenate to the Toxin

The aqueous toxin solution was used in this assay to induce lipid peroxidation and consisted of 0.5 mM hydrogen peroxide solution, 0.488 mM iron(III)chloride solution and 0.14 mM ascorbic acid solution in a ratio of 2:1:1. The final concentration of the positive control was 1 mM Trolox in 10% DMSO. The assay was performed as for the TMP standard curve (Section 7.2.3) and the absorbance measured at 532 nm.

7.2.5.2 Exposure of Rat Brain Homogenate to Quinolines, Flavones and 2-Phenylquinolin-4(1H)-ones

A series of 1 mM, 0.1 mM and 0.01 mM drug concentrations in 10% DMSO solutions were assayed. To ascertain its effect on inhibition of lipid peroxidation, 10% DMSO was tested in parallel and treated exactly as the test compounds.

7.2.6 Data Collection

The absorbance values noted were converted to the amount malondialdehyde equivalents produced, using the MDA Calibration Curve (Fig 7.2). The extent of lipid peroxidation was assessed by calculating nanomoles MDA produced per 1 milligram tissue.

7.2.7 Statistical Analysis

The *in vitro* experiment was repeated 5 times on 2 rat brains. A toxin group was included in every assay and used as a point of reference. Graphpad Prism was used to statistically analyse data in the Unpaired Student-Newman Keuls t-test and to determine the standard error of the means (S.E.M.). Results were expressed as the mean value \pm S.E.M of the 10 runs. Significant differences were obtained when $p < 0.05$.

7.3 Results

In this experiment good antioxidant activity is indicated by a lower absorbance value of MDA, indicating lipid peroxidation inhibition. The ability of the studied compounds to inhibit toxin-induced lipid peroxidation is presented in table 7.1 and figure 7.3. Graphs depicting the three groups of test compounds at three concentrations are included in Appendix E.

Table 7.1 Lipid Peroxidation of Rat Brain Homogenate in the presence of Quinolines, Flavones and 2-Phenylquinolin-4(1*H*)-one (N=10).

Test Compound	Concentration (mM)	Lipid Peroxidation (nmol MDA/mg tissue)	± SEM
Blank	-	0.2737	± 0.0793
Toxin	-	1.0880	± 0.0650
10% DMSO	-	0.9293	± 0.0890
Trolox (Positive control)	0.01	0.1711	± 0.0133
	0.1	0.0397	± 0.0051
	1	0.0295	± 0.0042
Quinoline (1)	0.01	0.8042	± 0.03933
	0.1	0.7723	± 0.05254
	1	0.727	± 0.02963
4-Hydroxyquinoline (2)	0.01	0.933	± 0.03432
	0.1	0.9162	± 0.04642
	1	0.765	± 0.03810
8-Hydroxyquinoline (3)	0.01	0.9269	± 0.05968
	0.1	0.8285	± 0.06771
	1	0.07339	± 0.02445
Flavone (4)	0.01	0.778	± 0.02278
	0.1	0.8262	± 0.04203
	1	0.8745	± 0.04248
6-Hydroxyflavone (5)	0.01	0.824	± 0.07079
	0.1	0.7942	± 0.04815
	1	0.6317	± 0.06794
7-Hydroxyflavone (6)	0.01	0.4154	± 0.0720
	0.1	0.4840	± 0.0872
	1	0.9141	± 0.0352

2-Phenylquinolin-4(1 <i>H</i>)-one (7)	0.01	0.9072	± 0.0417
	0.1	0.8694	± 0.04638
	1	0.8657	± 0.05932
6-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (8)	0.01	0.4830	± 0.0841
	0.1	0.3125	± 0.0650
	1	0.0511	± 0.0179
7-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (9)	0.01	0.691	± 0.08784
	0.1	0.7114	± 0.08479
	1	0.7181	± 0.07297
8-Hydroxy-2-phenylquinolin-4(1 <i>H</i>)-one (10)	0.01	0.5807	± 0.1155
	0.1	0.4908	± 0.0909
	1	0.2676	± 0.0418

7.4 Discussion

Reactive oxygen species are responsible for lipid peroxidation of the brain and malondialdehyde production in neurodegenerative disease (Markesbery *et al.*, 2001; Coyle and Puttfarcken, 1993). Antioxidant drugs are therefore necessary to inhibit oxidative damage. The toxin showed an increase in malondialdehyde (MDA) equivalent production of more than double that found in the blank homogenate ($\#p < 0.0001$), indicating that the combination of hydrogen peroxide, iron(III)chloride and vitamin C was effective in inducing lipid peroxidation. The toxin was used in all sample-containing groups to induce lipid peroxidation in the presence of test compounds, displaying the ability of the compounds to act as chain-breaking antioxidants by scavenging hydroxyl radicals. All the test compounds showed significant ($***p < 0.0002$) toxin-induced lipid peroxidation inhibition. It is noted however that the solvent DMSO also showed lipid peroxidation inhibition and was therefore taken into account when analysing the results. This experiment indicated that all the test compounds, dissolved in DMSO, have the ability to significantly inhibit production of thiobarbituric acid-reactive substances (TBARS). Certain compounds only showed slightly better inhibition than DMSO and for the purposes of this study, those compounds had little ability to inhibit lipid peroxidation, since it is assumed that DMSO was largely responsible for the observed antioxidant activity.

The quinolines (1 to 3) (Fig 7.4, Appendix E) all showed slight but concentration dependent inhibition of toxin-induced lipid peroxidation, with only 1 mM 8-hydroxyquinoline (3) showing good inhibition, comparable to that of 0.01 – 0.1 mM Trolox, and significantly lower than the blank ($\$p = 0.0217$). The 0.01 mM and 0.1 mM concentrations of the 4- and

8-hydroxyquinoline (**2** and **3**) did not differ significantly from the DMSO inhibition. Flavone (**4**) and 7-hydroxyflavone (**6**) at 1 mM concentration showed only slightly better inhibition of toxin-induced lipid peroxidation than DMSO, indicating poor lipid peroxidation inhibition and even toxicity at 1 mM concentration. The 6-hydroxyflavone (**5**) showed concentration dependent inhibition, with the 0.01 mM and 0.1 mM 7-hydroxyflavone (**6**) showing better inhibition and 0.01 mM 7-hydroxyflavone performing the best of the flavones. The decreasing inhibition at increased concentrations for flavones (**4** and **6**) might also be attributed to the fact that the DMSO-soluble compounds were insufficiently dissolved, precipitating in the hydrophilic environment of the assay. The unsubstituted 2-phenylquinolin-4(1*H*)-one (**7**), also showed very slight inhibition when compared to the inhibition caused by DMSO. All 2-phenylquinolin-4(1*H*)-ones however showed concentration dependant increase in inhibition, except for the 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**), which concentrations did not differ significantly. The 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) showed good inhibition at 1 mM, reducing peroxidation to below that of the blank, completely reversing the effect of the toxin and enhancing the natural antioxidant enzymes present in the homogenate. The 6-hydroxyl-2-phenylquinolin-4(1*H*)-one (**8**) showed the best inhibition in this assay at 1 mM concentration to below that of 0.01 mM Trolox and far lower than the blank homogenate ($\neq p=0.0104$).

8-Hydroxyquinoline (**3**) and 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**) showed the best inhibition of TBARS production of the test compounds at 1 mM, better than 0.01 - 0.1 mM Trolox. MDA equivalent production was lowered to the level, or below that of the blank homogenate by 1 mM 8-hydroxyquinoline (**3**) and 6- and 8-hydroxy-2-phenylquinolin-4(1*H*)-one, (**8** and **10**), indicating complete reversal of the toxin-induced peroxidation. The hydroxyl substituted 2-phenylquinolines (**8** to **10**) as a group, had the best ability to inhibit TBARS production.

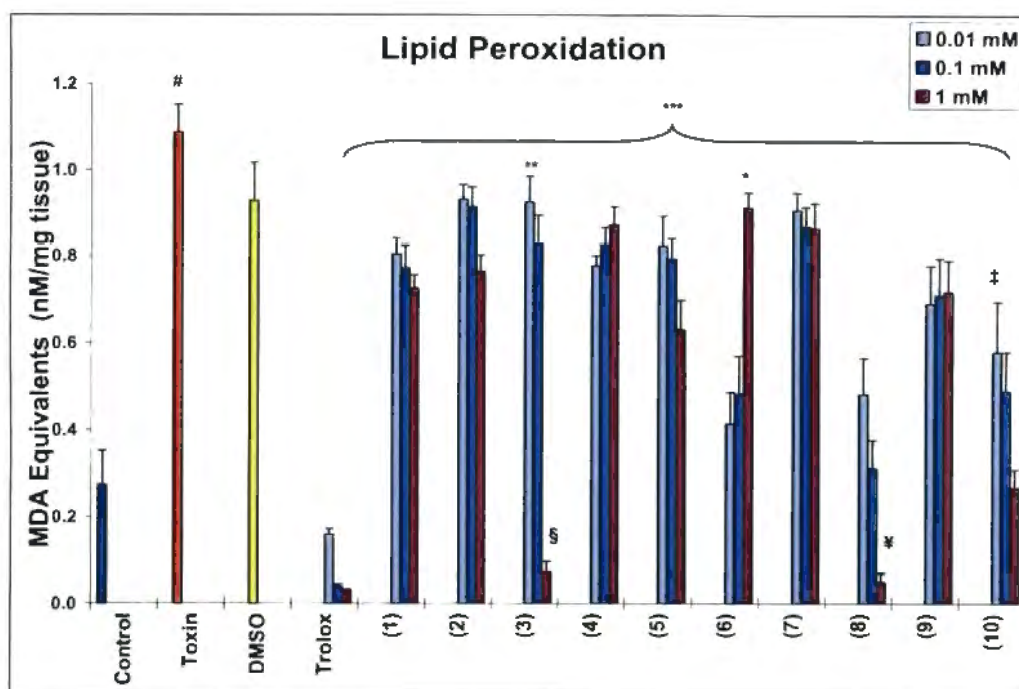


Figure 7.3 Lipid peroxidation inhibition of all test compounds at three concentrations. Each bar represents the mean \pm S.E.M.; N=10; # $p < 0.0001$ vs. blank; *** $p < 0.0002$ vs. Toxin; ** $p = 0.0014$ vs. Toxin; § $p = 0.0217$ vs. blank; * $p = 0.1902$ vs. Toxin; ‡ $p = 0.0104$ vs. blank; † $p = 0.0013$ vs. Toxin.

The unsubstituted flavone (4) however showed slightly better inhibition than the unsubstituted 2-phenylquinolin-4(1*H*)-one (7). As the 6- and 8-hydroxyl substituted compounds showed better activity, the position of hydroxyl substitution seemed to be an important factor in determining activity and concentration dependency. The significance of the 8-hydroxyl substitution of (3 and 10) in donating a hydrogen atom was also displayed in this experiment. It appeared that for the 2-phenylquinolin-4(1*H*)-ones the 6-hydroxyl (8) substitution had better lipid peroxidation inhibition activity than the 8- and 7-hydroxyl substitutions (10 and 9), while 7-hydroxyflavone (6) performed better than 6-hydroxyflavone (5) due to the stabilisation afforded by the *para* carbonyl substitution. In the flavone and 2-phenylquinolin-4(1*H*)-one series, the unsubstituted structures (4 and 7) showed less activity than their hydroxyl substituted counterparts, indicating the importance of the hydrogen donating group on the structures. This was however not the case with the quinolines and could be attributed to keto-enol tautomers formed by 4-hydroxyquinoline in solution (Tokay and Ögretir, 2002).

The ability of the compounds to cross the blood brain barrier however remains to be evaluated. It is postulated that a lipophilic side-chain will increase the membrane permeability

of the 4-quinolone structure, contributing to the possibility of utilising the 2-phenylquinolin-4(1*H*)-ones (**8** to **10**) in neuroprotection.

CHAPTER 8: CONCLUSION

Reactive oxygen species are a group of oxygen-derived molecules responsible for an ongoing destructive chain of oxidation reactions, causing oxidation of lipid cellular structures, proteins essential for cell function and DNA responsible for coding proteins and determining the activities of the cell (Markesbery *et al.*, 2001). Oxidative damage therefore has a wide range of repercussions that is best avoided. Unfortunately the body's endogenous antioxidant systems are unable to effectively keep the increasing amount of radicals at bay when the organism ages and mitochondria start producing more radicals and less ATP (Silva and Schapira, 2001). This age-related overload of antioxidants and the resulting damage to tissues is seen in neurodegenerative disorders and is a contributing factor to deterioration of the brain (Fu *et al.*, 1998). It is therefore desirable to curb the destruction caused by surplus oxygen radicals before the damage to brain tissue becomes evident, thereby effectively removing one contributing factor in neurodegeneration and slowing the onset and progression of deterioration induced by reactive oxygen species.

A series of synthesised compounds was proposed to mimic the antioxidant activity observed for the natural plant-derived flavonoids, thereby establishing structure-activity relationships for the different antioxidant reactions in the chain of oxidation. Flavones were selected as lead compounds for this study since antioxidant activity for these structures (Laguerre *et al.*, 2007) and the underlying mechanism of action has been well established (Silva, 2009). The effect of the oxo group in the heterocyclic aromatic structure of flavone, together with the multitude of hydrogen donating substituents observed in nature, formed the basis of the study. The oxo group was substituted for a proton donating amine group and hydrogen donating hydroxyl groups, were substituted. The structure derived from these structural changes was a 2-phenylquinolin-4(1*H*)-one skeleton with hydroxyl substitution on positions 6, 7 and 8, which were compared to the similarly substituted flavones. The effect of the carbonyl on C-4 and the resulting protonated amine, was also compared to that of the hydroxyl-substituted C-4 and its resulting deprotonated amine, to establish the effect of the amine on antioxidant activity. Since an 8-hydroxyl substituted flavone was not commercially available 8-hydroxyquinoline was included to ascertain the effect of the 8-substitution on antioxidant activity and to fully display the effect of hydroxyl substitution. The three basic compounds – quinoline, flavone and 2-phenylquinolin-4(1*H*)-one – were included in this study to form a complete series of compounds assessed for antioxidant activity (Fig 8.1).

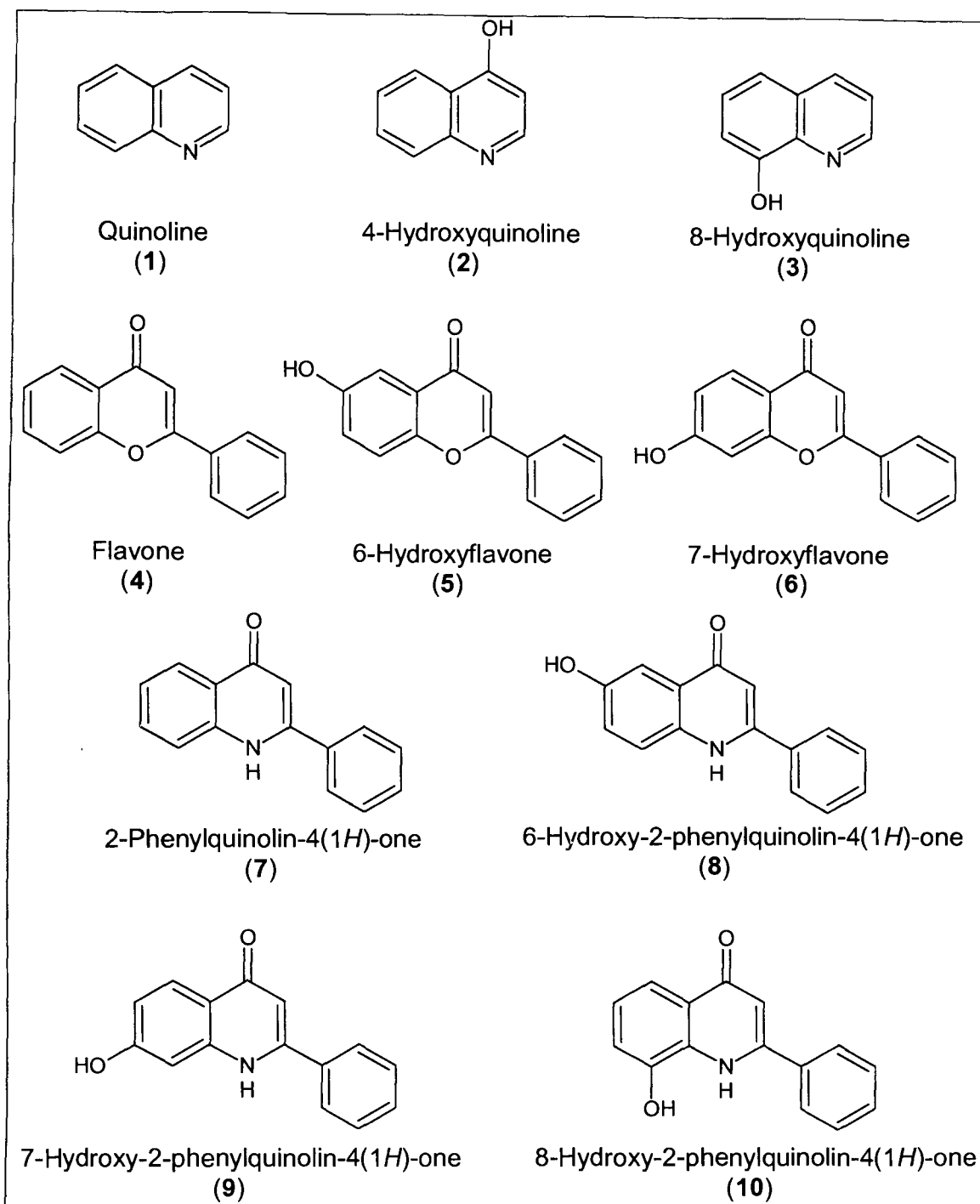


Figure 8.1 Complete Series of Test Compounds

The synthesis of 2-phenylquinolin-4(1*H*)-ones (7 to 10) was completed successfully using to the Conrad-Limpach method described by Somanathan and Smith (1981) and was characterised by NMR, IR and MS techniques (Section 3.4.2).

Evaluation of antioxidant activity was established by employing both chemical and biological *in vitro* assays and determined the ability to scavenge superoxide anions, peroxy and

hydroxyl radicals and to reduce ferric iron (Pulido *et al.*, 2000). These oxidants are present in the oxidative chain at the initiation and propagation stages and therefore give an estimate of what could be achieved by the test compounds at certain stages of oxidation. An overall, total antioxidant screening could however only be done by *in vivo* administration of the compounds to assess the ability to cross the blood brain barrier, as well as the metabolites formed. It could also give an indication of the mechanism of action and the transformation the compound underwent inside the organism. The in-depth clarification of the chemical mechanism was not in the scope of this study and was therefore discussed only briefly.

