The antioxidant properties of the methanol extract of *Cotyledon orbiculata* L. var *orbiculata* (Haw.) DC. Leaves.

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Die antioksidant aktiwiteit van die metanol ekstrak van *Cotyledon orbiculata* L. var *orbiculata* (Haw.) DC. Blare.

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LIST OF ABBREVIATIONS

ACN	Acetonitrlie
ADP	Adenosine diphosphate
BHT	Butylated hydroxytoluene
DMEM	Dulbecco's modified eagle medium
GAA	Glacial acetic acid
GABA	Gamma amino buturic acid
GPX	Glutathione peroxidase
GSR	Glutathione reductase
GSR	Glutathione reductase
GS-SG	Glutathione co-factor
GST	Glutathione-S-transferase
H_3PO_4	Phosphate buffer
HPLC	High pressure liquid chromatography
IR	Infrared spectroscopy
KCN	Potassium cyanide
LC/QTOF	Accurate-Mass Time-of-Flight spectrometer
LPO	Lipid peroxidation
MDA	Malondialdehyde
MDA	Malondialdehyde
MS	Mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	Nitroblue tetrazolium
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance spectroscopy
NO•	Nitric oxide radical

NOS	Nitric oxide synthase
NWU	Northwest University
$O_2\bullet^-$	Superoxide radical
$OH\bullet$	Hydroxyl radical
$ONOO^-$	Peroxynitrate
PBS	Phosphate buffer
PenStrep	Penicillin and streptomycin
RNS	Reactive Nitrogen species
R-OOH	Organic hydroperoxides
ROS	Reactive oxygen species
SDBS	Spectral database for organic compounds
SOD	Superoxide dismutase
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
UV	Ultra violet
WHO	The World Health Organisation

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ABSTRACT

South Africa is a country of great diversity. Different climate zones and a host of different habitats make South Africa the perfect platform for rich floral diversity. This floral diversity lends itself to the study of natural products by discovering new “natural drugs” that can be used in the treatment of many illnesses.

Studies into the antioxidant properties of plants that are used in traditional medicine are an important aspect of research to determine the rationale of the use of plants by traditional healers.

Many neurodegenerative diseases, like epilepsy, Parkinson’s and Alzheimer’s diseases, are linked to oxidative stress. Antioxidants could play a major role as neuroprotective agents and could alter the progression of these diseases.

Epilepsy is one of the world’s most prevalent central nervous system disorders and affects more than seventy per one thousand children in South Africa. Most of these cases are people in rural areas of South Africa where communities rely on the use of traditional medicine.

Cotyledon orbiculata L. var *orbiculata* (Haw.) DC. is widely used in traditional medicine to treat epilepsy and other central nervous system disorders. The need to screen these plants for activity and toxicity is very important to understand the complex mechanism of action in the treatment of patients.

In this study the methanol extract and three different fractions of the methanol extract of *Cotyledon orbiculata* were used to test for antioxidant activity and toxicity towards neuroblastoma cells.

The freeze dried leaves of *Cotyledon orbiculata* were extracted with methanol using a Soxhlet apparatus. The concentrated extracts were analysed using HPLC (high pressure liquid chromatography) and three major peaks were selected for isolation.

Three assays were performed to assess the antioxidant activity and toxicity of the isolated compounds.

The thiobarbituric acid assay (TBA) quantifies the extent of the inhibition of lipid peroxidation in rat brain homogenates by the isolated fractions.

All of the samples were able to attenuate lipid peroxidation as seen from the results obtained from the TBA assay. The methanol extract showed the best attenuation of lipid peroxidation in the rat brain homogenate with fraction 1 and 2 showing greater attenuation of lipid peroxidation than fraction 3.

The nitroblue tetrazolium assay (NBT) quantifies the ability of the fractions to scavenge superoxide radicals in a rat brain homogenate.

All samples were able to scavenge superoxide radicals as indicated by the NBT assay. The methanol extract showed the best superoxide scavenging abilities in the assay whereas fraction 1 showed better scavenging abilities than fraction 2 and 3.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) indicates the toxicity of the fractions towards neuroblastoma cells.

The methanol extract and fraction 2 in the highest concentration of 10 mg/ml were the only samples that showed toxicity towards neuroblastoma cells.

The molecular structure of a compound from fraction 2 was determined by using nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy (IR), and mass spectroscopy (MS). This compound was identified as diethyl malate. Diethyl malate is an artefact that is generated in HPLC procedures in the presence of malic acid (which naturally occurs in the leaves of *Cotyledon orbiculata*) and ethanol.

The methanol extract of *Cotyledon orbiculata* has high antioxidant activity and could be due to the presence of malic acid in the leaves of the plant. The rationale in the use of *Cotyledon orbiculata* in the treatment of epilepsy could not be determined due to the isolation of an artefact, diethyl malate, obtained from the fraction.

Further research should include methods to prevent artefact formation and purification of the samples that are obtained.

Key terms: *Cotyledon orbiculata*, methanol extract, HPLC, antioxidants, toxicity.

OPSOMMING

Verskillende klimaatstreke en ŉ verskeidenheid van habitattipes skep die perfekte omstandighede vir Suid-Afrika se ryk plantegroei. Hierdie diversiteit dra by tot die studie van natuurlike produkte deur medisinale navorsing met die oog op die ontdekking van nuwe “natuurlike geneesmiddels” wat kan gebruik word in die behandeling van verskillende siektetoestande.

Studies van natuurlike antioksidante vanaf plante wat gebruik word in tradisionele medisyne is belangrik om die gebruik van hierdie plante in die behandeling van siekte toestande te verstaan.