In this study a full *in vitro* screening was done with the aid of the oxygen radical absorbance capacity (ORAC), ferric reducing/antioxidant power (FRAP), superoxide anion (NBT) and lipid peroxidation (TBARS) assays.

The results obtained in the oxygen radical absorbance capacity (ORAC) assay for 0.001 mM concentrations (table 4.1 and Figure 4.4) showed increasing radical absorbance in the order quinolines, 2-phenylquinolin-4(1*H*)-ones and flavones, excepting 7-hydroxyflavone (**6**), which had the lowest activity in this assay. Conversely, 6-hydroxyflavone (**5**) performed the best. Flavone (**4**) also performed better than 2-phenylquinolin-4(1*H*)-one (**7**) at 0.1 mM concentration (Fig 4.5), further indicating the superiority of the flavones in this assay. In general, hydroxyl substitution increased peroxy scavenging activity in this experiment. For substitution of 2-phenylquinolin-4(1*H*)-one, activity increased from 8-hydroxyl (**10**), 6-hydroxyl (**8**) to 7-hydroxyl (**9**) substitution, with the 7-hydroxyl (**9**) performing second best in this experiment. This trend was ascribed to the stabilising effect of the C-4 carbonyl in the case of 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**), which enhanced proton donating ability, while the pK_a of the amine group of the quinolines and 2-phenylquinolin-4(1*H*)-ones in the basic environment, detracted from the amine-containing compounds' peroxy scavenging ability due to decreased proton donating ability. The peroxy radical quenching ability was increased in a concentration dependent manner for most of the test compounds (Fig 4.3), illustrating that at higher concentrations, increased activity might be observed, especially for 8-hydroxyquinoline (**3**), 6-hydroxyflavone (**5**) and 6-, 7- and 8-hydroxy-2-phenylquinolin-4(1*H*)-ones (**8** to **10**), which were not able to yield acceptable data at higher concentrations due to the inability of AAPH to overpower their antioxidant activities (Section 4.1). 4-Hydroxyquinoline (**2**) and 2-phenylquinolin-4(1*H*)-one (**7**) (Fig 4.3) were comparable, indicating that the deprotonated and protonated amine groups did not enhance scavenging activity, which does not correlate well with the hydrogen donor mechanism of action. The correlating activities of the 8-hydroxyl substituted 2-phenylquinolin-4(1*H*)-one (**10**) and quinoline (**3**) at 0.001 mM concentration also further

indicated that the amine group was not responsible for scavenging activity in this assay, but rather the 8-hydroxyl substitution. This fact is further confirmed in that all unsubstituted compounds displayed poor scavenging activity. Of the unsubstituted compounds 2-phenylquinolin-4(1*H*)-one (**7**) performed the worst (table 4.1), but displayed the greatest increase in activity with hydroxyl substitution as observed for the 7-hydroxyl-2-phenylquinolin-4(1*H*)-one (**9**). The ability to inhibit lipid peroxidation through the scavenging of peroxy radicals was thus confirmed and was shown to be primarily affected by hydroxyl substitution.

The ferric reducing/antioxidant power (FRAP) assay determined the chemical ability of test compounds to reduce ferric ions in an acid medium, thereby establishing the ability of the test compounds to inhibit the Haber-Weiss reaction by reducing the ferric catalyst and inhibiting hydroxyl radical production. In the FRAP assay the ferric reduction obtained in the fixed time of 33 minutes (Fig 5.2) illustrated that the quinolines and flavones had low activity in reducing ferric ions, while the 2-phenylquinolin-4(1*H*)-ones showed moderate to good activity when compared to Trolox. The best activity was seen for 8-, then 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**10** and **8**), followed by 8-hydroxyquinoline (**3**) and 7-hydroxyflavone (**6**). Only the 2-phenylquinolin-4(1*H*)-ones showed prominent activity. Better activity might have been observed for the above-mentioned compounds had it been possible to extend the reaction time (Appendix C). At its best concentration flavone (**4**) did not outperform 2-phenylquinolin-4(1*H*)-one (**7**), indicating the superiority of the amine group over the oxo group in this experiment. The activity was also increased with the protonated amine group in the 2-phenylquinolin-4(1*H*)-ones (**7**), when compared to the deprotonated group in the structurally related 4-hydroxyquinoline (**2**) and quinoline (**1**). Hydroxyl substitution increased activity considerably, but erratically since the compound that performed the worst in this experiment was 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**). The same observation of inconsistency was made for the activities of the hydroxylated flavones (**5** and **6**), where the 7-hydroxyflavone (**6**) showed increased activity. The exact opposite of the observed ORAC activity for the 7-hydroxyl substituted compounds were observed in the FRAP assay. However, as observed in the ORAC assay, the 8-hydroxyl substitution was once again a key factor in increasing scavenging activity, since both 8-hydroxyquinoline (**3**) and 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) performed well in their separate groups. This might be caused by the location of the hydroxyl group in close proximity to the amine group. The activity of 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**) might then also be explained by the proximity of the C4-carbonyl group, which might have had a positive effect on the ionisability of the compound. The effect of the amine and aromatic substitution on C2 was also indicated, showing activity comparable to that of Trolox for

8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**). The compounds showing concentration dependant increase in activity, 8-hydroxyquinoline (**3**), 7-hydroxyflavone (**6**) and 6-, 7- and 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**8** to **10**) all had a hydroxyl group able to donate electrons; however this mechanism of action was not exclusively proved as three hydroxyl-substituted compounds, (**2**, **5** and **9**) displayed poor activity. This occurrence might be explained by poor solubility, inability to ionise in the acidic medium or iron(II) chelation – removing iron from the reaction and yielding low FRAP-values.

The results obtained in the superoxide anion nitro-blue tetrazolium (NBT) assay (Fig 6.4, Appendix D) indicated the ability of the test compounds to scavenge superoxide anions and showed the order of decreasing scavenging activity to be: hydroxyquinolines (**2** and **3**), followed by flavone (**4**), 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**), 2-phenylquinolin-4(1*H*)-one (**7**), hydroxyflavones (**6** and **5**), quinoline (**1**) and hydroxy-2-phenylquinolin-4(1*H*)-ones (**8** and **10**). Remarkably, the compounds that demonstrated good activity in the FRAP assay, (**10** and **8**), showed the worst superoxide anion scavenging activity, indicating that either the same mechanism of action was not present or that the results obtained in the chemical assay were not reproducible in this biological assay. The exact trend was observed as for the 2-phenylquinolin-4(1*H*)-ones in the ORAC assay, demonstrating that the 7-hydroxyl substitution was stabilised by the *para* carbonyl and led to increased hydrogen donor activity. The 0.01 mM and 0.1 mM concentrations of (**9**) performed better than Trolox at the same concentrations. In this assay the hydroxylated quinolines (**2** and **3**) performed the best followed by the hydroxylated flavones (**5** and **6**) and 2-phenylquinolin-4(1*H*)-ones (**8** to **10**). This differed from the ORAC assay, where the quinolines performed comparably to the 2-phenylquinolin-4(1*H*)-ones and the flavones performed the best. In contrast, flavone (**4**) and 2-phenylquinolin-4(1*H*)-one (**7**) performed better than their respective hydroxylated compounds, except for 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**), which performed slightly better than (**7**). With the exclusion of 4-hydroxyquinoline (**2**) and 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**), it appeared that hydroxyl substitution on the structure decreased superoxide anion scavenging activity in this experiment, which goes against the hydrogen donor mechanism of action concept. It is unclear why this should be the case, since the same trend was observed in hydroxyl substitution position for 2-phenylquinolin-4(1*H*)-ones as in the ORAC assay, which is based on a hydrogen donating mechanism. Higher concentrations of the test compounds may in some way have had a toxic effect on cells, or poor solubility may have been a contributing factor. It is however possible that the hydroxylated compounds were unable to donate their hydrogen atoms in the hydrophilic medium due to hydrogen bonds formed (Pedrielli *et al.*, 2001; Pinelo *et al.*, 2004).

When comparing the unsubstituted compounds, flavone (4) performed better than 2-phenylquinolin-4(1*H*)-one (7) and quinoline (1), displaying a different trend to that observed for the hydroxylated compounds. This demonstrated the effect of the hydroxyl groups and that of the functional groups on the compounds, where the protonated amine did perform better than the deprotonated amine, the opposite of which was observed with the hydroxylated compounds. Complete reversal of the effect of cyanide was observed for 4- and 8-hydroxyquinoline (2 and 3), flavone (4), 7-hydroxy-2-phenylquinolin-4(1*H*)-one (9) and 2-phenylquinolin-4(1*H*)-one (7), however the hydroxyl-substituted compounds (5, 6, 8 and 10) demonstrated less scavenging activity than that of the blank brain homogenate. The best compound, 4-hydroxyquinoline (2) indicated that the amine, together with one hydroxyl group gave the best superoxide anion scavenging activity. The mechanism of action in the NBT and FRAP assays are clearly not related since the compounds showing the best ferric reducing power (8 and 10) was the worst superoxide anion scavengers.

Lipid peroxidation *in vivo* is a consequence of the amount of superoxide anions produced further up the cascade of oxidative events leading to hydroxyl radical production. Even though the studied drugs may not show the best superoxide inhibition, they might still act as antioxidants by scavenging the hydroxyl radical in the lipid peroxidation (TBARS) assay. The results were in decreasing order of activity: 6-hydroxy-2-phenylquinolin-4(1*H*)-one (8), 8-hydroxyquinoline (3), 8-hydroxy-2-phenylquinolin-4(1*H*)-one (10), 7- then 6-hydroxyflavone (6 and 5), 7-hydroxy-2-phenylquinolin-4(1*H*)-one (9), quinoline (1), 4-hydroxyquinoline (2), flavone (4) and 2-phenylquinolin-4(1*H*)-one (7). In general this assay showed that hydroxyl substitution of compounds increased their hydroxyl radical scavenging activity. The 6-hydroxy-2-phenylquinolin-4(1*H*)-one (8) performed the best of the 2-phenylquinolin-4(1*H*)-ones, followed by the 8- (10) then 7-hydroxyl (9) substitutions, which does not follow the trend exactly as seen in the FRAP assay, but still demonstrated the 7-substitution to be the worst. Of the flavones, the 7-substitution (6) performed better than the 6-substitution (5), possibly indicating the positive effect of the *para* carbonyl. MDA equivalent production was reduced to below that of the blank brain homogenate by 8-hydroxyquinoline (3) and the 6- and 8-hydroxy-2-phenylquinolin-4(1*H*)-ones (8 and 10), indicating complete reversal of the toxin-induced peroxidation and the significance of the 8-hydroxyl substitution in donating a hydrogen atom (Fig 7.6 Appendix E). This indicated that the basic, deprotonated amine on the structure (3) gave better hydrogen donating ability to the hydroxyl group than the acidic, protonated amine group (10) in this medium. The unsubstituted 2-phenylquinolin-4(1*H*)-one (7), showed very slight inhibition and performed worse than quinoline (1) or 4-hydroxyquinoline (2), further suggesting that the amine group was not as important in this assay as hydroxyl substitution. As postulated from the FRAP

assay results, the 6- and 8-hydroxy-2-phenylquinolin-4(1*H*)-ones (**8** and **10**) displayed good activity in the lipid peroxidation assay, indicating that the FRAP assay correlated well with the data obtained in this biological TBARS assay, since hydroxyl radical production caused by the Haber-Weiss reaction ultimately lead to lipid peroxidation initiation. The ability to reduce ferric iron and inhibit lipid peroxidation was linked in these two assessments and it may therefore be assumed that compounds (**8** and **10**) will exhibit the ability to modify all hydroxyl radical associated oxidative reactions following the Haber-Weiss reaction and leading up to inhibition of initiation of lipid peroxidation.

The results obtained were used to compare 2-phenylquinolin-4(1*H*)-ones to flavones and to derive antioxidant structure-activity relationships. Compounds displaying favourable activity might prove useful in discovering appropriate antioxidant compounds to combat neurodegenerative disease resulting from oxidative damage. Overall, the flavones (**4** to **6**) performed better in the ORAC assay than the 2-phenylquinolin-4(1*H*)-ones (**7** to **9**), though the 2-phenylquinolin-4(1*H*)-ones indicated superior activity in the FRAP, NBT and TBARS assays, indicating that the 2-phenylquinolin-4(1*H*)-ones were in general slightly better antioxidants than the flavones in these experiments. Substitution with hydroxyl groups was also demonstrated to increase antioxidant activity in the ORAC, FRAP and TBARS assays. In addition, the importance of the 8-hydroxyl substitution in quinolines and 2-phenylquinolin-4(1*H*)-ones were established, as well as the relative unimportance of the amine, which only demonstrated an advantage in the FRAP assay. The FRAP and TBARS assays favoured the 8- and 6-hydroxyl substituted 2-phenylquinolin-4(1*H*)-ones, (**8** and **10**), while in the ORAC and NBT assays the C4-carbonyl group enhanced the stability of the 7-substitution (**9**) causing increased hydrogen donation and antioxidant activity.

The 2-phenylquinolin-4(1*H*)-ones acted as chain-breaking antioxidants in these assessments, able to slow the progression of oxidation, rather than to inhibit initiation of oxidation. Further study into the iron chelating ability of the compounds may prove the ability of the test compounds to remove heavy metals associated with oxidative stress. Iron chelating ability of the compounds might also have had a direct effect on the results obtained in the FRAP assay, causing a possible misrepresentation of the ability of individual compounds to act favourably upon the iron-catalysed oxidative reactions. However, the FRAP and TBARS assays correlated well and the likelihood of iron chelation was therefore decreased. Also, the necessary 3-hydroxyl substitution (Déziel *et al.*, 2004) was not present in the assessed series. Establishing the general toxicity of these compounds which were derived from antibacterial agents, might also prove informative with regards to the low antioxidant values obtained for certain compounds.

Antioxidant screening of the test compounds revealed the importance of the 8-hydroxyl substitution and the lesser importance of the amine and C-2 aromatic ring for these chain-breaking antioxidants. However, as neither the chemical or biological *in vitro* assays are representative of what might occur in the living organism, the *in vivo* assessment of compounds will be a more valuable approach to estimating antioxidant activity in the human brain. It was noted that a clearer trend in structure-activity relationships was observed in the chemical assays ORAC and FRAP, than in the biological evaluations, NBT and TBARS. The reason for these less consistent group trends in the biological assays was the many contributing factors in the brain homogenate that cannot be simulated in chemical assays. Therefore biological assays provided a better estimate of what activity might be expected *in vivo*. It is thus necessary to further study the test compounds *in vivo* to assess their ability to cross the blood brain barrier, to establish the metabolic processes present in the living brain and to estimate the antioxidant activity on the organism. Only then may conclusions be drawn and structure-activity relationships for the human brain be interpreted, demonstrating the impact these chain-breaking compounds might have on the neurodegeneration occurring in the elderly.

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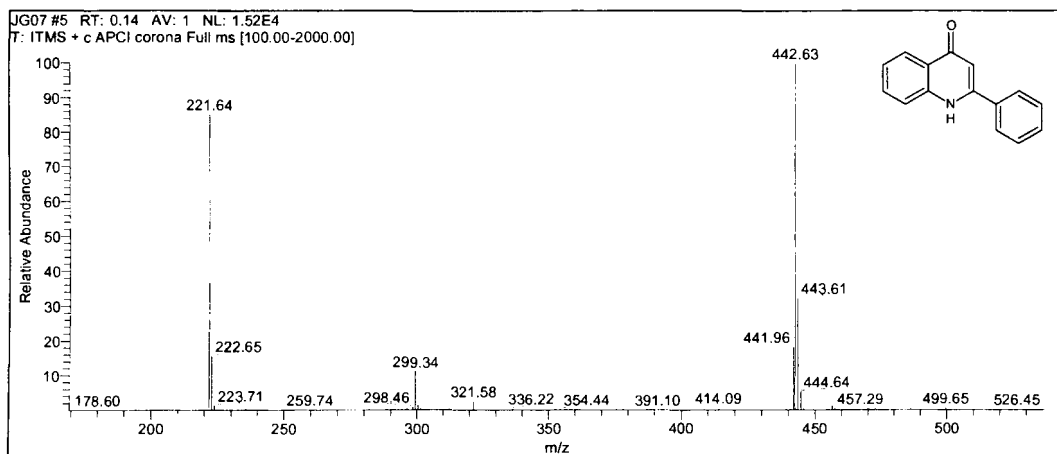
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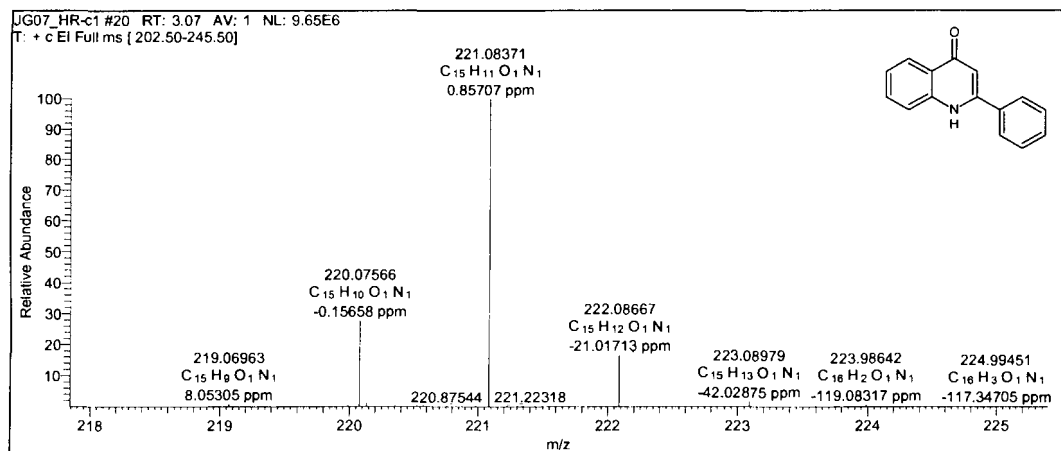
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APPENDIX A

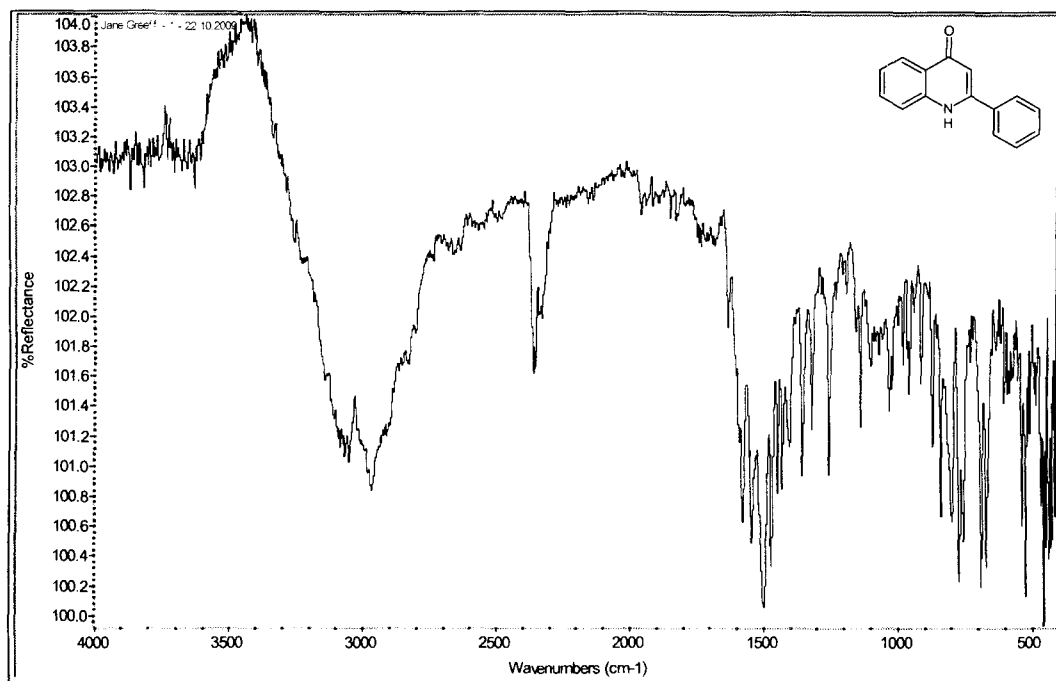
Spectrum 1 APCI MS of 2-Phenylquinolin-4(1H)-one (7)