Baie sentralesenuweestelselsiektes soos Parkinsonisme, Alzheimer se siekte en epilepsie is as gevolg van ŉ wanbalans van pro-oksidante en antioksidante. Hierdie wanbalans staan bekend as oksidatiewe stres.

Epilepsie is een van die algemeenste sentralesenuweestelselsiektes en beïnvloed meer as sewentig uit eenduisend kinders in Suid-Afrika. Die meeste gevalle is onder die inwoners van die afgeleë gebiede van Suid-Afrika waar hulle staat maak op die gebruik van tradisionele medisyne.

Cotyledon orbiculata L. var *orbiculata* (Haw.) DC. word algemeen gebruik deur tradisionele helers om sentralesenuweestelselsiektes, asook epilepsie te behandel. Die sifting van plante ten opsigte van aktiwiteit en toksisiteit is belangrik om die korrekte behandeling van pasiënte te verseker.

In hierdie studie was die metanolekstrak en drie fraksies van ŉ metanolekstrak van *Cotyledon orbiculata* gebruik om te toets vir antioksidantaktiwiteit en toksisiteit teenoor neuroblastomaselle.

Die gevriesdroogde blare van *Cotyledon orbiculata* was geëkstraheer met metanol in ‘n Soxhlet-apparaat. Die gekonsentreerde ekstrakte was daarna geanaliseer met die gebruik van HDVC (hoëdrukvloeistofchromatografie) en drie verskillende pieke was geïsoleer.

Drie biologiese toetse was uitgevoer om die antioksidantaktiwiteit en die toksisiteit van die monsters te bepaal.

Die thiobarbituraatsuuranalise (TBA) kwantifiseer die omvang van die inhibisie van lipiedperoksidasie in rotbreinhomogenate deur die monsters.

Al die monsters van *Cotyledon orbiculata* het lipiedperoksidasie geïnhibeer soos gesien uit die resultate van die TBA analise. Die metanolekstrak het lipiedperoksidasie die meeste inhibeer in die rotbreinhomogenaat waar fraksie 1 en 2 hoër inhibisie getoon het as fraksie 3.

Die nitrobloutetrasoliumanalise (NBT) kwantifiseer die vermoë van die fraksies om superoksiedradikale in ‘n rotbreinhomogenaat op te ruim.

Al die monsters van *Cotyledon orbiculata* kon superoksiedradikale opruim in die rotbreinhomogenaat soos gesien uit die NBT analise. Die metanolekstrak het die hoogste opruiming getoon met fraksie 1 wat beter aktiwiteit getoon het as fraksie 2 en 3.

Die 3-(4,5-dimetielthiasool-2-yl)-2,5-difenieltetrasoliumbromiedanalise (MTT) bepaal die toksisiteit van die monsters teenoor neuroblastomaselle.

Die metanolekstrak en fraksie 2 by die hoogste konsentrasie van 10 mg/ml was die enigste monsters wat toksisiteit teenoor die neuroblastomaselle getoon het.

Die molekulêre struktuur van ‘n verbinding van fraksie 2 was bepaal deur kernmagnetiseresonansspektroskopie (KMR), infrarooispektroskopie (IR) en massaspektroskopie (MS). Die aktiewe verbinding is geïdentifiseer as diëtielmalaat. Diëtielmalaat kan ontstaan in HDVC-metodes in die teenwoordigheid van maliensuur (wat natuurlik voorkom in die blare van *Cotyledon orbiculata*) en etanol.

Die metanolekstrak van *Cotyledon orbiculata* het hoë antioksidantaktiwiteit getoon, waarskynlik as gevolg van die hoë konsentrasie van maliensuur teenwoordig in die vakuole van die blare. Die gebruik van *Cotyledon orbiculata* kon nie met enige sekerheid aanbeveel word vir die behandeling van epilepsie nie, aangesien ‘n artefak uit die metanolekstrak geïsoleer is. Verdere studies in die veld word aanbeveel wat kan rus op die voorkoming van artefak-vorming, en beter tegnieke om die monsters te skei en te isoleer word ook aanbeveel.

Sleutel terme: *Cotyledon orbiculata*, metanolekstrak, HDVC, anti-oksidant, toksisiteit.

CHAPTER 1: INTRODUCTION AND AIM OF STUDY

1.1 Introduction

The free radical theory of ageing is identified by Sorg (2004) as multiple genetic theories of ageing and the accumulation of damage into cellular components such as lipids, proteins and DNA. The latter being described as a process of developing “metabolic waste” that the organism cannot eliminate properly. This “metabolic waste” or catabolites can act as oxidants for other molecules and produce free radicals.

Ageing is seen as a progressive, inevitable process partially related to the accumulation of oxidative damage of biomolecules like nucleic acids, lipids, proteins, or carbohydrates. This damage is due to an imbalance of pro-oxidants and antioxidants, favouring the former.

Two more factors contribute to the ageing process, the chemical composition of the brain and the poor ability of the brain to eliminate free radicals. The brain contains high concentrations of polyunsaturated fatty acids that serve as targets for lipid peroxidation. In addition, the brain also has lower concentrations of glutathione peroxidase and catalase compared to other organs (Mariani *et al*, 2005).

The increase of free radicals and oxidants and the diminished antioxidant defence system in the brain can lead to multiple neurodegenerative diseases like Parkinson’s disease, Alzheimer’s disease and epilepsy (Mariani *et al*, 2005).

The progression of neurodegenerative diseases can be decelerated by targeting the antioxidant defence system or by limiting free radical production and oxidative stress. Antioxidants can combat oxidative stress by reducing the amount of free radicals formed *in vivo*. Many synthetic antioxidants have been developed, but the use of some have since been discontinued due to toxicity or ineffectiveness and therefore antioxidants from natural sources have received much attention in recent years.