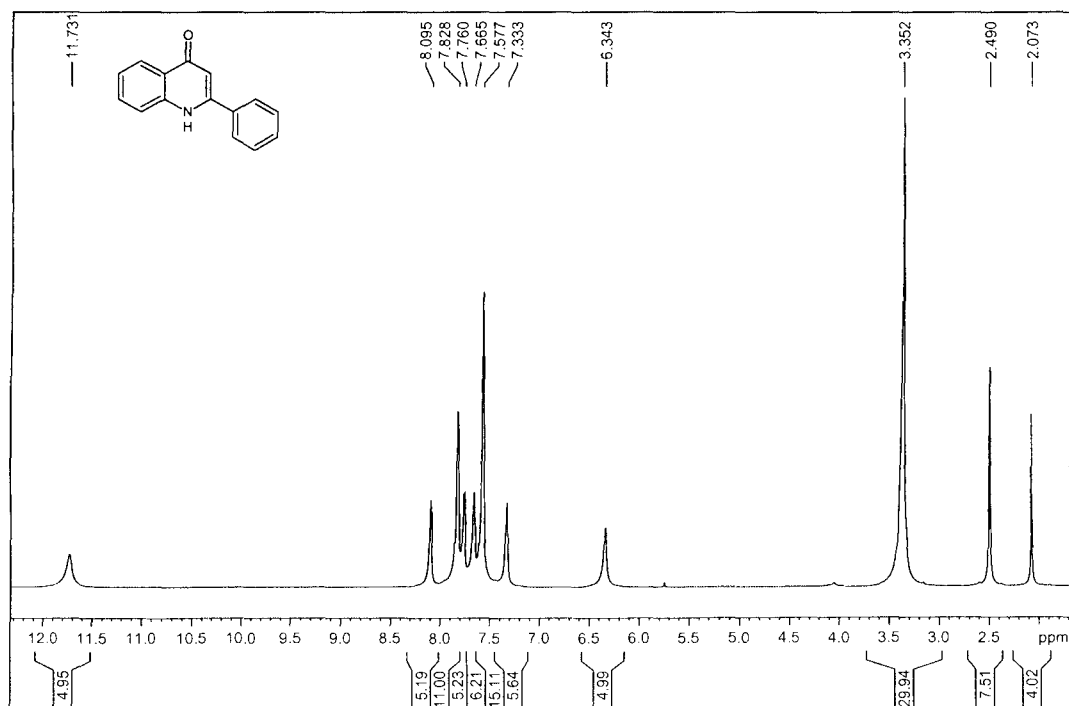
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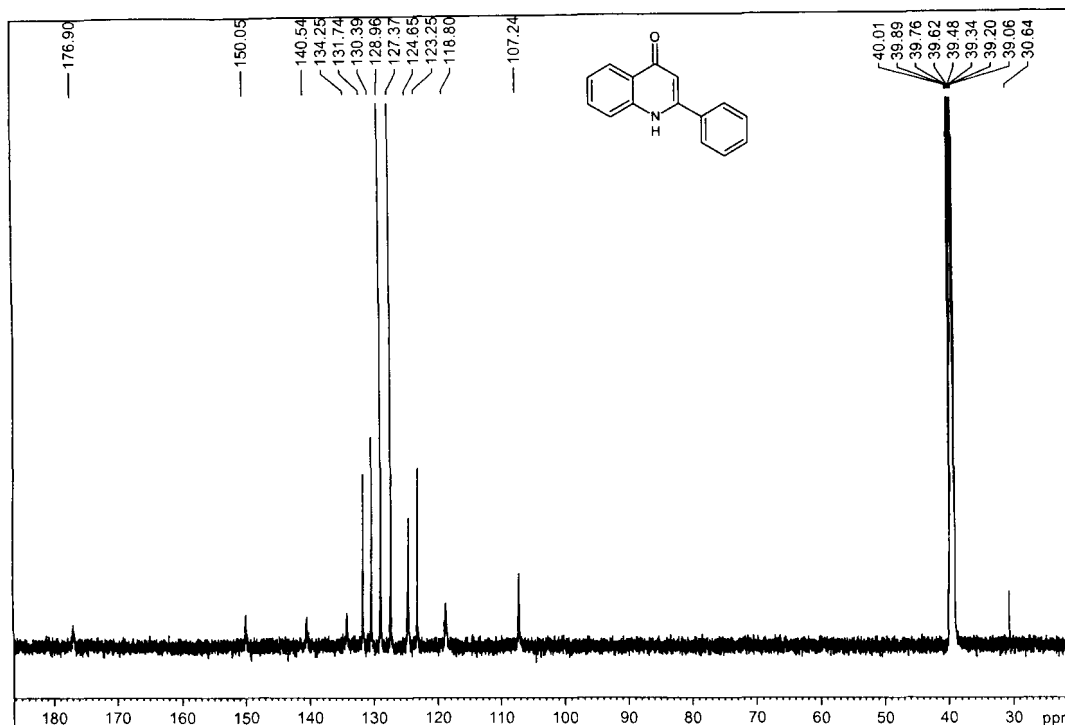
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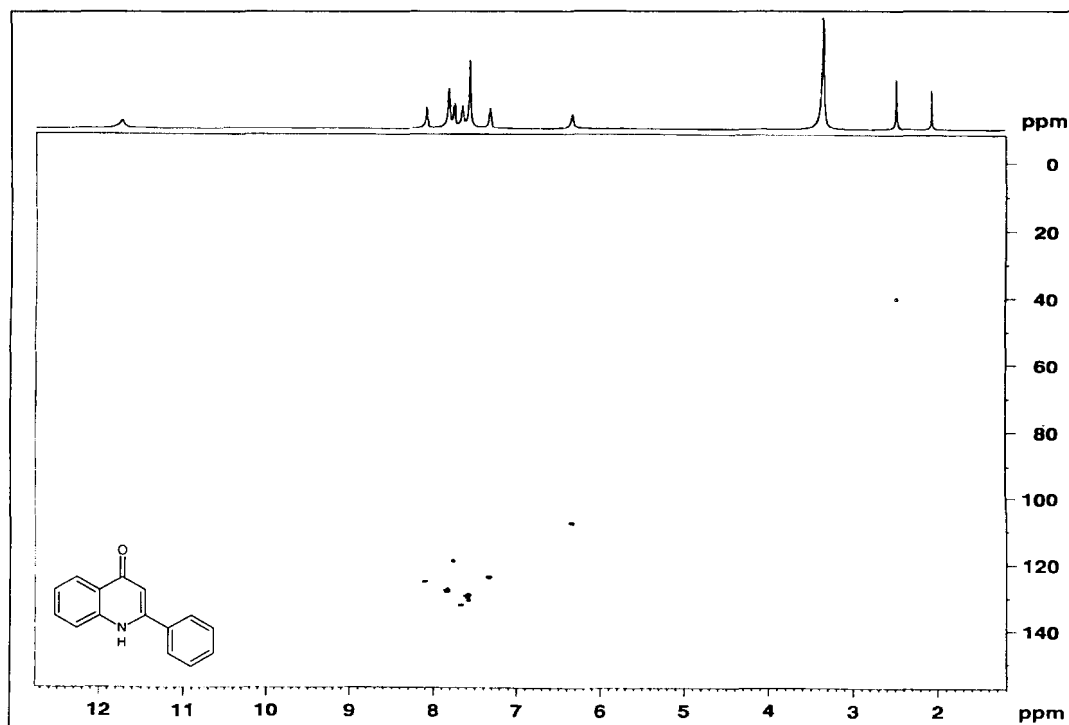
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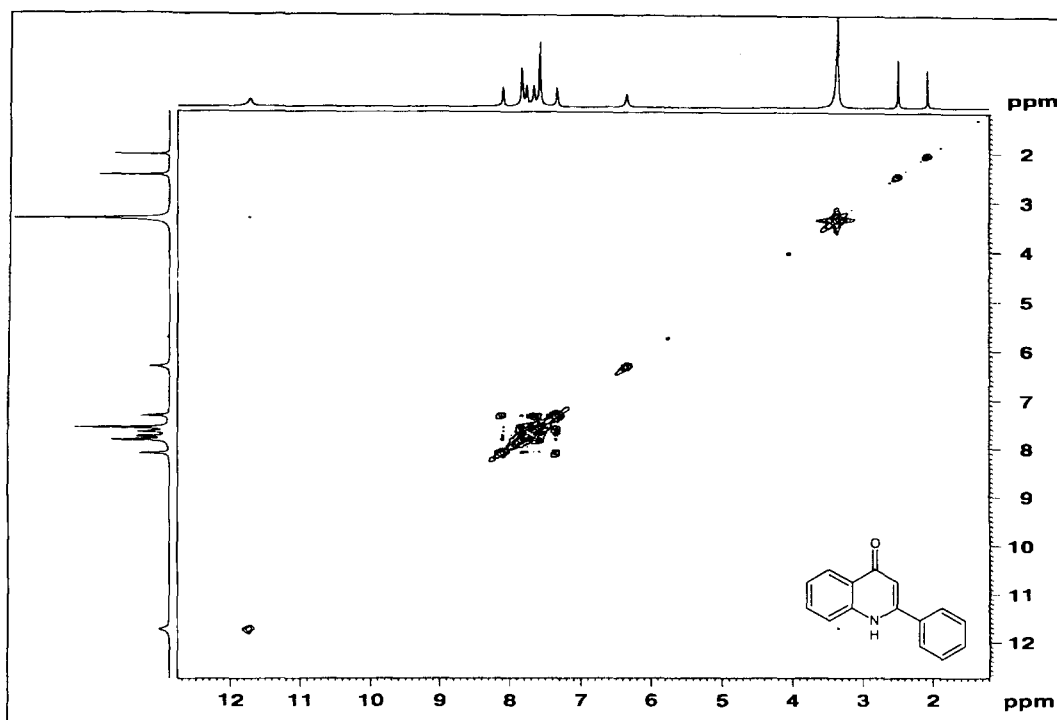
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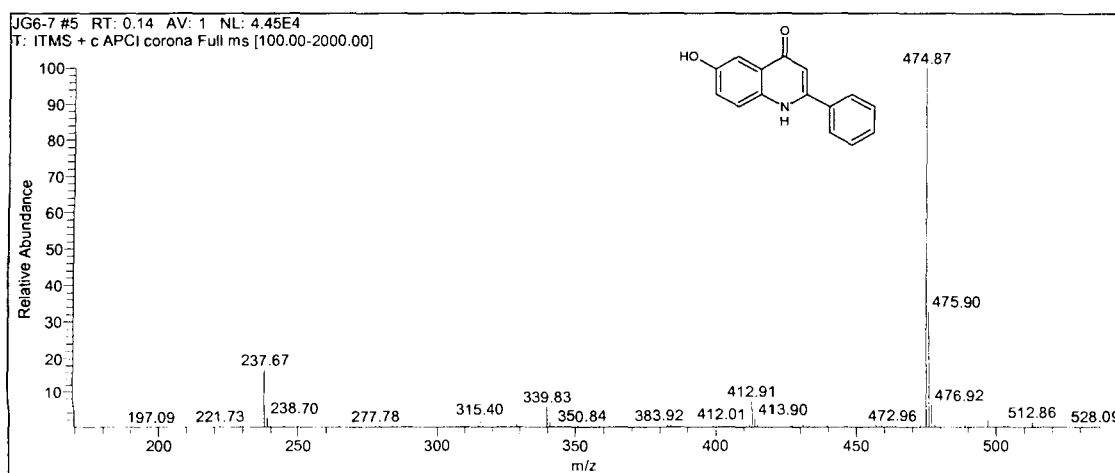
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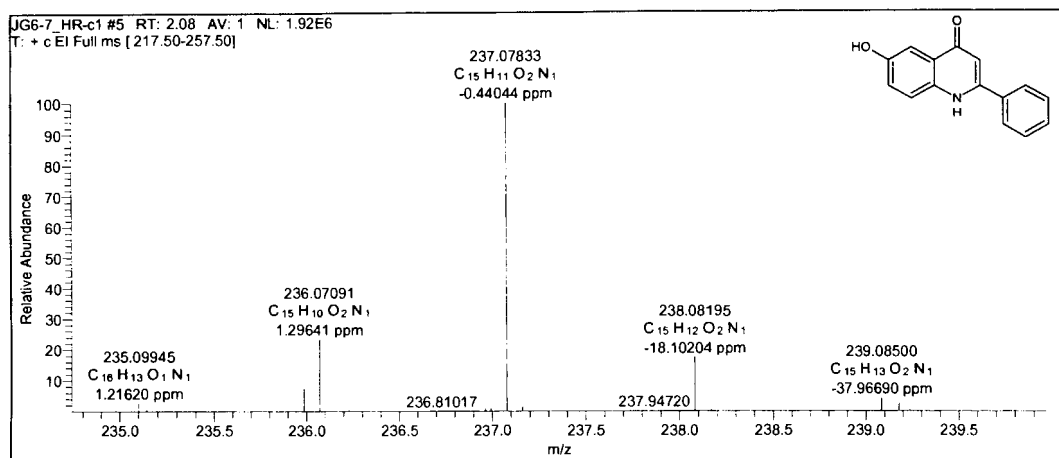
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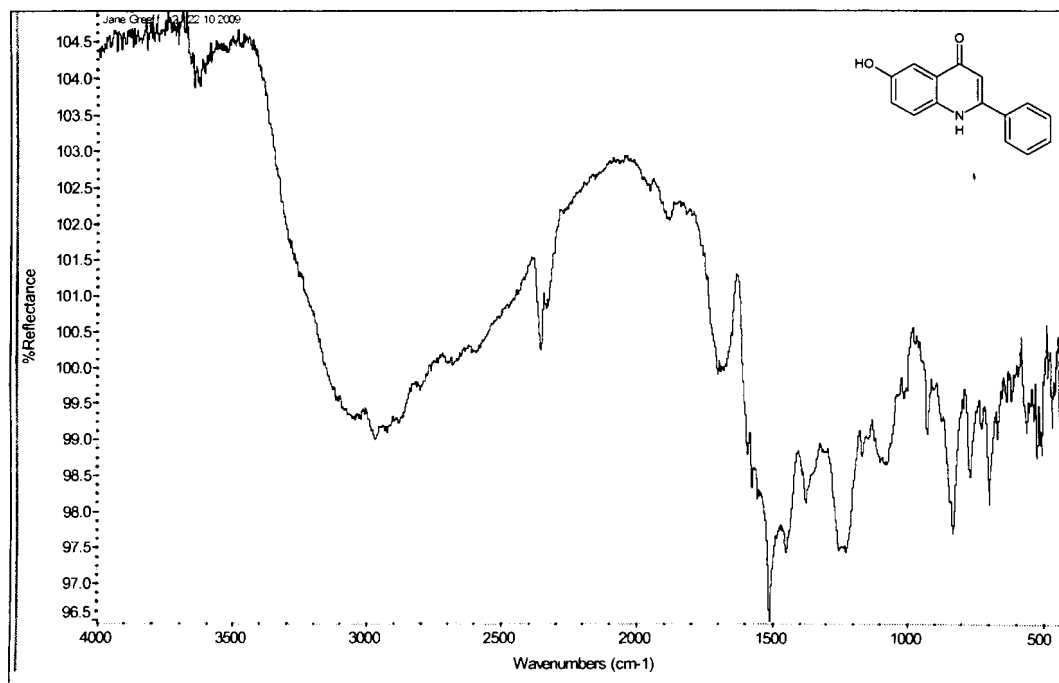
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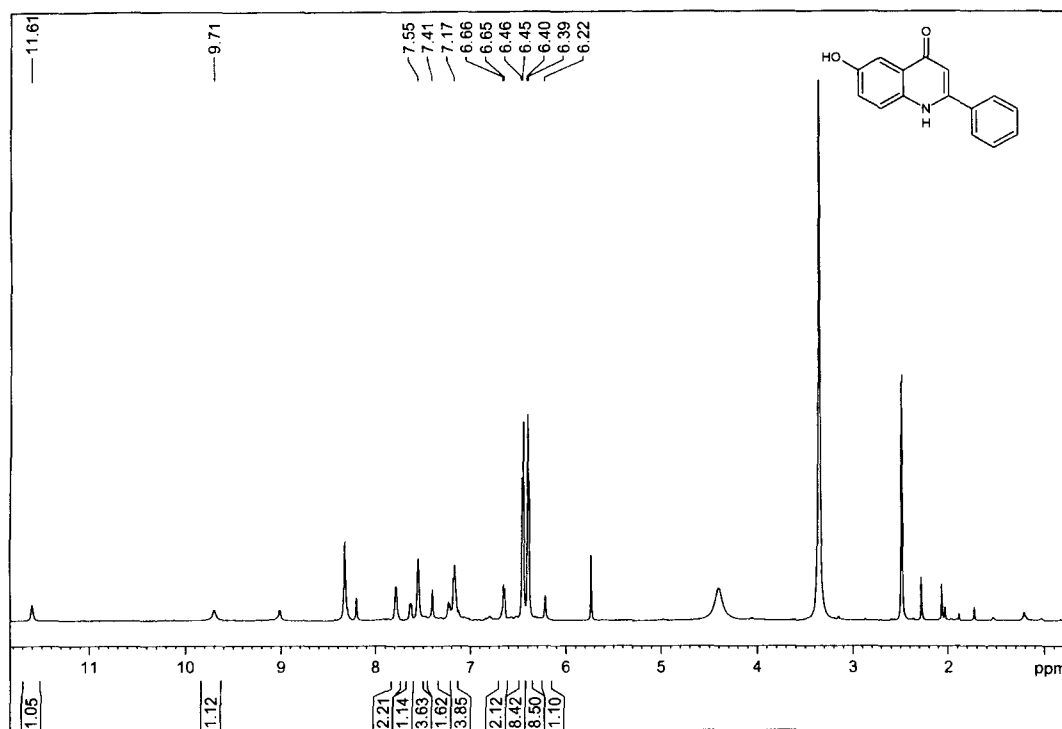
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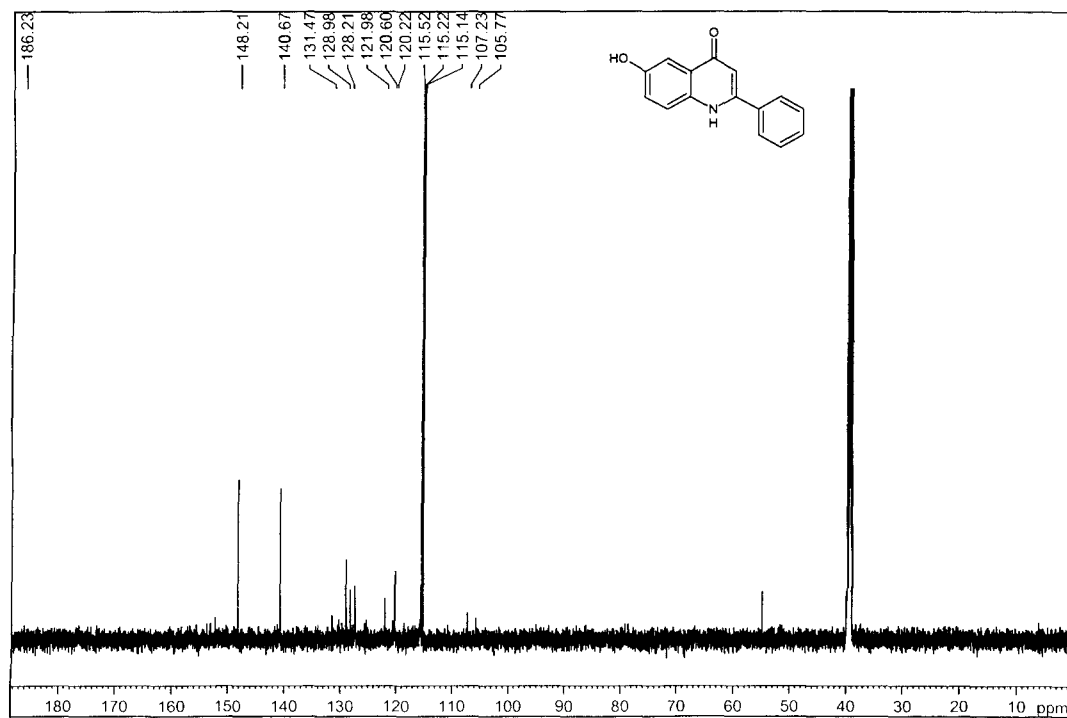
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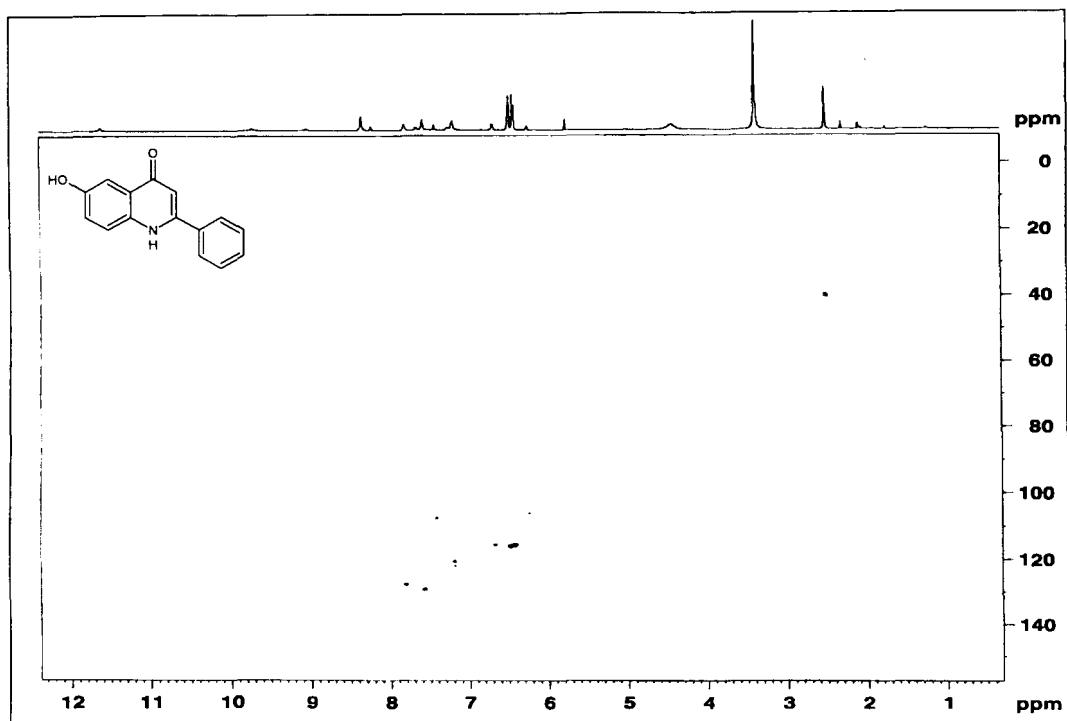
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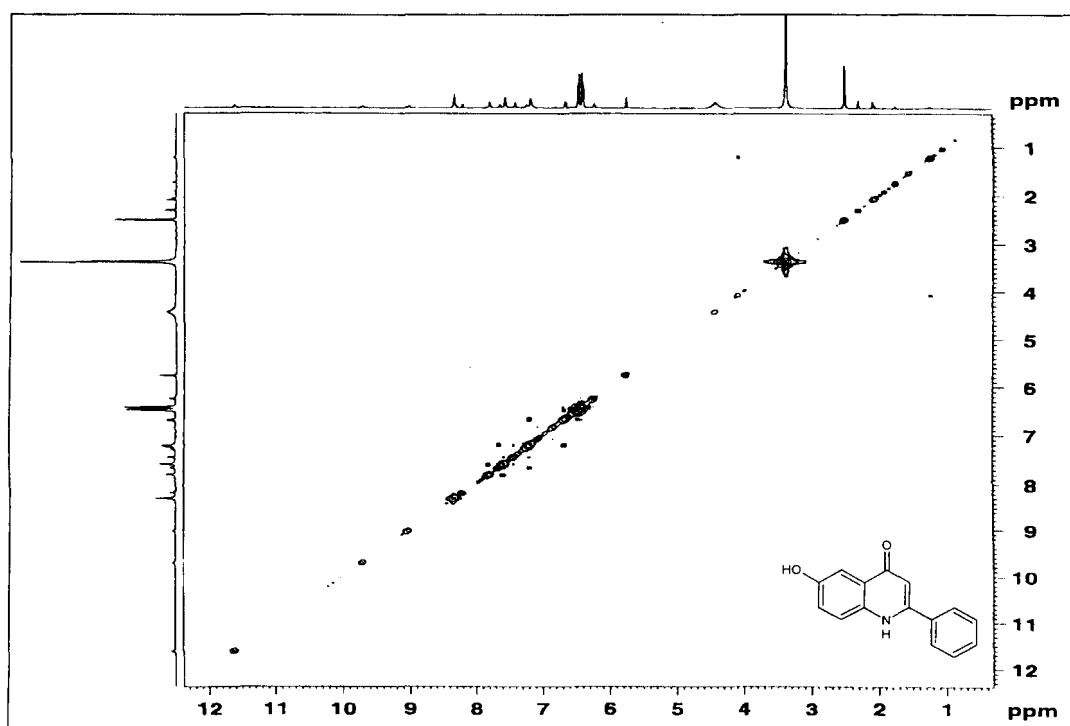
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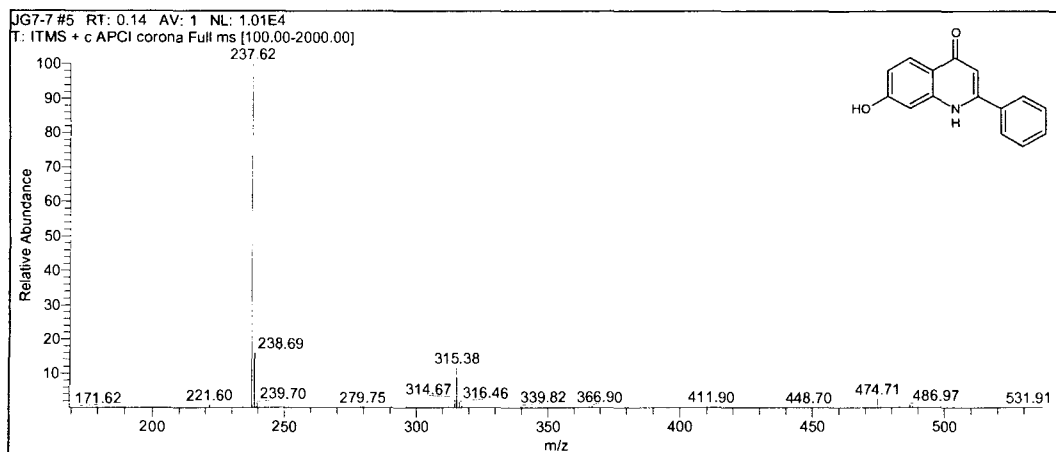
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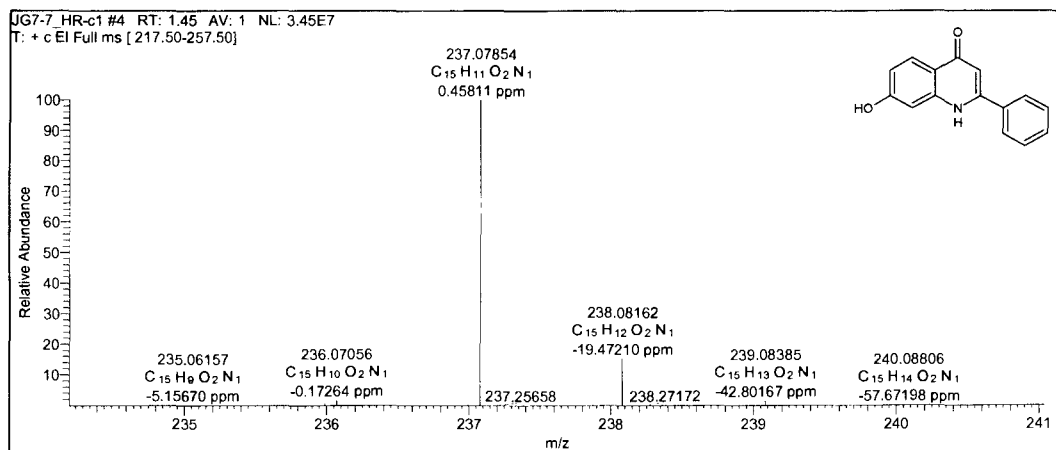
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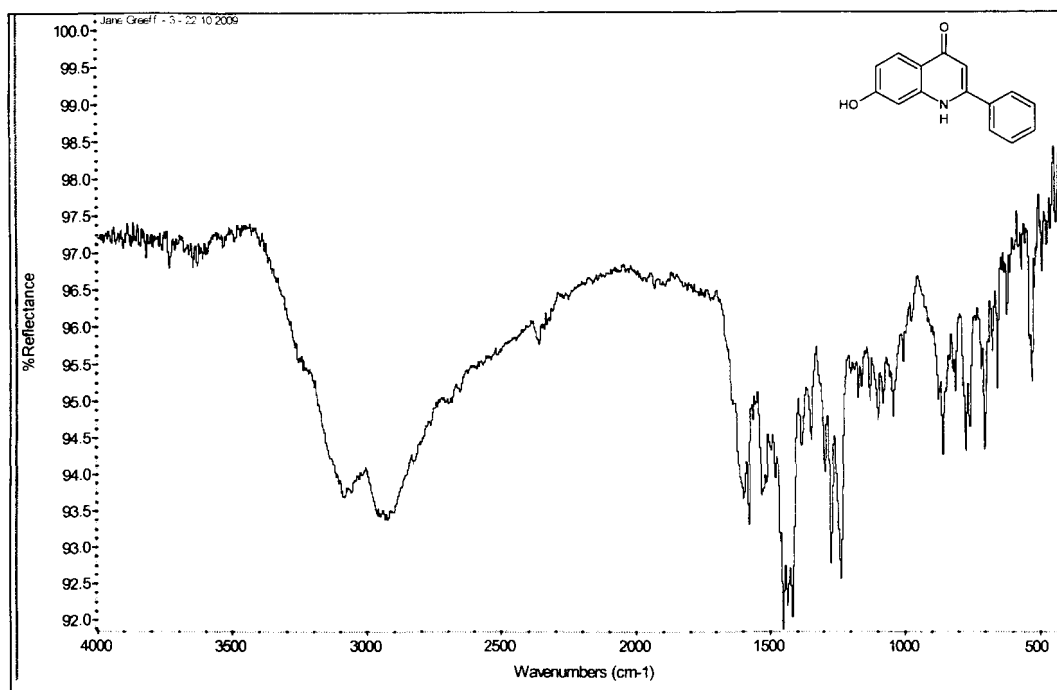
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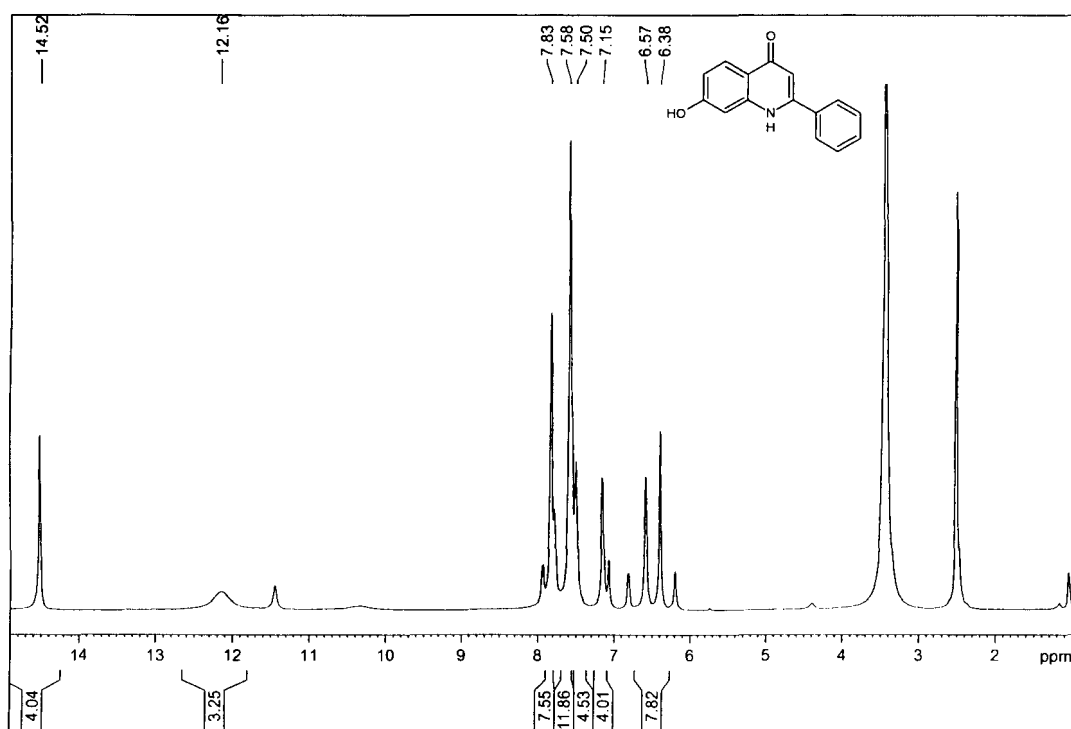
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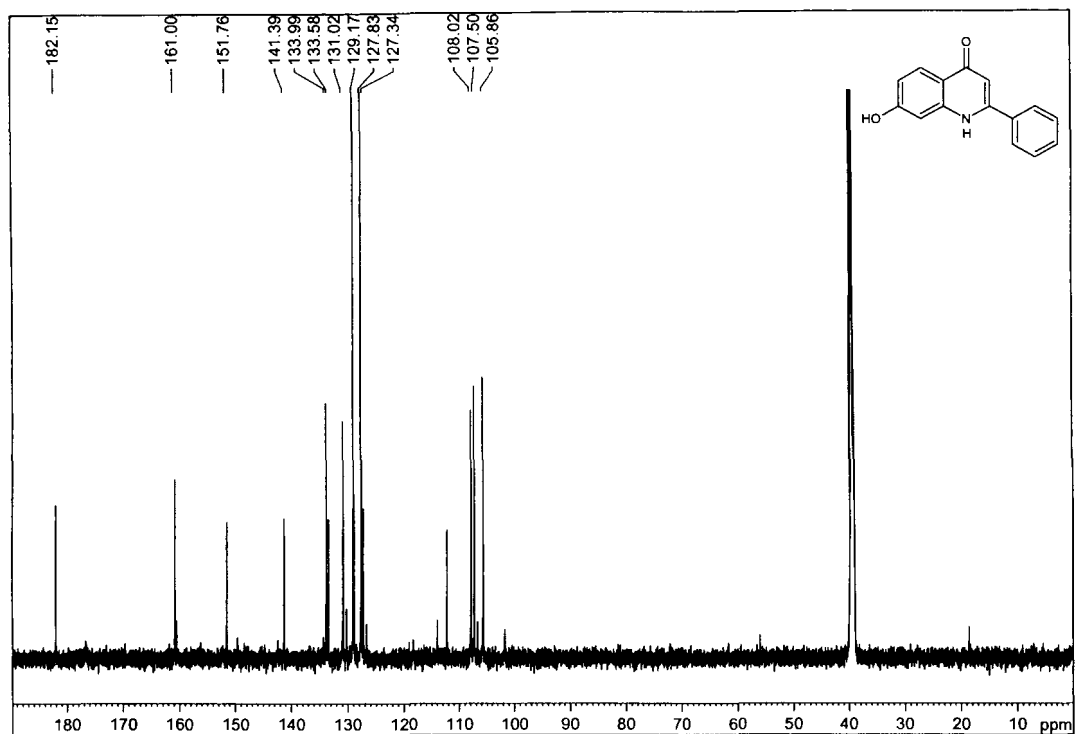
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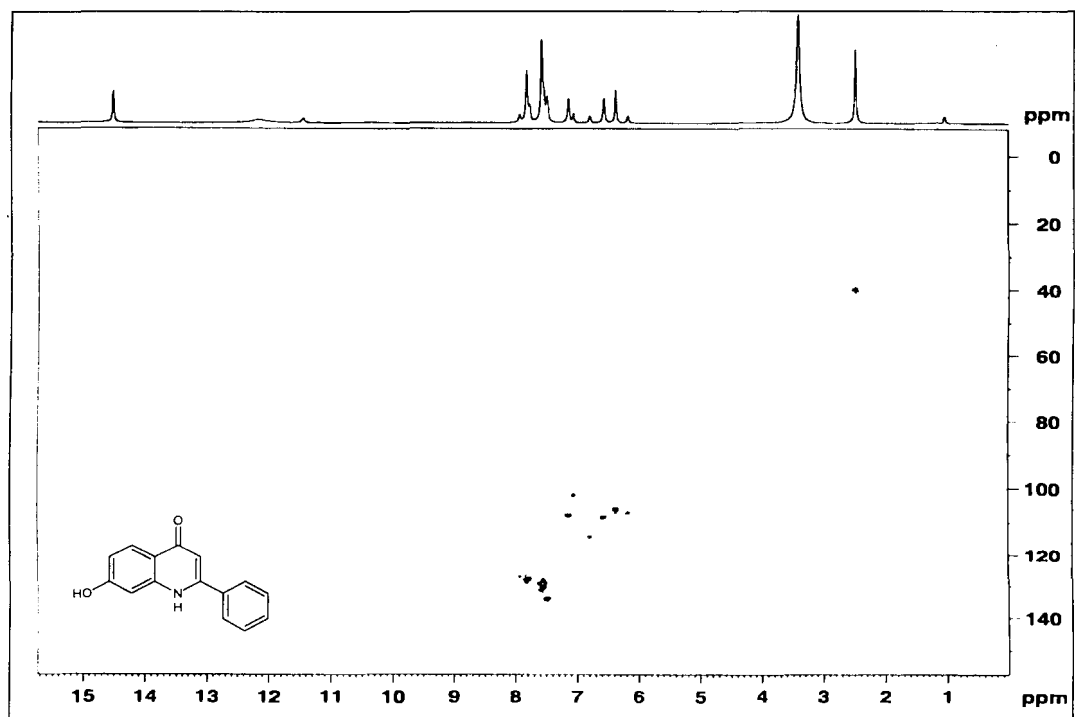
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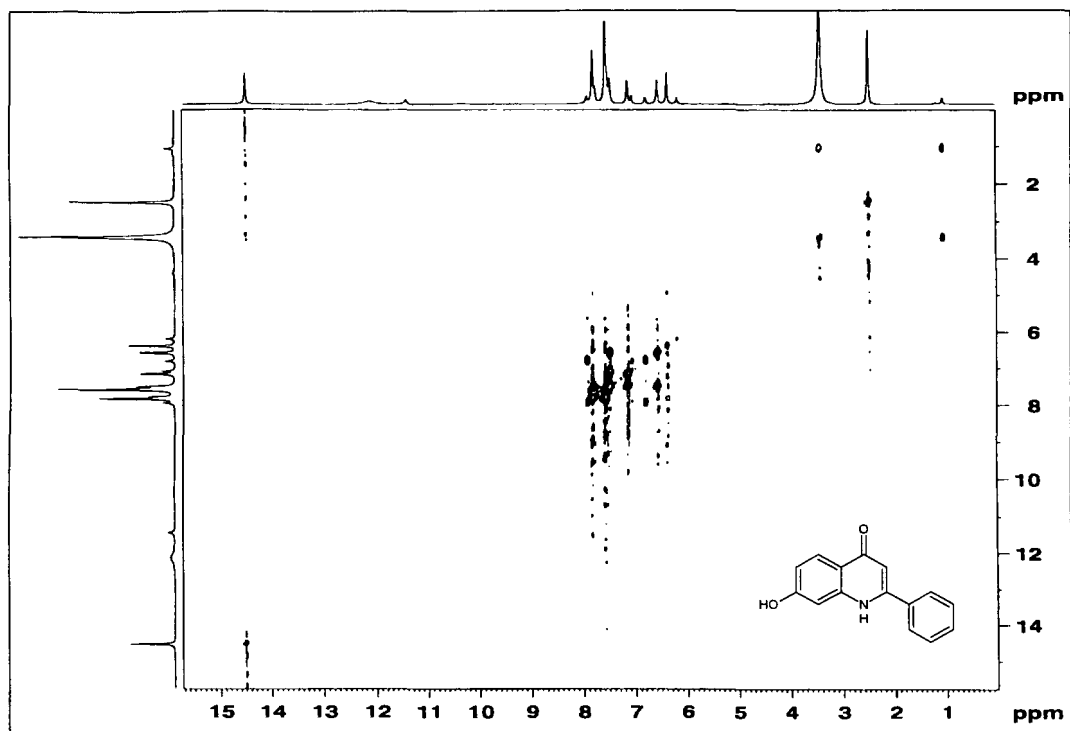
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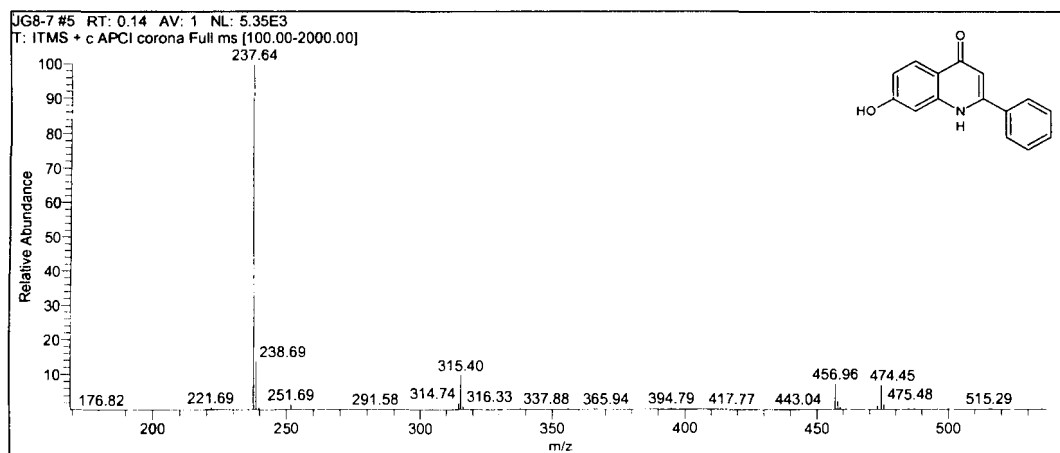
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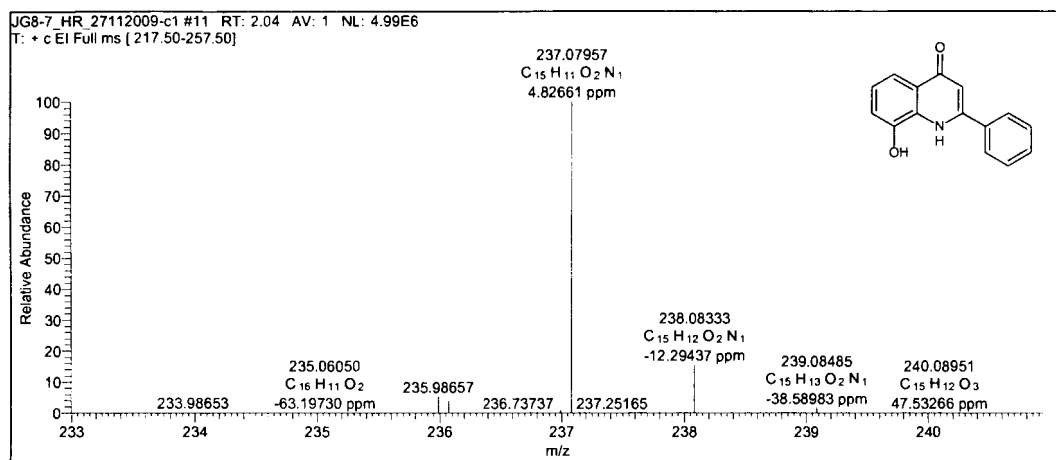
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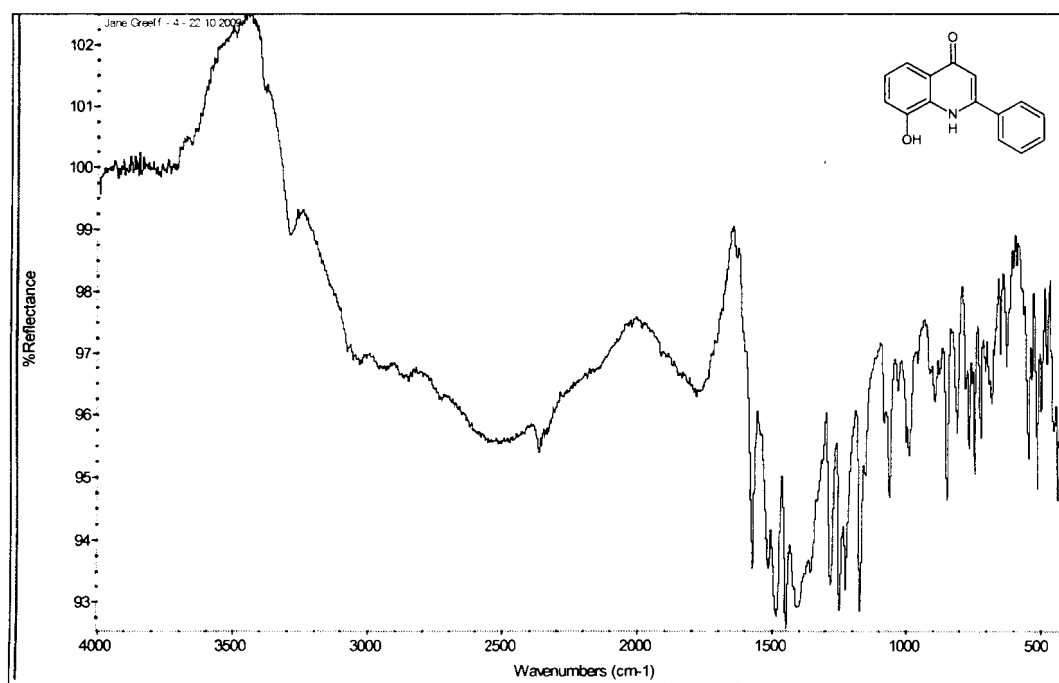
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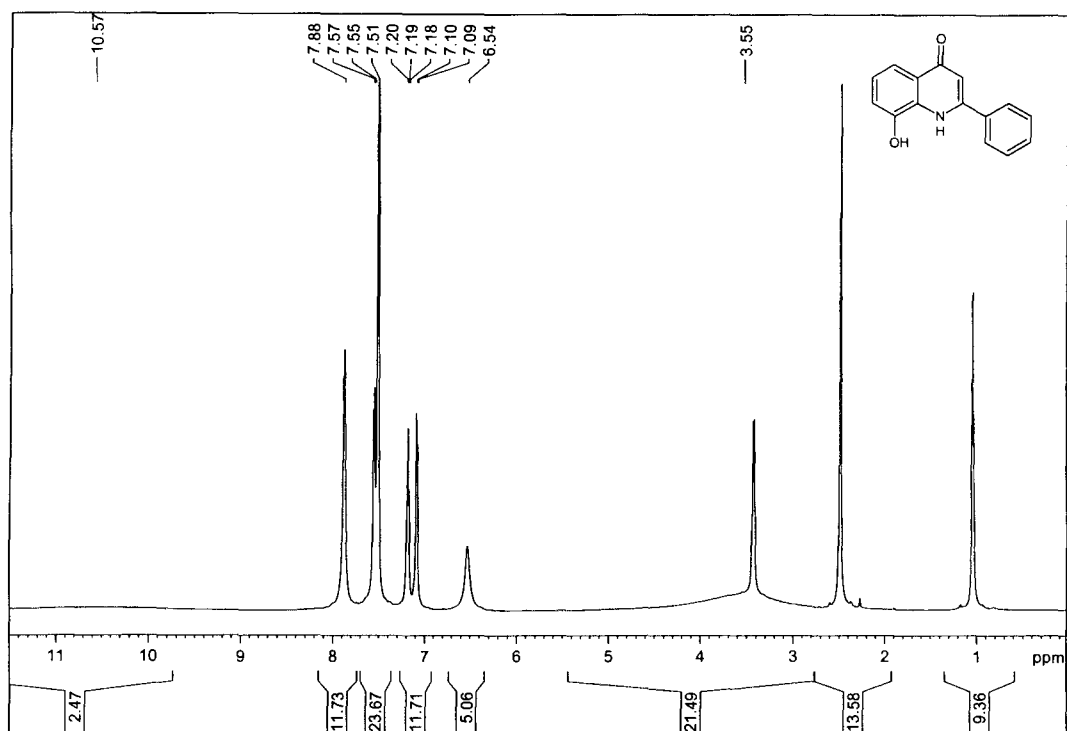
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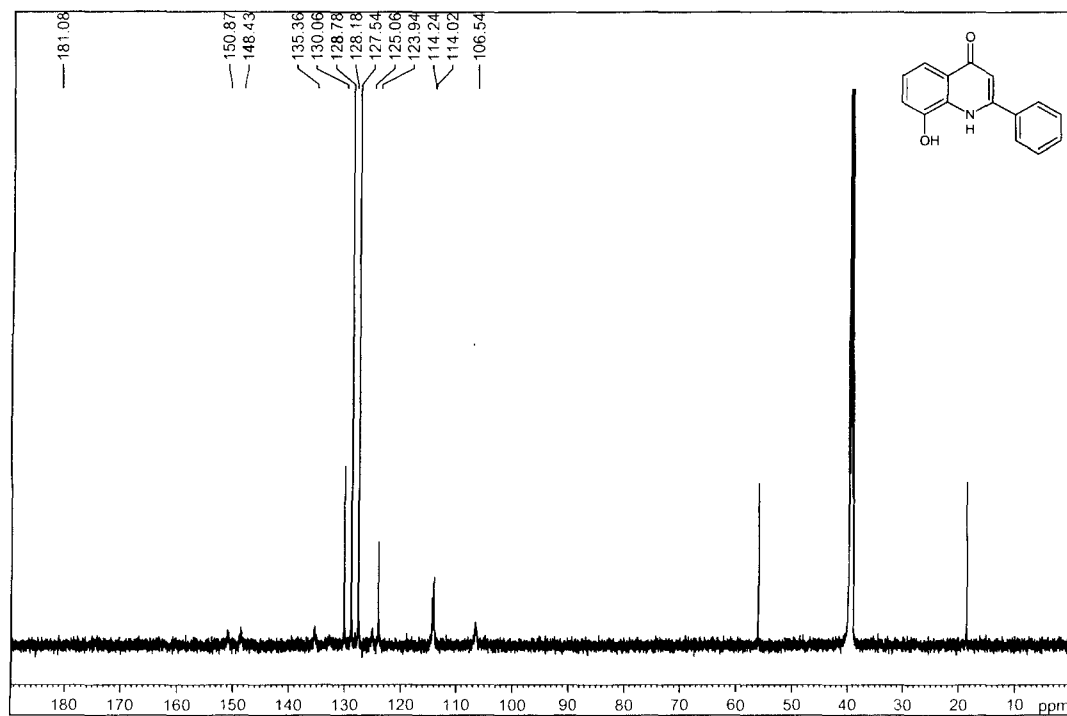
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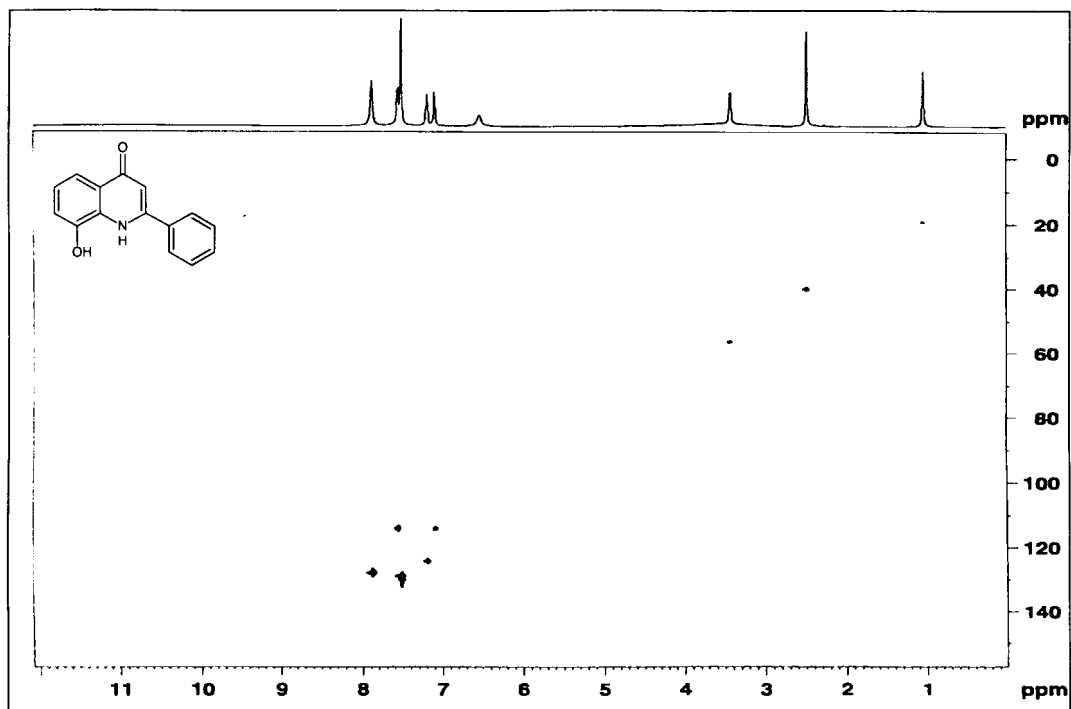
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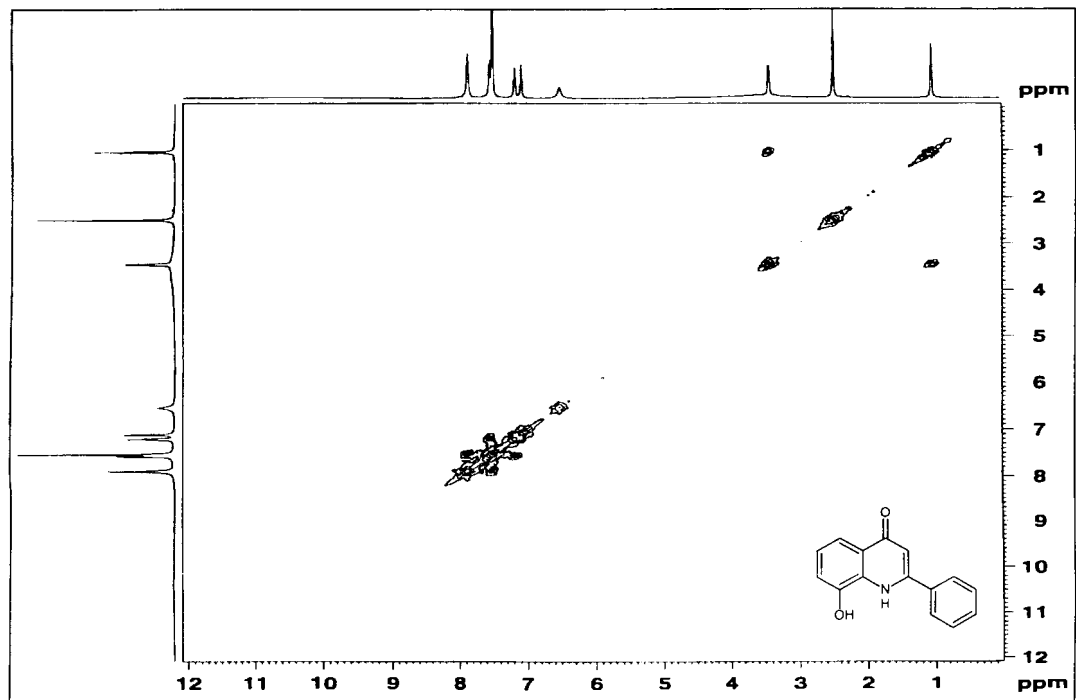
Spectrum 26 ^{13}C NMR of 8-Hydroxy-2-phenylquinolin-4(1H)-one (10)