A radical approach to healthcare in many countries is the use of natural sources such as plants to treat multiple health issues. This practice is known to us as traditional medicine (Mariani *et al*, 2005).

The World Health Organisation (WHO) defines traditional medicine as "diverse health practices, approaches, knowledge and beliefs incorporating plant, animal and/or mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness" (WHO, 2002).

In South Africa a large number of plants are used in traditional medicine which has led to a great reliance by rural communities on this source to fulfil their daily medicinal needs (Amabeoku *et al*, 2007). Many neurodegenerative diseases are currently being treated with traditional medicine in South Africa. Traditional healers in South Africa use a wide variety of plants which consist of over 60 families and 150 species to treat central nervous system disorders (Stafford *et al*, 2008).

One of the plants used by traditional healers is *Cotyledon orbiculata* L. var *orbiculata* (Haw.) DC. which is a member of the family *Crassulaceae*. This succulent shrub is widely used in South African traditional medicine to treat epilepsy and other central nervous system disorders (Strijbos *et al*, 1994).

Many neurodegenerative diseases, like Parkinson's disease, Alzheimer's disease and epilepsy, are linked to oxidative damage and oxidative stress in the brain. Antioxidants could play a major role as neuroprotective agents and could alter the progression of these diseases.

Free radicals are produced in the brain by numerous methods. The deposition of iron and copper ions into neuronal cells after head injury produces hydroxyl radicals and can cause lipid peroxidation (Sharma *et al*, 2005). Neurotransmitters like dopamine and glutamate which are extensively used by the brain can also produce free radicals, like superoxide and nitric oxide (Volterra *et al*, 1994). The influx of calcium into neuronal cells leads to depolarisation of the cells and can cause the formation of epileptic focus development (Strijbos *et al*, 1994).

Amebeoku (2007) stated that the methanol extract of *Cotyledon orbiculata* had higher anti-convulsant effects when compared to the aqueous extract, and according to Mori *et al* (1999), many natural occurring antioxidants prevent epileptogenic focus formation and post traumatic induced seizures in the iron injected rat brain. With this

data it could be deduced that the methanol extract may contain more potent antioxidants than the aqueous extract.

In a study done by Louw (2009) the antioxidant properties of extracts from *Cotyledon orbiculata* were compared. It was found that the methanol extract had high antioxidant effects. This was then selected as a target to extract, isolate and identify an active component responsible for the antioxidant properties.

1.2 Aim of study

The role of free radicals and reactive oxygen species in neurodegenerative diseases, and the role of antioxidants from various sources to treat these illnesses, and the safe use of traditional medicine have created an area of research into natural products and plants as a source of antioxidants to treat central nervous system disorders.

The aim of this study is then to determine the antioxidant properties and toxicity of the methanol extract of *Cotyledon orbiculata* leaves and to identify an active compound responsible for the results.

To achieve this aim the following objectives were set:

- To screen the methanol extract and fractions from the extract of the plant for antioxidant properties by using the appropriate assays.
- To test the methanol extract and fractions from the extract for toxicity using the MTT assay, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.
- To characterise the compound(s) responsible for the activity and toxicity.

CHAPTER 2: LITERATURE REVIEW

2.1 Free radical theory of ageing

Throughout the years hundreds of theories of how an organism ages have been postulated, but most of them have been dismissed due to the lack of support or outrageous claims. Few have remained, as viable theories, in the molecular ageing process (Salmon *et al*, 2010). One of these theories, according to Sorg (2004), is the free radical theory of ageing.

Ageing is seen as a progressive, inevitable process partially related to the accumulation of oxidative damage into biomolecules like nucleic acids, lipids, proteins, or carbohydrates. This damage is due to an imbalance of pro-oxidants and antioxidants, favoring the former.

Two other factors contribute to the ageing process of the brain, the chemical composition of the brain and its poor ability to eliminate free radicals. The brain serves as a target for lipid peroxidation due to the high concentration of polyunsaturated fatty acids. Furthermore, lower concentrations of glutathione peroxidase and catalase contribute to the ageing of the brain (Mariani *et al*, 2005).

Sorg (2004) lists two theories to explain ageing; genetic theories of ageing and the theory of accumulation of cellular damage. The latter is described as a process of developing “metabolic waste” that the organism cannot eliminate properly. This “metabolic waste” or catabolites can act as oxidants for other molecules and produce free radicals.

In normal brain ageing, the brain undergoes morphological and functional changes affecting dendritic trees and synapses. Processes like neurotransmission, circulation and metabolism are also affected. This influences the motor and sensory system, sleep, memory and learning.

Impaired brain function as a result of ageing can be related to oxidative stress and free radicals. This oxidative stress is largely due to the increased vulnerability of the brain to the damaging effects of oxidative stress and the diminished capacity of the brain to defend itself against these factors (Mariani *et al*, 2005).

2.2 What is a free radical?

A free radical is any chemical species that consists of one or more unpaired electrons. These free radicals have an altered, mostly increased, chemical activity than when bound in the original molecule. This increase in activity is due to the ability of the specie to "steal" electrons from other molecules. This loss of electrons is called oxidation and most free radicals are known as oxidizing agents, because they accept donated electrons from other molecules or atoms (Gilgun-Sherki *et al*, 2001).

All aerobic organisms use oxygen to generate energy that is essential for life. Oxygen is easily absorbed and transported throughout an organism due to its availability and can easily diffuse through all cellular membranes. Oxygen can also be toxic and mutagenic through the production of reactive oxygen species (Buonocore *et al*, 2010).