Spectrum 27 Hsqc NMR of 8-Hydroxy-2-phenylquinolin-4(1H)-one (10)

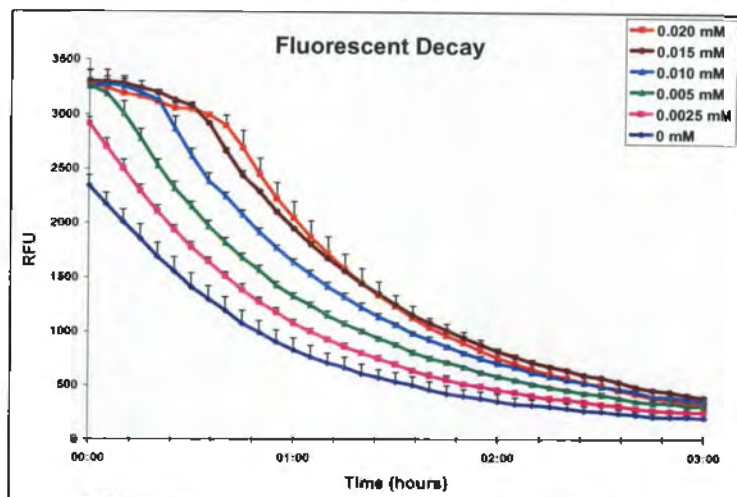


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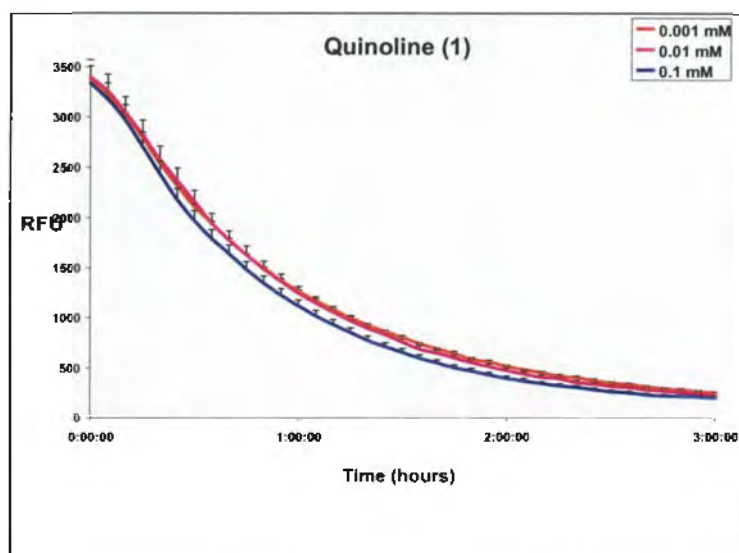


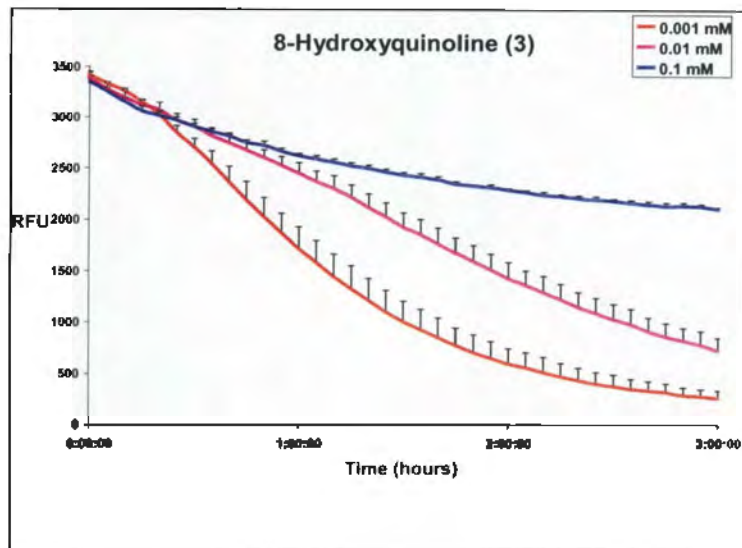
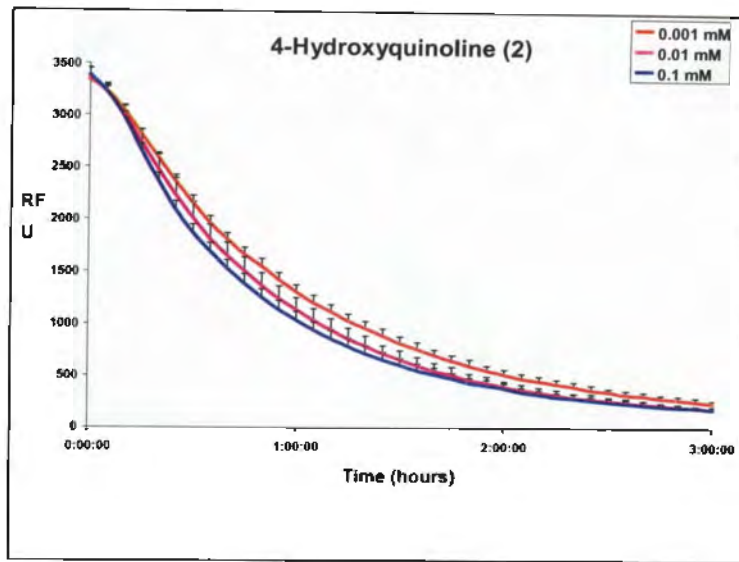
APPENDIX B

Fluorescence Decay observed for Trolox Standard in the presence of AAPH

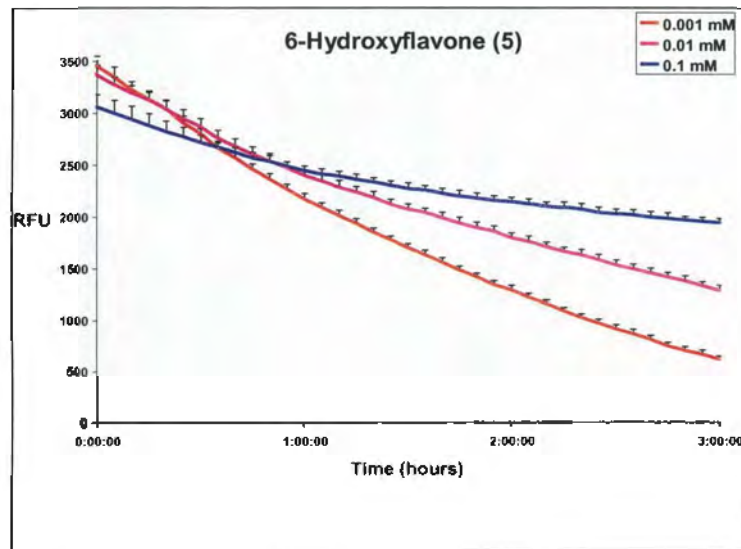
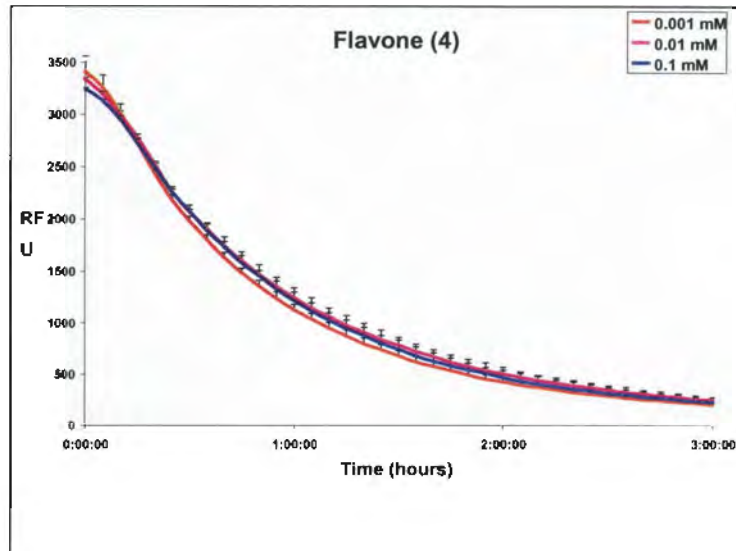


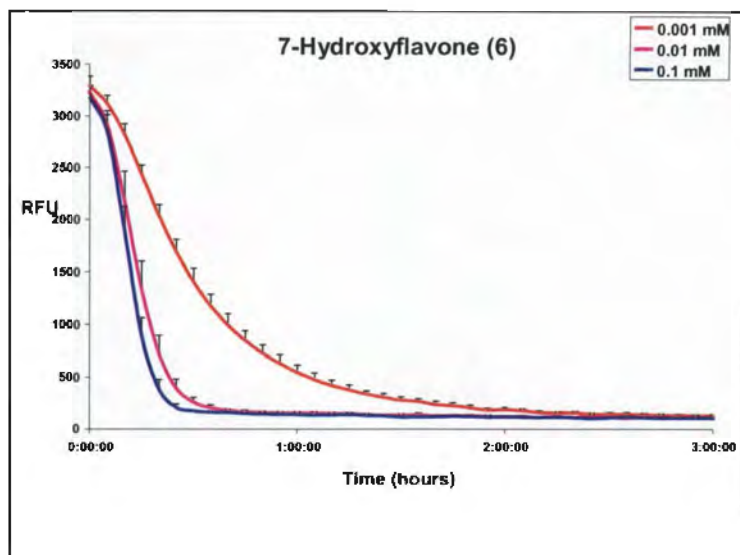
Fluorescence Decay observed for Quinolines (1 to 3) in 3 Concentrations in the presence of AAPH



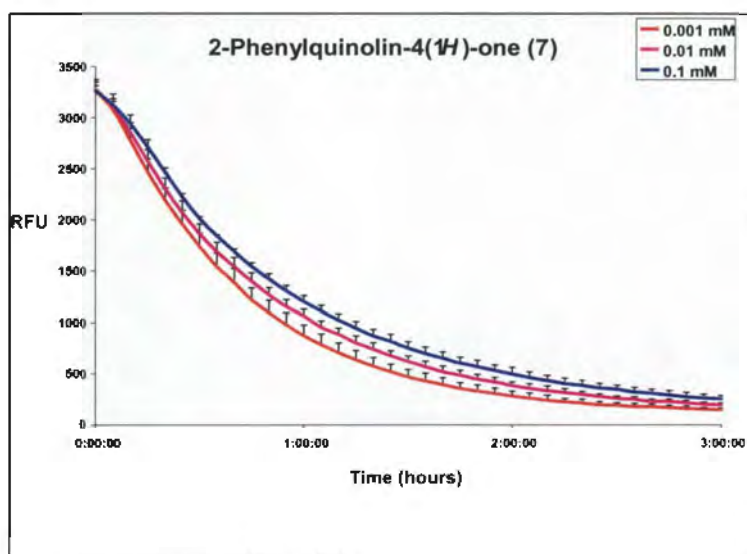


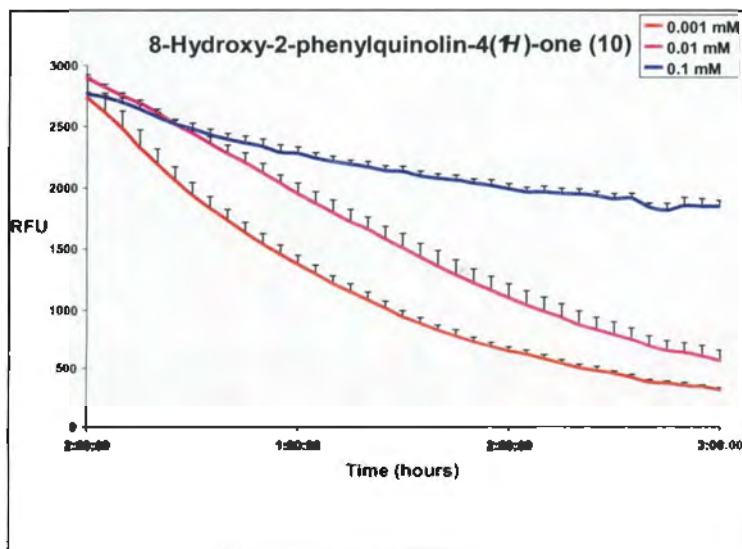
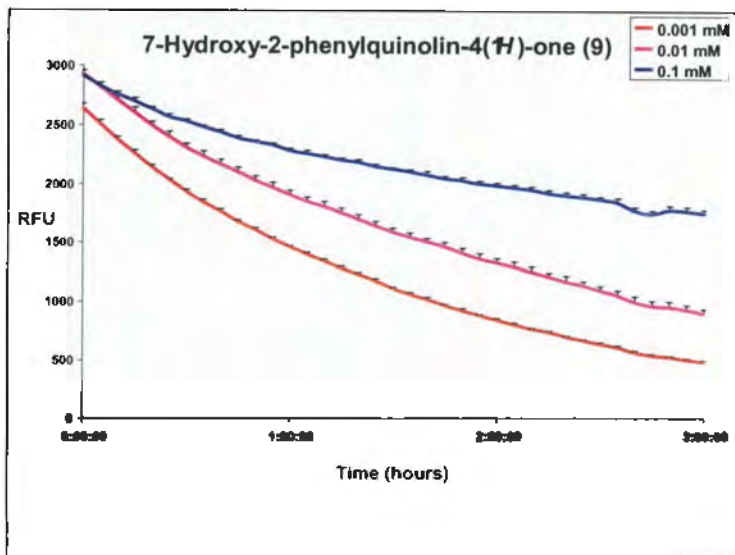
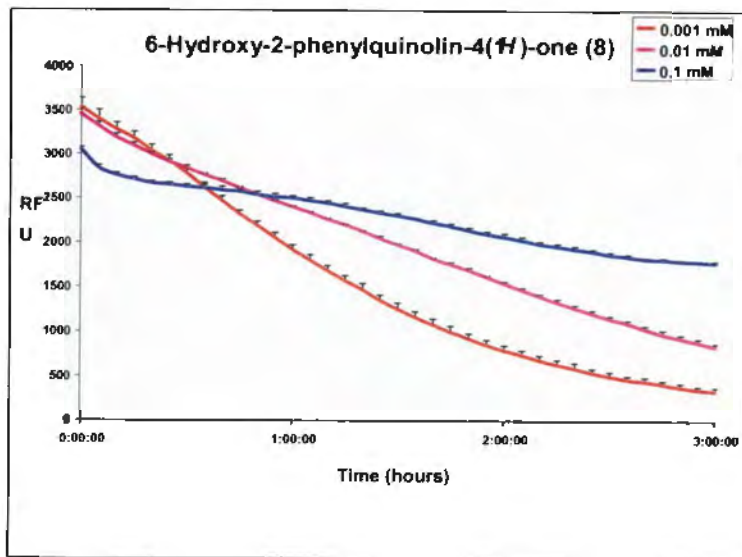
Fluorescence Decay observed for Flavones (4 to 6) in 3 Concentrations in the presence of AAPH