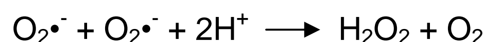
2.3 Types of free radicals

2.3.1 Singlet oxygen

The singlet oxygen radical is produced by an input of energy that rearranges the electrons of biological oxygen. This input of energy removes the spinning restrictions of the electrons and greatly increases the oxidizing activity of oxygen. This increased activity easily oxidizes proteins, DNA and lipids (Buonocore *et al*, 2010).

2.3.2 Superoxide radical ($O_2\bullet^-$)

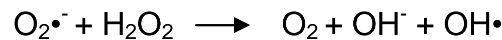
Large amounts of superoxide radicals are produced in the mitochondria through various processes including the electron transport complex and the reduction of certain co-enzymes. It is estimated that 1 – 3% of all molecular oxygen is converted to superoxide radicals that can be very dangerous and toxic to cellular function (Linnane *et al*, 2007).



$$O_2\bullet^- + O_2\bullet^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

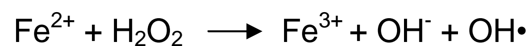
2.3.3 Hydroxyl radical

The hydroxyl radical (OH•) is formed when molecular oxygen is reduced by three electrons in the presence of hydrogen peroxide. This state of oxygen is extremely reactive and can react with any biological molecule. The most common source of hydroxyl radicals is the metal catalyzed Haber-Weiss reaction.



$$O_2\bullet^- + H_2O_2 \longrightarrow O_2 + OH^- + OH\bullet$$

Another reaction commonly associated with the formation of hydroxyl radicals is the Fenton reaction (Martinez-Cayuela, 1994).



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

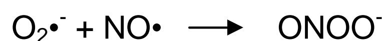
Four main sources of OH• production is transition metal catalysis (especially copper and iron), background exposure to radiation, reaction of $O_2\bullet^-$ with NO• to produce peroxynitrate and the reaction of HOCl with $O_2\bullet^-$ (Halliwell, 1995).

2.3.4 Nitric oxide

NO• is a poorly reactive gaseous free radical and is produced in vascular endothelium cells and neutrophils and macrophages by using the enzyme nitric oxide synthase (NOS) (Fouad, 2008).

2.3.5 Peroxynitrate

Peroxynitrate ($ONOO^-$) is not a free radical, but like hydrogen peroxide can result in the formation of free radicals through various chemical reactions (Gilgun-Sherki *et al*, 2001). $ONOO^-$ is most commonly formed by a radical-radical reaction of NO• and $O_2\bullet^-$ (Halliwell, 1995).



$$O_2\bullet^- + NO\bullet \longrightarrow ONOO^-$$

Table 2.1 Table showing molecules mediating oxidative stress; directly extracted from Sorg (2004).

Name	Structure	Main reactions
Superoxide	•O–O−	Catalysis of Haber–Weiss reaction by recycling Fe2+ and Cu+ ions; formation of hydrogen peroxide or peroxynitrate
Hydrogen peroxide	HO–OH	Formation of hydroxyl radical, enzyme inactivation and oxidation of biomolecules.
Hydroxyl radical	•OH	Hydrogen abstraction, production of free radicals and lipid peroxides and oxidation of thiols
Ozone	−O–O+=O	Oxidation of almost all biomolecules especially those containing double bonds and formation of ozonides and cytotoxic aldehydes.
Singlet oxygen	O=O	Reaction with double bonds, formation of peroxides and decomposition of amino acids and nucleotides.
Nitric oxide	•N=O	Formation of peroxynitrate and reaction with other radicals.
Peroxynitrate	O=N–O–O−	Formation of hydroxyl radical, oxidation of thiols and aromatic groups, conversion of xanthine dehydrogenase to xanthine oxidase and oxidation of biomolecules.
Hypochlorite	ClO−	Oxidation of amino and sulphur-containing groups and formation of chlorine.
Radical	R•	Hydrogen abstraction, formation of peroxyl radicals and other radicals and decomposition of lipids and other biomolecules.
Peroxyl radical	R–O–O•	Hydrogen abstraction, formation of radicals and decomposition of lipids and other biomolecules.
Hydroperoxide	R–O–OH	Oxidation of biomolecules and disruption of biological membranes.
Copper and iron ions	Cu2+, Fe3+	Formation of hydroxyl radical by Fenton and Haber–Weiss reactions.

The most common free radicals identified are the hydroxyl radical (OH•), superoxide radical ($O_2•^-$) and nitric oxide radical (NO•). Hydrogen peroxide and peroxynitrate are reactive species and not free radicals but precursors to the formation of free radicals. These groups (free radicals and precursors) are commonly referred to as reactive oxygen species (ROS) (Gilgun-Sherki *et al*, 2001).

2.4 Free radical production by biological systems

Free radicals are very important in the correct functioning of multiple biological reactions of every organism. There are multiple intracellular systems that produce oxygen free radicals (Martinez-Cayuela, 1994).

2.4.1 Small cytoplasmic molecules

The autoxidation of small cytoplasmic molecules produce free radicals by reducing O_2. Some of these molecules are catecholamines, flavones, quinones and thiols (Porter *et al*, 1995).

2.4.2 Cytoplasmic proteins

Cytoplasmic enzymes generate free radicals as byproducts from their catalytic processes of these proteins. Two of these enzymes are xanthine oxidase and aldehyde dehydrogenase. Heamoglobin is another protein that generates free radicals in its metabolism (Fang *et al*, 2002).

2.4.3 Membrane enzymes

Common enzymes like lipoxygenase and cyclooxygenase generate free radicals in their catalytic reactions in the formation of leukotrienes, thromboxanes and prostaglandins (Martinez-Cayuela, 1994).

2.4.4 Mitochondrial electron transport system

Oxygen is usually catalyzed by cytochrome c oxidase to water in the mitochondrial electron transport system, but when the process is dependent on ADP (adenosine diphosphate) for energy, superoxide radicals are freely produced (Liu *et al*, 2002).