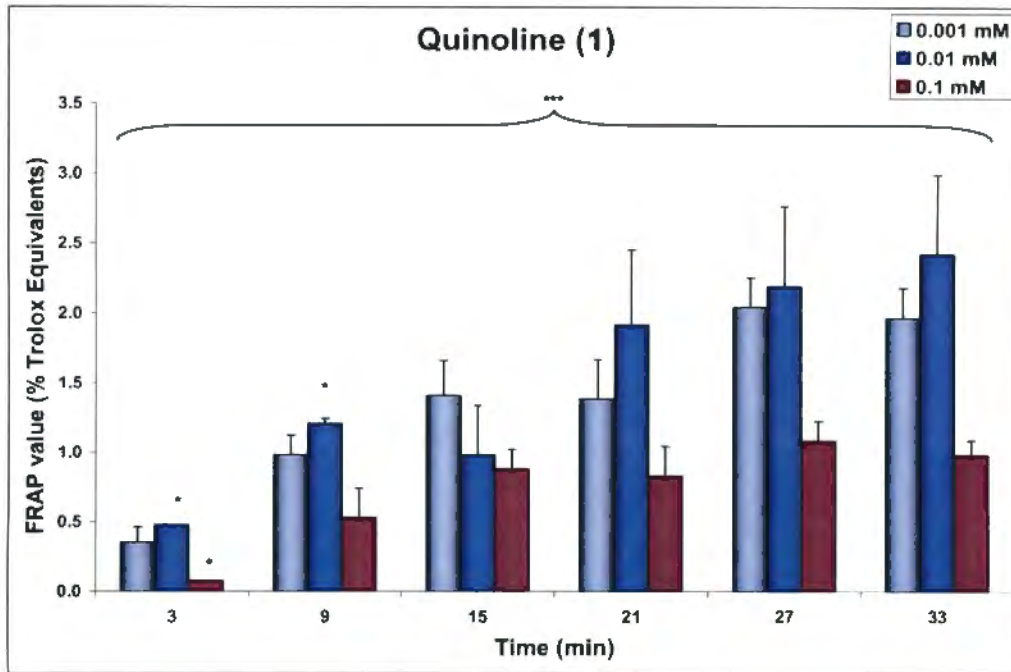


Fluorescence Decay observed for 2-Phenylquinolin-4(1*H*)-ones (7 to 10) in 3 Concentrations in the presence of AAPH

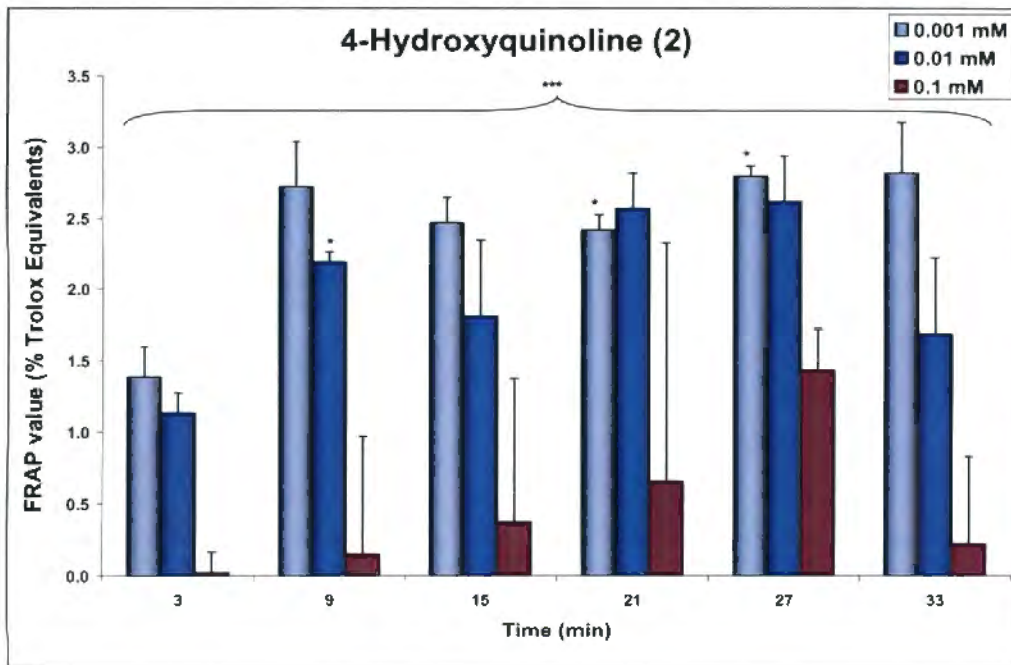




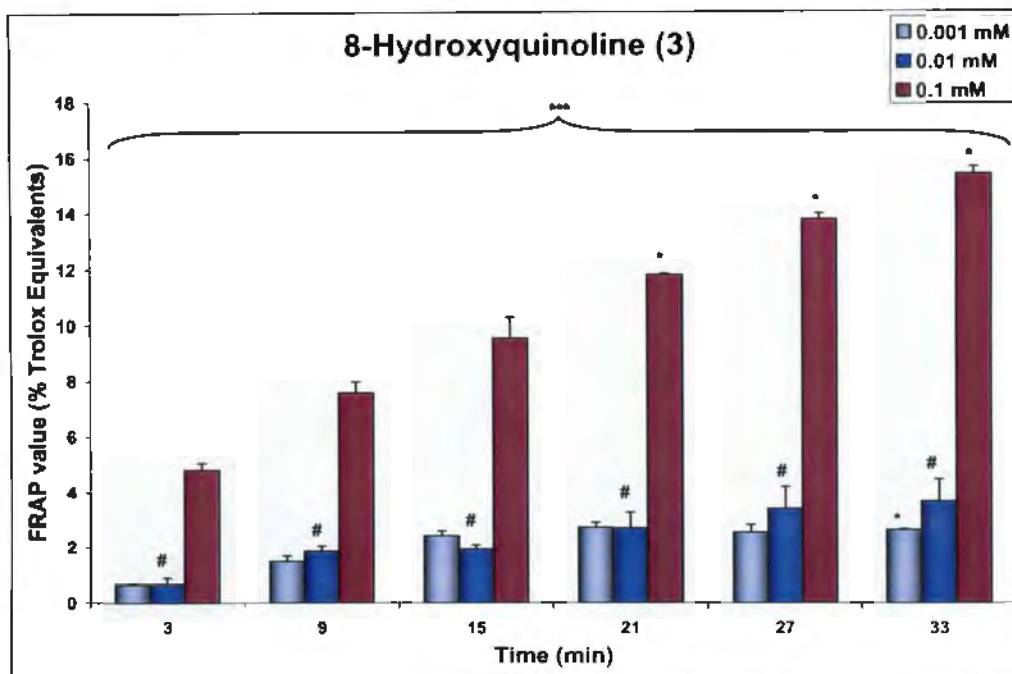
APPENDIX C



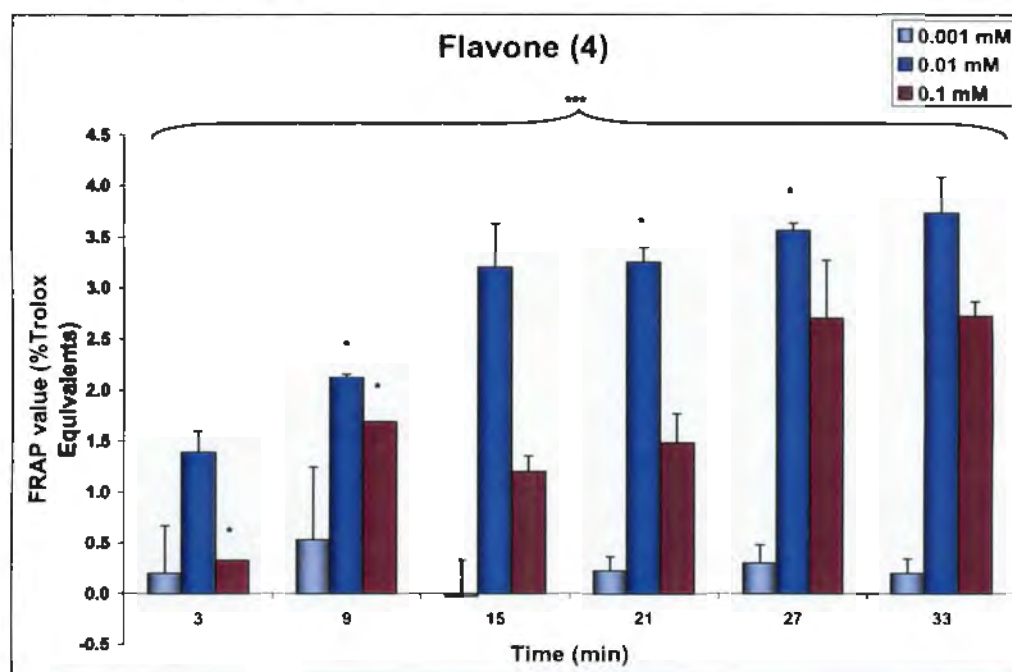
FRAP-values obtained for three concentrations Quinoline (1). Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox (Paired t-test).



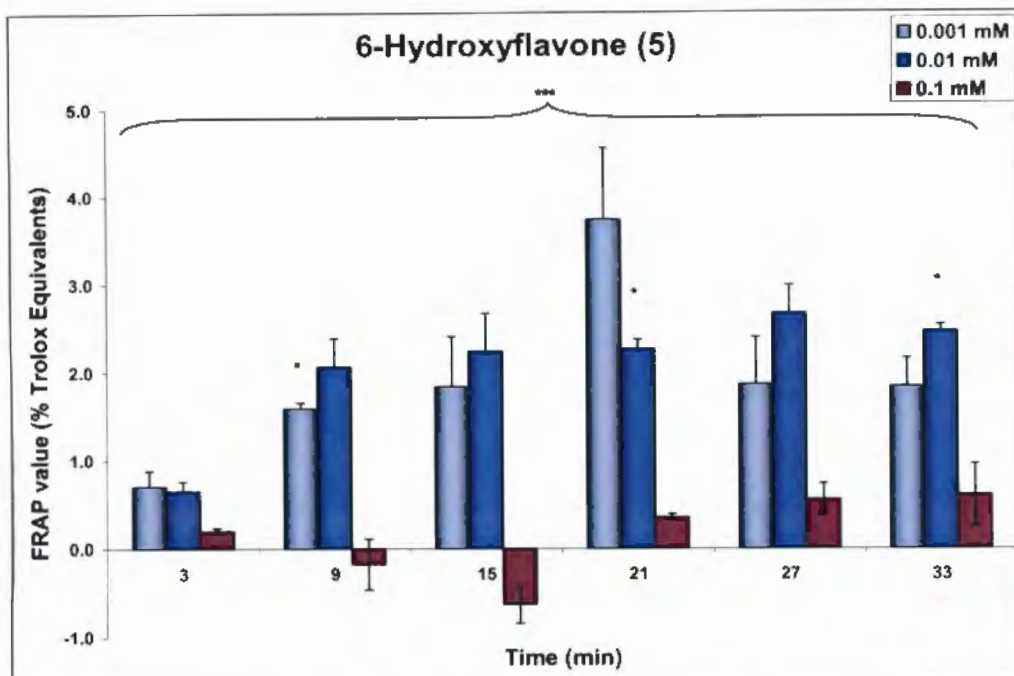
FRAP-values obtained for three concentrations 4-Hydroxyquinoline (2). Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox (Paired t-test).



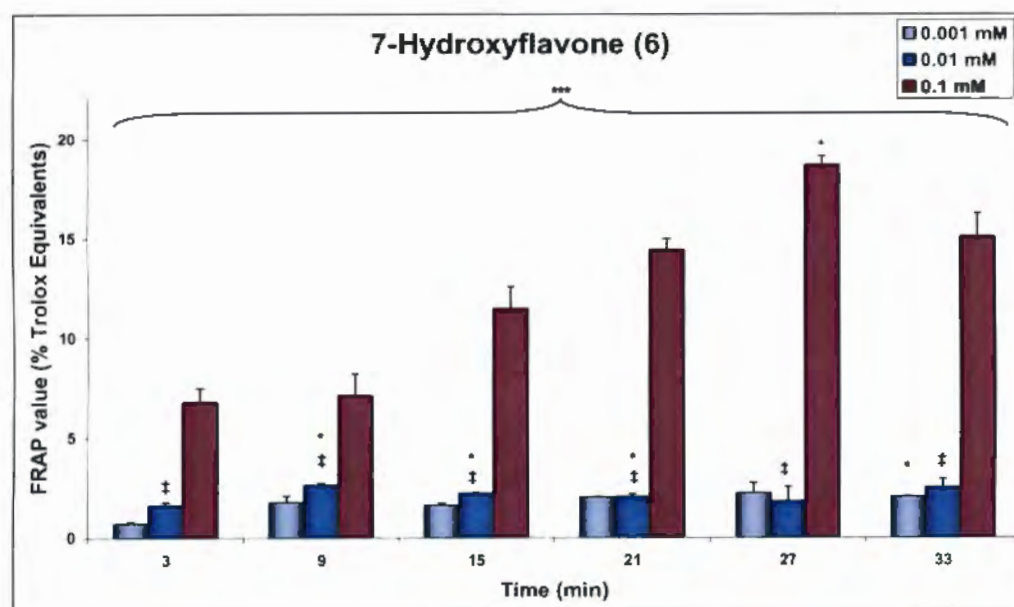
FRAP-values obtained for three concentrations 8-Hydroxyquinoline (3). Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox; #p=0.001 vs. 0.1 mM 8-hydroxyquinoline (Paired t-test).



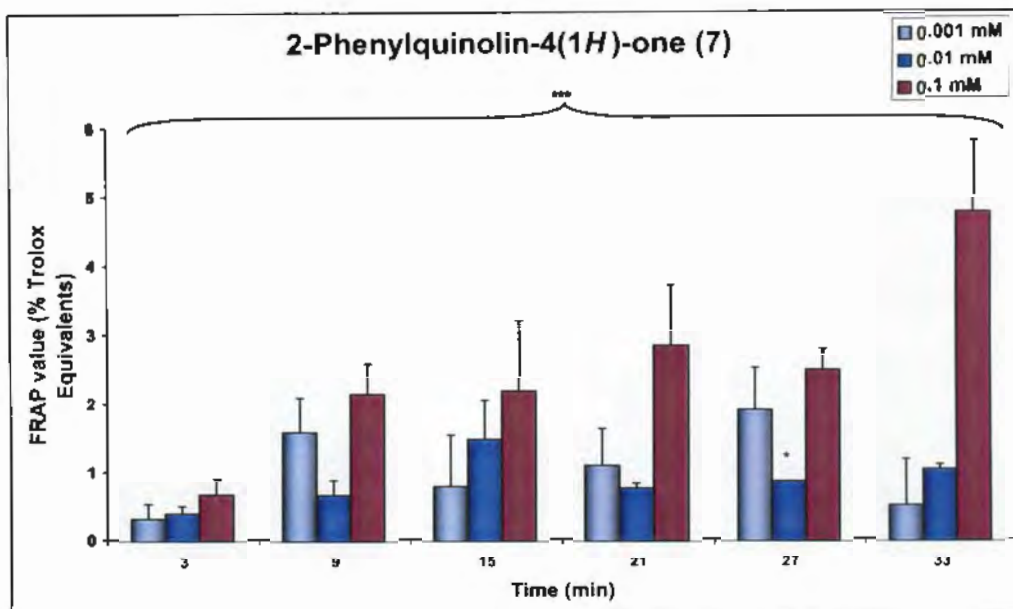
FRAP-values obtained for three concentrations Flavone (4). Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox (Paired t-test).



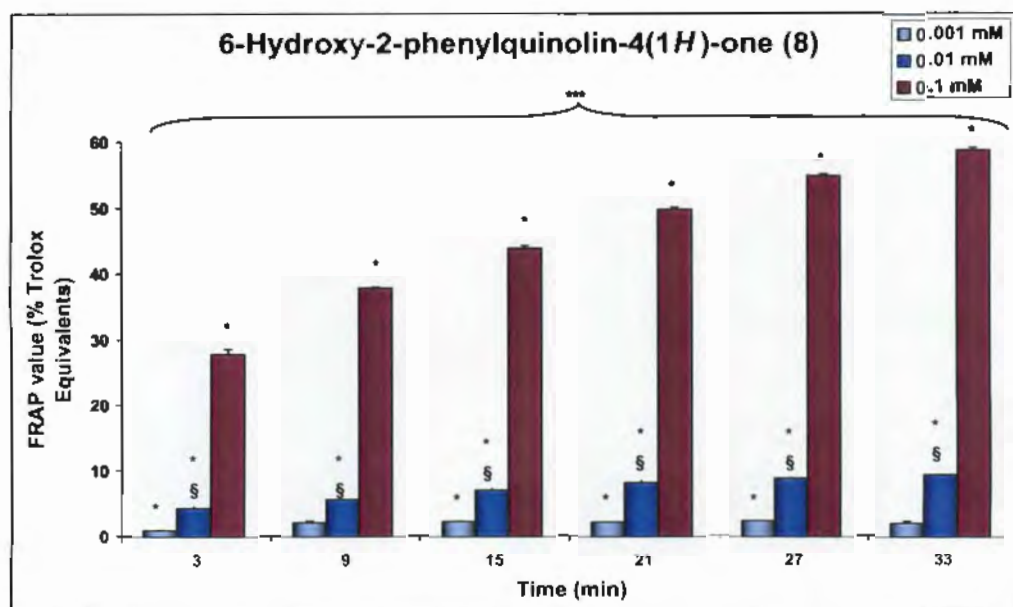
FRAP-values obtained for three concentrations 6-Hydroxyflavone (5). Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox (Paired t-test).



FRAP-values obtained for three concentrations 7-Hydroxyflavone (6). Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox; ‡p=0.0035 vs. 0.1 mM 7-hydroxyflavone (Paired t-test).