2.4.5 Microsomic electron transport systems

In the catalysis of cytochrome P_{450} and -b_5, electrons are redirected from the normal redox cycle to circulating molecular oxygen, forming free radicals. In this process, hydrogen peroxide and superoxide radicals are produced (Martinez-Cayuela, 1994).

2.5 Cytotoxicity of free radicals

Free radicals usually have a very high reactivity, but very short half-lives. When free radicals react with non-radical molecules, more free radicals are produced which can then react again and initiate a cascading effect in free radical production, reactions and interactions. This can lead to free radical effects in other targets, far from the original point of origin, of the free radical.

All cellular compounds can be targets of free radical damage which leads to metabolic and cellular disturbances. These compounds include lipids, proteins, nucleic acids and carbohydrates (Martinez-Cayuela, 1994).

2.5.1 Effects on lipids

The brain consists of high concentrations of polyunsaturated fatty acids and other membrane lipids responsible for lipid peroxidation. This lipid peroxidation causes severe damage to the membrane structure and influences the ability of the membrane to function correctly (Gill, 2010). Lipid peroxidation leads to the forming of alcohols, aldehydes, volatile hydrocarbons and hydroperoxides which inhibit synthesis of certain proteins and also change the vascular permeability and the inflammatory response. Lipid peroxidation also contributes to the cross-linking and polymerization of membrane components to DNA bases (Martinez-Cayuela, 1994).

2.5.2 Effects on proteins

Amino acids such as tyrosine, phenylalanine, tryptophane and histidine can react with free radicals. These reactions with the free radical and the unsaturated or sulphur groups of the amino acids lead to cross linking of amino acids. Free radicals can also cause protein fragmentation by peptide bond hydrolysis (Martinez-Cayuela, 1994).

2.5.3 Effects on nucleic acids

The majority of damage to the nucleic acids occurs to the bases and their deoxyribose sugars. In the case of the double helix conformation of DNA, the deoxyribose sugars are mostly targeted by free radicals due to the external positions of these sugars (Gill, 2010).

2.5.4 Effects on carbohydrates

Glycosylated proteins and carbohydrates are also targeted by free radicals. Oxidation of monosaccharides and polysaccharides leads to the depolymerization of these carbohydrates (Martinez-Cayuela, 1994).

2.6 What is an antioxidant?

According to Halliwell (1995) an antioxidant is: “any substance that when present at low concentration compared with those of an oxidazable substrate significantly delays or prevents oxidation of that substrate.”

Antioxidants have the ability to scavenge reactive oxygen species of oxygen and nitrogen. Precursors to free radicals are also terminated by antioxidants. The protective properties of the antioxidants depend on the type of the reactive specie, the place of generation and the severity of the damage.

2.7 Antioxidant defenses

The antioxidant defenses can be classified into two groups: enzymes and low molecular weight antioxidants. The enzymes consist of superoxide dismutase (SOD), catalase and peroxidase. The low molecular weight antioxidants are grouped into indirect acting (chelating agents) and direct acting (scavengers and chain breakers) antioxidants (Gilgun-Sherki *et al*, 2001).

These groups of antioxidants all have different mechanisms of action. The chelating agents bind transition metals to render them no longer available to serve as a precursor to the generation of reactive oxygen species. The chain breaking antioxidants act in the lipid phase to trap reactive species that can spread to neighboring cells to cause oxidative damage. Scavengers trap free radicals and reactive species and their precursors in the aqueous phase to eliminate any potential oxidative damage to the cellular environment (Foaud, 2008). Another way that antioxidants work is to up-regulate the endogenous antioxidant defenses of the body (Cui *et al*, 2004).

Table 2.2 Table of endogenous antioxidants; directly extracted from Sorg, (2004).

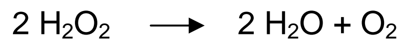
Antioxidant	Phase	Action
Superoxide dismutases (SOD)	Hydrophilic	Dismutation of O_2^- into H_2O_2 and O_2.
Catalase	Hydrophilic	Dismutation of H_2O_2 into H_2O and O_2.
Glutathione peroxidases (GPX)	Hydrophilic lipophilic	Reduction of R–OOH into R–OH.
Glutathione reductase (GSR)	Hydrophilic	Reduction of oxidised glutathione.
Glutathione-S-transferases (GST)	Hydrophilic	Conjugation of R–OOH to GSH (→GS–OR).
Metallothioneins	Hydrophilic	Binding to transition metals (neutralisation).
Thioredoxins	Hydrophilic	Reduction of R–S–S–R into R–SH.
Glutathione	Hydrophilic	Reduction of R–S–S–R into R–SH, Free radical scavenger, Cofactor of GPX and GST.
Ubiquinol	Lipophilic	Free radical scavenger (prevents LPO)
Dihydrolipoic Acid	Amphiphilic	ROS scavenger, Increases antioxidant and phase II enzymes.
Ascorbic acid (vitamin C)	Hydrophilic	Free radical scavenger, Recycles tocopherols (vitamin E), Maintains enzymes in their reduced state.
Retinoid (vit. A) and carotenoids	Lipophilic	Free radical scavengers, Singlet oxygen (1O2) quencher.
Tocopherols (vitamin E)	Lipophilic	Free radical scavenger (prevents LPO), Increases selenium absorption.
Selenium	Amphiphilic	Constituent of GPX and thioredoxins.

Abbreviations: GPX – glutathione peroxidase; GSR - glutathione reductase; GST - glutathione-S-transferase; LPO - lipid peroxidation; SOD - superoxide dismutase.

Defences against oxidative stress rely on the ability of the antioxidant defences to trap reactive species before they get the opportunity to react and oxidise biomolecules. It is important to have these defences in both the hydrophilic and lipophilic phases (Sorg, 2004).

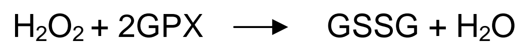
The most effective antioxidants are endogenous enzymes that catalyse the reduction of the reactive species. These enzymes include superoxide dismutase (SOD), catalase and glutathione peroxidase. Superoxide dismutase catalyses the reaction of superoxide ($O_2\bullet^-$) to dioxygen and hydrogen peroxide (H_2O_2) (Uriu-Adams *et al*, 2005).

Catalase is an enzyme that removes water from the cell and is responsible for the metabolism of hydrogen peroxide into water and oxygen.

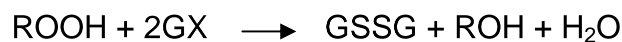


$$2\,H_2O_2 \longrightarrow 2\,H_2O + O_2$$

Glutathione peroxidase (GPX) consists of four selenium atoms and is found in the cytoplasm of all eukaryotic cells (Martinez-Cayuela, 1994). GPX is responsible for the reduction of hydrogen peroxide and organic hydroperoxides (R-OOH) into a glutathione co-factor (GS-SG) which is further reduced by glutathione reductase (GSR) (Sorg, 2004).



$$H_2O_2 + 2GPX \longrightarrow GSSG + H_2O$$



$$ROOH + 2GX \longrightarrow GSSG + ROH + H_2O$$

Metallothioneins are proteins that bind to metal ions to detoxify the metals and prevent the damaging effects of reactive oxygen species (Nath *et al*, 2000).

Lipoic acid is abundant in green vegetables and is rapidly absorbed from the diet and transported to cells and reduced to dihydrolipoic acid. These molecules easily cross through the blood–brain barrier and are considered to be potent antioxidants capable of scavenging free radicals (Moraes *et al*, 2010).

L-Ascorbic acid (vitamin C) is a low molecular weight antioxidant that scavenges free radicals and also recycles vitamin E which is a very potent membrane antioxidant (Patra *et al*, 2001).

Carotenoids that are very closely related to vitamin A are also free radical scavengers and play an important role in the prevention of oxidative stress in the eye. The carotenoids are singlet oxygen quenchers, and prevent the formation of singlet oxygen in the retina (Stahl, 2004).

Tocopherols (vitamin E) are the most important antioxidants in the lipophilic phase. When tocopherols are oxidized they become radicals themselves and these radicals are speedily converted into its original functional groups by ascorbic acid (Sorg, 2004).

Table 2.3 Antioxidants and ROS scavengers in neurodegenerative disorders (Gilgun-Sherki, 2001).

Antioxidants and ROS scavengers groups
Endogenous enzymes - superoxide dismutase (SOD), catalase, glutathione peroxidase
Low molecular weight antioxidants - Glutathione, tocopheroles (vitamin E), ascorbic acid (vitamin C), retinoic acid (vitamin A), melatonin, uric acid, lipoic acid.
Endogenous antioxidant cofactors - coenzyme Q10.
Precursors and derivatives of endogenous antioxidants compounds and enzymes - acetylcysteine, carotenoids.
Naturally occurring plant substances - flavonoids.
Synthetic free radical compounds - Euk-8.

2.8 What is oxidative stress?

Oxidative stress is directly linked to an imbalance between the rate of oxidant production and that of their metabolism. Oxygen is used in the mitochondria for energy production via a four electron reduction reaction. When this reduction is not completed, reactive oxygen species are produced. Other sources of reactive oxygen species are the environment (air pollutants), UV radiation from the sun and our diet. The organism is equipped with a very strong self-defense mechanism to eliminate most of these reactive species, but when the processes cannot complete fully, and the few undesirable reactions escape the repair and prevention systems, they can accumulate over time, damaging the organism.

Almost all natural processes in the body generate reactive oxygen species (ROS) and, if the organism fails to neutralize them, these reactive oxygen species (ROS) accumulate and can react with other biomolecules and substrates in the body, creating the undesirable situation of oxidative stress (Sorg, 2004).

2.9 Oxidative stress in neurodegenerative diseases

The human brain contributes to about 2% of the total body mass of a person, but uses over 20% of all oxygen obtained from respiration (Sorg, 2004). Throughout the entire life, the brain is exposed to oxidative stress. Gilgun-Sherki (2001) states that

certain diseases of the central nervous system are thought to be caused directly by free radical processes and oxidative stress.

The brain itself produces very high levels of super oxide and nitric oxide radicals and in some cases these free radicals cannot react with the needed substrates to eliminate them quickly and effectively. For these reasons it is believed that oxidative damage has a very important role in the development of neurodegenerative diseases (Sorg, 2004).

At this stage it is not certain if oxidative stress is the primary event in neurodegeneration or if it is a secondary effect related to other pathological pathways. Oxidative stress is however directly linked to the propagation of cellular damage that is seen in neurodegenerative diseases (Mariani *et al*, 2005).

2.10 Oxidative stress in Alzheimer's disease

Alzheimer's disease is one of many diseases that cause the gradual loss of brain cells and is the leading cause of dementia in elderly patients (Sorg, 2004). Alzheimer's disease is clinically characterized by memory dysfunction, loss of lexical access, spatial and temporal disorientation and impairment of judgment. The histopathological signs of Alzheimer's disease include synaptic and nerve cell loss, extracellular β-amyloid protein deposition and hyperphosphorylated tau protein (Mariani *et al*, 2005).

Age is another risk factor in the development of Alzheimer's disease. Metabolic defects that occur within the normal ageing process contribute to the dysfynction of the mitochondria and the accumulation of oxidative damage. Oxidative damage leads to the aggregation of the β-amyloid tau protein (Smith et al, 2000).