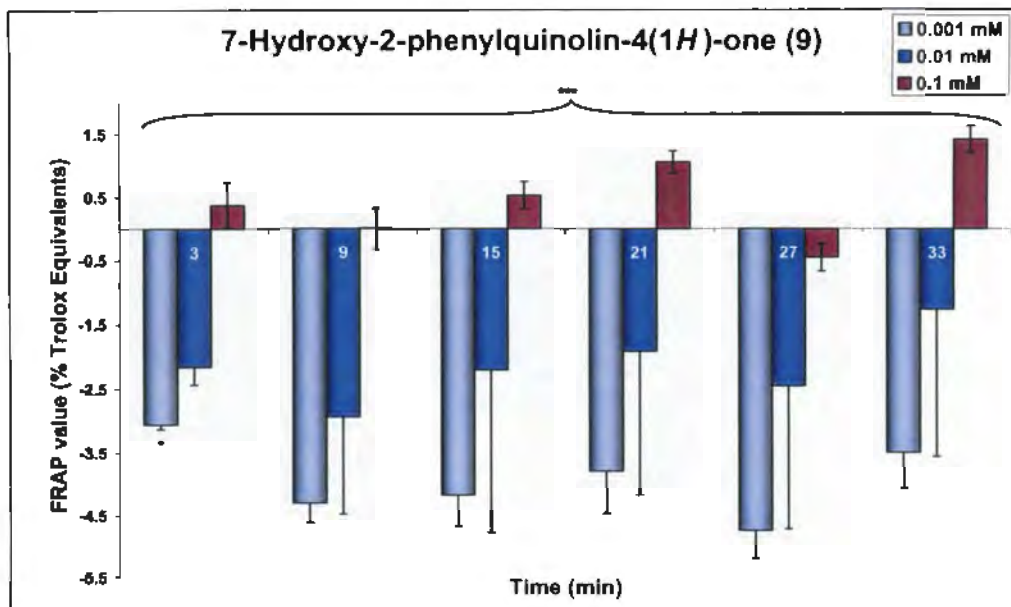
FRAP-values obtained for three 2-Phenylquinolin-4(1H)-one (7) concentrations. Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox (Paired



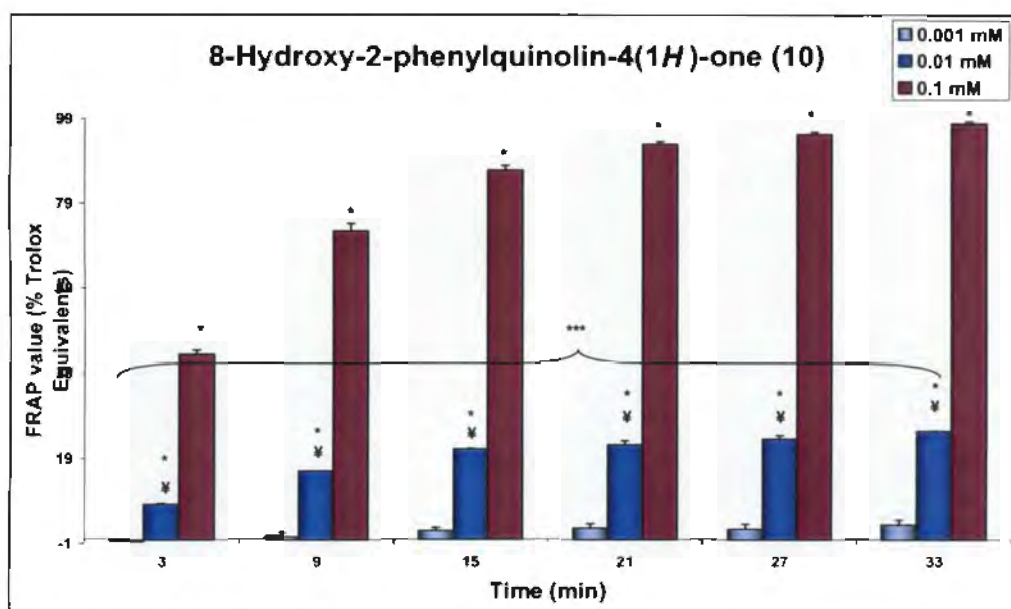
Hydroxy-2-phenylquinolin-4(1H)-one (8) concentrations. Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox; §p=0.0002 vs. 0.1 mM 6-hydroxy-2-phenylquinolin-4(1H)-one (Paired t-test).

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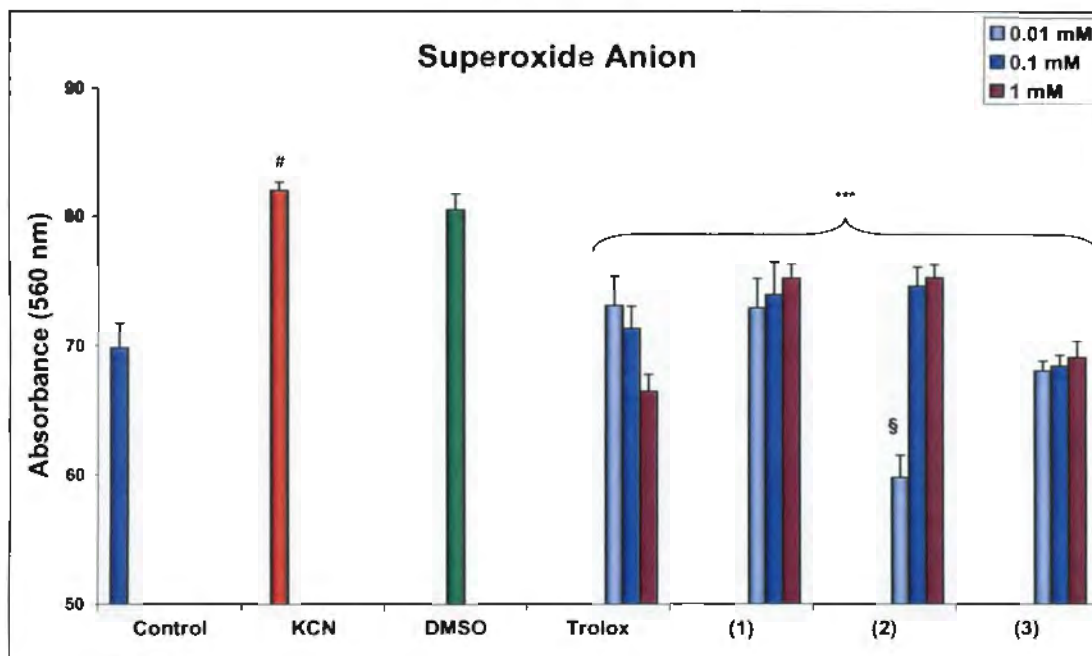


FRAP-values obtained for three 7-Hydroxy-2-phenylquinolin-4(1H)-one (9) concentrations. Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox (Paired t-test).

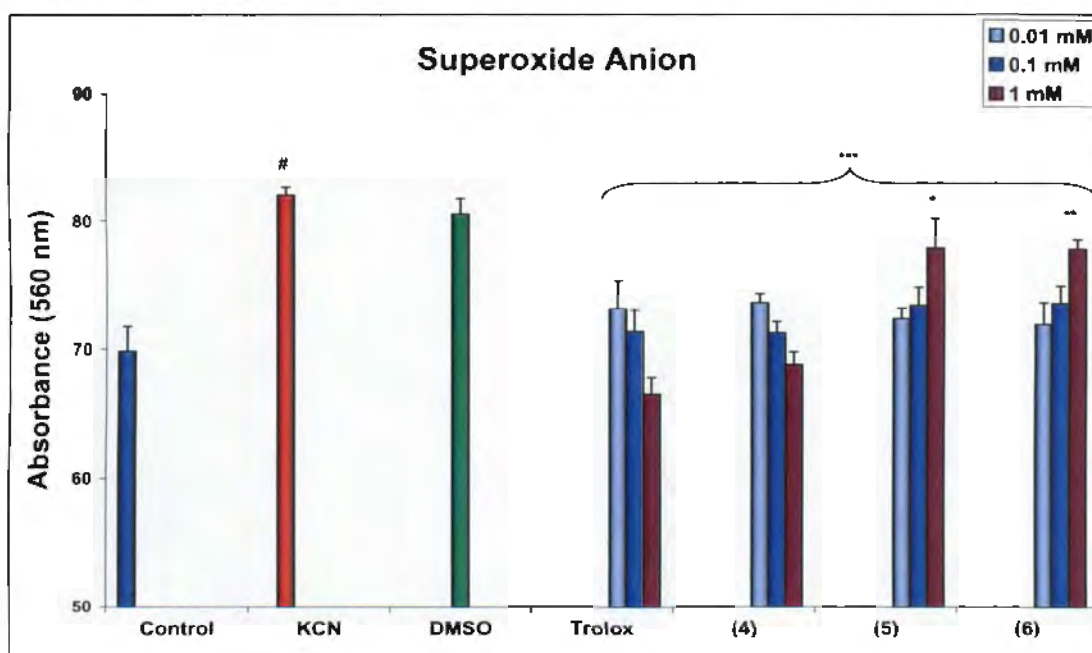


FRAP-values obtained for three 8-Hydroxy-2-phenylquinolin-4(1H)-one (10) concentrations. Each bar represents the mean \pm S.D.; *R.S.D. < 5%; N=3; ***p<0.0001 vs. 0.1 mM Trolox; †p<0.0001 vs. 0.1 mM 8-hydroxy-2-phenylquinolin-4(1H)-one (Paired t-test).

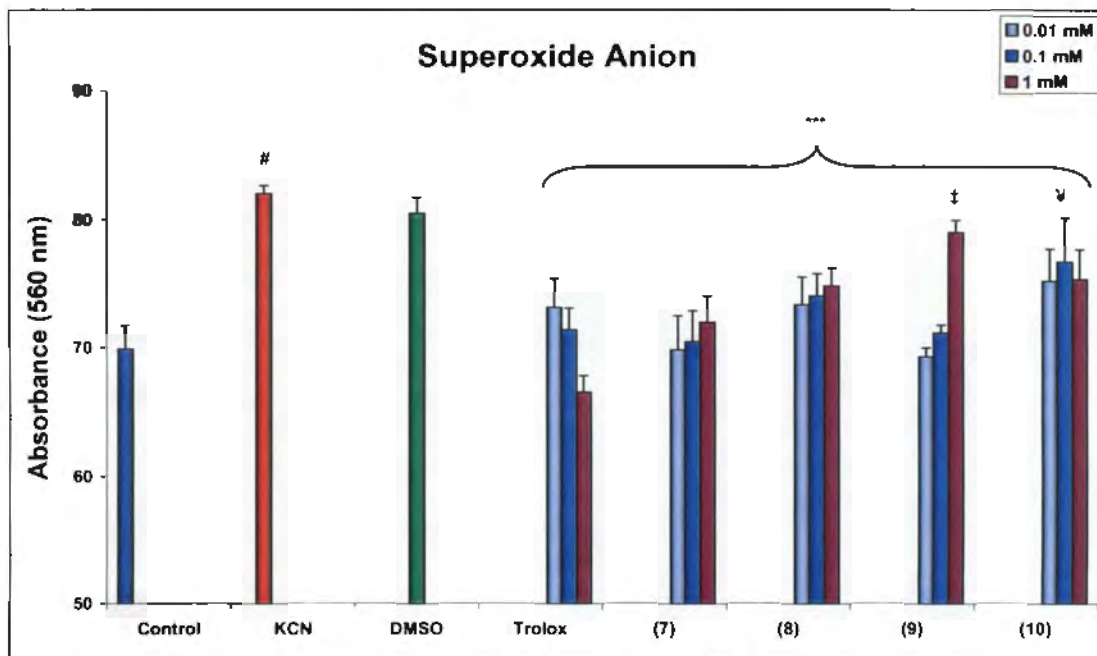
APPENDIX D



The effect of Quinolines (1 to 3) on superoxide anion production by KCN in rat brain homogenate. Each bar represents the mean \pm S.E.M.; N=10; # p <0.0001 vs. blank, *** p <0.0001 vs. KCN; § p =0.0015 vs. blank.

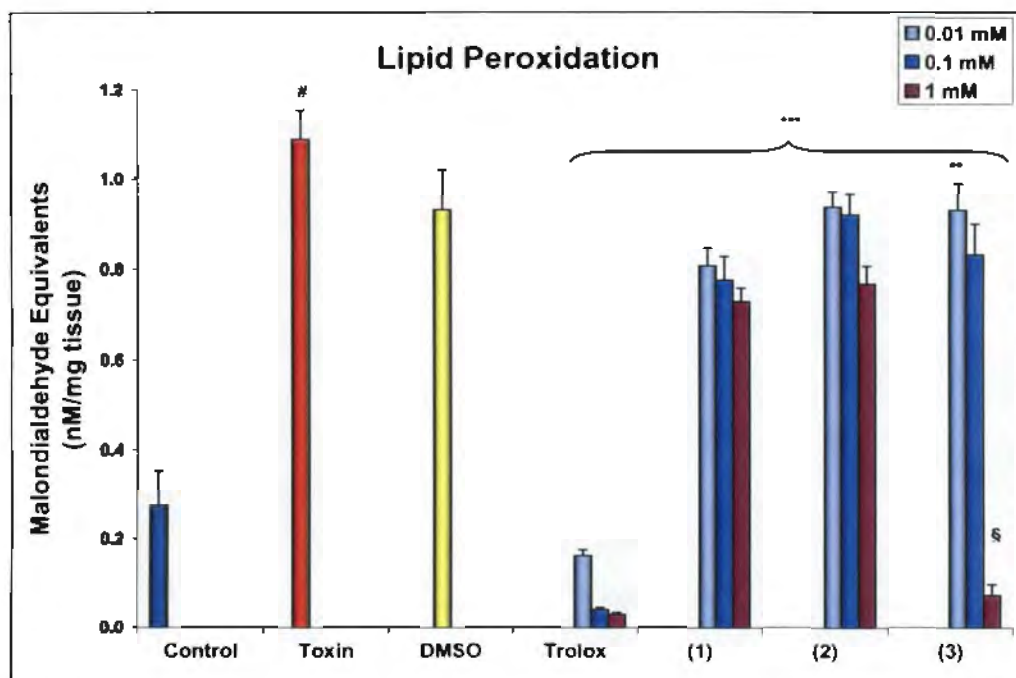


The effect of Flavones (4 to 6) on superoxide anion production by KCN in rat brain homogenate. Each bar represents the mean \pm S.E.M.; N=10; # p <0.0001 vs. blank, *** p <0.0001 vs. KCN, ** p =0.0047 vs. KCN, * p =0.0237 vs. KCN.

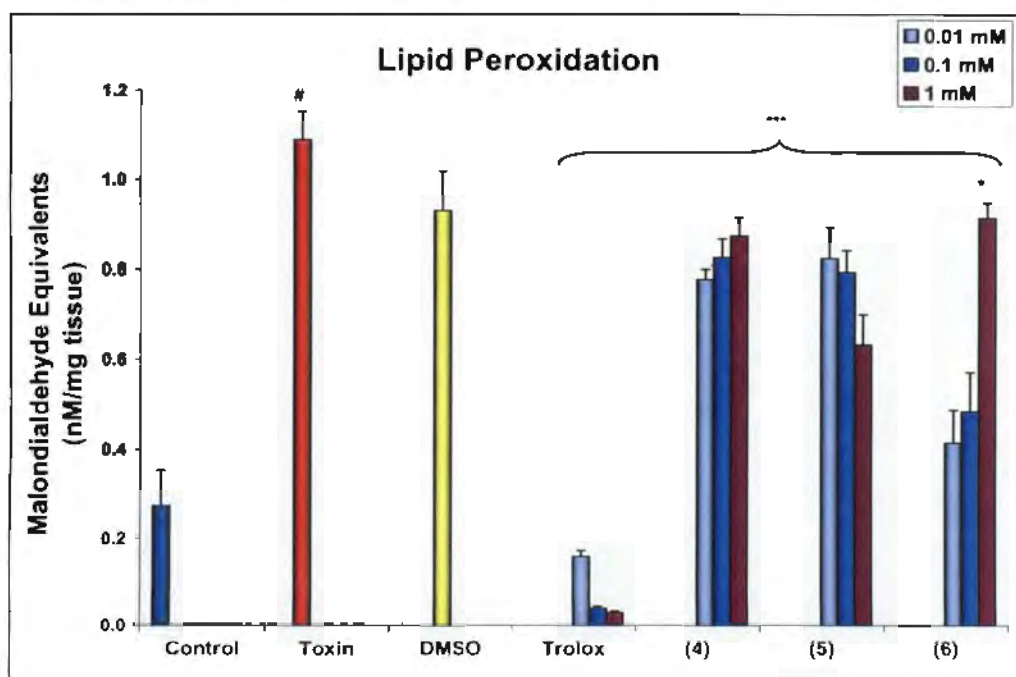


The effect of 2-Phenylquinolin-4(1*H*)-ones (7 to 10) on superoxide anion production by KCN in rat brain homogenate. Each bar represents the mean \pm S.E.M.; N=10; # p <0.0001 vs. blank, *** p <0.0004 vs. KCN, ‡ p =0.0394 vs. KCN, ¥ p =0.0155 vs. KCN.

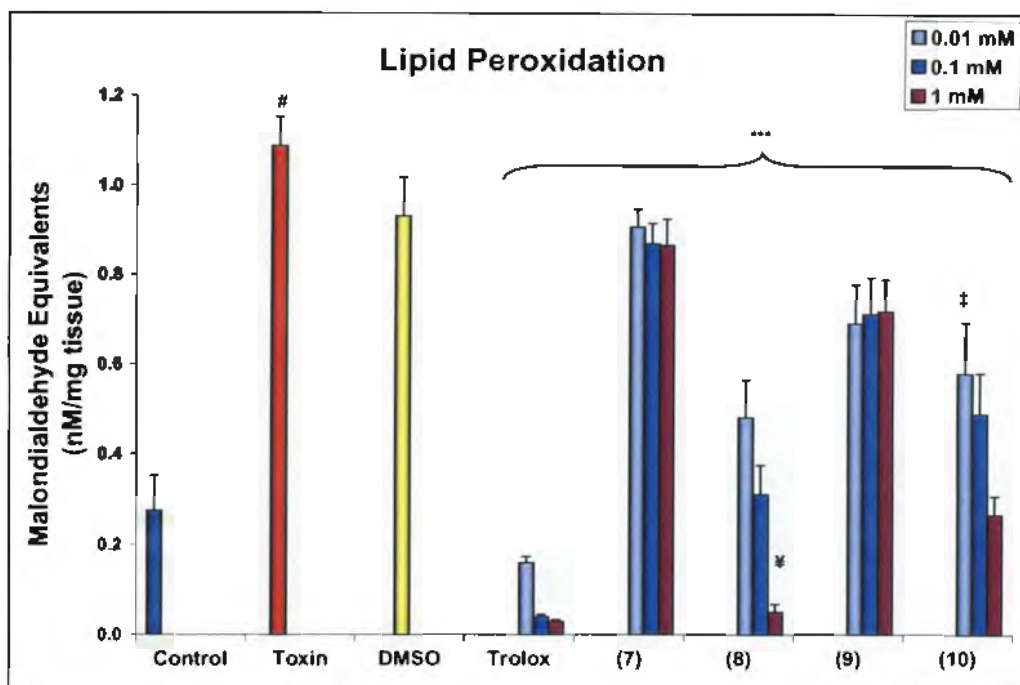
APPENDIX E



Lipid Peroxidation Inhibition of three concentrations of Quinolines (1 to 3). Each bar represents the mean \pm S.E.M.; N=10; #p<0.0001 vs. blank; ***p<0.0001 vs. Toxin; **p=0.0014 vs. Toxin; §p=0.0217 vs. blank.



Inhibition of three concentrations of Flavones (4 to 6). Each bar represents the mean \pm S.E.M.; N=10; #p<0.0001 vs. blank, ***p<0.0001 vs. Toxin; *p=0.1902 vs. Toxin.



Inhibition of three concentrations of 2-Phenylquinolin-4(1*H*)-ones (7 to 10). Each bar represents the mean \pm S.E.M.; N=10; #p<0.0001 vs. blank, ***p<0.0002 vs. Toxin, †p=0.0104 vs. blank; ‡p=0.0013 vs. Toxin.