Increased activity of catalase, superoxide dismutase and glutathione was recorded in the brains of patients with Alzheimer's disease. These findings implicate higher levels of free radicals and possible damage due to oxidative stress. This process leads to neurodegeneration and possibly plaque formation in the central nervous system. Markers linked to oxidative stress were found in the brains of Alzheimer's disease patients and increase the severity of symptoms (Mariani *et al*, 2005).

2.11 Oxidative stress in Parkinson's disease

Parkinson's disease is the most common neurodegenerative movement disorder and is due to the degeneration of the substantia nigra (Sorg, 2004) degeneration of the striata and depletion of dopamine. The most common symptoms of Parkinson's disease are bradykinesia, postural instability, tremor and gait difficulty (Seet *et al*, 2010).

In the metabolism of dopamine a large quantity of reactive oxygen species are produced that has a major role in the oxidative stress theory of Parkinson's disease (Gilgun-Sherki *et al*, 2001). In the oxidation of dopamine, toxic semiquinones are produced which can also speed up the normal degeneration of the brain. Increased levels of lipid peroxidation and damage to DNA were also found in the brains of Parkinson's disease patients. Superoxide radicals, hydroxyl radicals and hydrogen peroxide are produced when dopamine is rapidly metabolized by monoamine-oxidase-B (Mariani *et al*, 2005).

Increased levels of oxidized glutathione was found in the substantia nigra of some Parkinson's disease patients and could be associated with the deficiency of the natural antioxidant defense system leading to degeneration of the nigral neurons in these patients (Gilgun-Sherki *et al*, 2001).

2.12 Background and history of epilepsy

Epilepsy is a very serious central nervous system disorder and affects millions of people worldwide. Epilepsy has a higher prevalence in third world countries than in well developed countries; a likely reason for this is linked to social depravation.

Studies show that people in a socio-economical deprived area are more likely to develop epilepsy due to malformations like tuberous sclerosis and other haematomas. Infections including meningitis and encephalitis and parasitic infections especially cysticercosis are common causes of epilepsy in Third World countries (Stafford *et al*, 2008).

Electrolyte disturbances due to lack of clean drinking water, which include hypernatraemia, hyponatraemia, hypocalcaemia and hypomagnesaemia can also

lead to the developing of epilepsy. Toxins, trauma and metabolic defects in infants are also a major risk factor for development of epilepsy later in life. In South Africa epilepsy is said to have a high prevalence in children with about 73 in 1000. Children in Africa are twice as likely as children in other parts of the world to die due to epilepsy.

The cultural aspect in the treatment of epilepsy is very important in third world countries. People in rural communities would rather consult a traditional healer than a university trained doctor with problems relating to epilepsy. 42.5 % of children suffering from epilepsy are treated with traditional remedies, while 34.6% of children receive no treatment at all (Stafford *et al*, 2008).

Epilepsy in South Africa has been labelled by the rural communities as a contagious disease evoking much fear under the people. Bewitchment, fear and evil spirits are said to be the cause of epilepsy. Epilepsy is a serious disease with social implications and is deeply smeared with stigma. Discrimination against sufferers is common and can be in the form of education, employment and marriage.

Infectious diseases like neurocysticercocis (*Teania solium* infection) and HIV, causing opportunistic infections can be an explanation of the high prevalence of epilepsy in South Africa (Stafford *et al*, 2008).

According to Engelborghs (2000) about 40% of patients suffering from epilepsy have a genetic background that contributes to the aetiology of epilepsy. Familial epilepsies like childhood absence epilepsy, myoclonic epilepsy and benign childhood epilepsy have complicated patterns of inheritance (Engelborgh *et al*, 2000). In more than 50% of patients suffering from epilepsy the actual cause is not known (Löscher, 2002).

Different types of epileptic symptoms occur, but the seizure most likely to cause death, both due to primary (direct effects of the seizure) and secondary (death not directly relating to the seizure) is *grand mal*. The epilepsy most frequently found in young children is *petit mal*, where absence or a “distant stare” is usually the characteristic symptom (Katzung, 2004). A short classification list of epilepsy follows.

Table 2.4 Classification of different types of epilepsy (Katzung, 2004).

Classification of different types of epilepsy
Partial seizures
Simple partial seizure
Complex partial seizure
Generalized partial seizure
Generalized seizures
Grand mal (tonic clonic seizure)
Petit mal (Absence seizure)
Tonic seizure
Atonic Seizure
Clonic and myoclonic seizure
Infantile spasm

2.13 Neurotransmitters in epilepsy

2.13.1 GABA (gamma-aminobutyric acid)

GABA is the main neurotransmitter involved in epilepsy. Engelborghs (2000) states that the GABA hypothesis of epilepsy implies, that a reduction of GABA-ergic inhibition results in epilepsy, whereas an enhancement of GABA-ergic inhibition results in an anti-epileptic effect.

In the continuous activation of cortical circuits a decrease in the amplitude of inhibitory post synaptic potentials are seen, this could be caused by a decrease in GABA released from the post synaptic terminals. The desensitization of GABA receptors is coupled to increases in Cl^- conductance or changes in the ionic gradient from intracellular accumulation of Cl^-. Passive redistribution is ineffective in this state. Furthermore, the Cl^- - K^+ co-transport becomes less effective in seizures as it depends on the K^+ gradient. The Cl^- - K^+ co-transport depends on metabolic processes and its effectiveness is affected by hypoxia and ischemia.

Endogenous agents like guanidino compounds and exogenous compounds like penicillin, picrotoxin and pilocarpine which are all convulsants, inhibit GABA-ergic transmission by interacting on a specific site on the post synaptic $GABA_a$ receptor or directly interfering in the GABA synthesis. These compounds block GABA inhibition and amplify the dendritic spike generating mechanism that involves Ca^{2+} (Engelborghs *et al*, 2000).

2.13.2 Glutamate

Activation of ionotropic and metabotropic postsynaptic glutamate receptors leads to convulsions. N-methyl-D-aspartate (NMDA) antagonists are all powerful anti-convulsants.

Genetic alterations in animals have been reported to be epileptogenic, but no mutations in humans are connected to epilepsy. There is however a link between changed NMDA receptor functions and epilepsy in rats and in man. Increased sensitivity to glutamate effects on the NMDA receptor is seen in brain slices taken from both rats and humans.

During synaptic activity increased volumes of Ca^{2+} enter the neurons. Alterations in the metabotropic glutamate receptor function also leads to epileptogenesis. Neuronal membranes which are exposed to an increased level of extracellular glutamate usually have an increased sensitivity for neuronal excitability. This can lead to absence seizures in most patients (Engelborghs *et al*, 2000).

2.13.3 Catecholamines

Abnormalities in the catecholamines of the central nervous system have been reported to play a role in epilepsy. In epileptic rats dopamine was decreased in the nucleus caudatus and noradrenalin was increased in the midbrain and brainstem. Lower concentrations of dopamine have been recorded in the epileptic foci of patients. In absence epilepsy in animals, convulsions are decreased by dopamine agonists and exaggerated by dopamine antagonists. It is suggested that decreased dopamine concentrations facilitate the occurrence of these seizures by lowering the threshold (Engelborghs *et al*, 2000).

Monoamine oxidase inhibitors, a drug class that inhibits the activity of the monoamine oxidase enzyme, prevents the breakdown of monoamine neurotransmitters (like dopamine and noradrenalin) and thereby increase their availability. There are two isoforms of monoamine oxidase; mono amine oxidase-A and mono amine oxidase-B. Dopamine is affected by both types and cause an increase in the concentration of dopamine (Katzung, 2004). The increased levels of these catecholamines can lead to an epileptic fit.

2.13.4 Opioid peptides

Opioids and opioid peptides both have convulsant and anti-convulsant activities. *Kappa* (κ) agonists lower spike waves in animal models of absence epilepsy. Peptides with *mu* (μ) agonist properties induce seizures when administered to patients due to the inhibition of interneurons. Mu (μ) receptor density is increased in patient with complex partial seizures (Engelborghs *et al*, 2000).

2.14 Oxidative stress and epilepsy

Iron-induced epilepsy due to head trauma is based on the deposition of iron and copper ions from the damaged tissue which can cause hydroxyl formation, lipid peroxidation and autoxidation of neurotranmitters (Sharma *et al*, 2005).

Many neurotransmitters in the brain, like dopamine, levodopa and noradrenaline can react with oxygen to produce reactive oxygen species (superoxide) that depletes gluthatione and can cause oxidative damage. Furthermore, glutamate is extensively used in the brain as neuritransmitter and can produce an excess of superoxide and nitric oxide in lipid peroxidation conditions (Volterra *et al*, 1994).

The brain contains high levels of microglia which produce superoxide anions and hydrogenperoxide when activated and can secrete cytokines, which in turn produce more reactive oxygen species and nitric oxide (Richter *et al*, 1999).

Reactive oxygen species can leak away from the catalytic intermediates in the cytochrome P_{450} cycle in the brain, which leads to superoxide and hydrogenperoxide generation (Patel, 2004).

Stimulation of NMDA receptors in the brain can result in the synthesis and release of nitric oxide and can cause neuronal cell damage. Nitric oxide also stimulates guanyl cyclase to increase intercellular cGMP which in turn surpresses $GABA_A$ activity (Mailly *et al*, 1999). This reduced activity leads to the influx of calcium ions into neuronal cells and can lead to depolarisation and epileptic focus generation (Strijbos *et al*, 1994).

Nitric oxide can also react with superoxide radicals to form peroxynitrate anions.

2.15 Anti-epileptic drugs and method of action

In the past decades there has been remarkable progress in the pharmacological field regarding epilepsy, several new drugs were introduced and better formulations of old drugs were developed. In spite of this progress about one third of patients are still resistant to the current treatments. Current anti-epileptic drugs do not affect the progression of the disease or prevent any form of epilepsy e.g. after a head injury (Löscher, 2002).

The $GABA_a$ receptor has a binding site for current anti-epileptic drugs (benzodiazepines, carbolines, barbiturates and certain steroids) that modify the chloride channel gating of GABA (Stafford *et al*, 2005).

Most of the current anti-epileptic drugs were discovered by screening with no rationale to the mechanism of action of the drug. As knowledge of epilepsy grew and the mechanisms of action were derived, it was obvious that most anti-epileptic drugs exert their anti-convulsant effects through only a couple of neurochemical mechanisms.

The mechanism of action of anti-epileptic drugs currently in use rests on the fact that they decrease neuronal membrane excitability by binding to the site of action exerting a change in neurotransmitter receptor complexes or interacting with ion channels. The ion channels affected are sodium and calcium channels. Drugs binding to the neurotransmitter complexes enhance the effects of GABA-ergic neurotransmission and also inhibit the effects of excitatory neurotransmitters (Engelborghs *et al*, 2000).

In recent years there have been many advances in the treatment of epilepsy, apart from the current and new anti-epileptics, other methods of treatment also exist. These new strategies include surgery to remove a seizure focus and vagal nerve stimulation which is a new non-pharmacological alternative treatment. By following a ketogenic diet, consisting of high fat intake and very little carbohydrates, a reduction in seizures could be established. By treating patients with antioxidants, oxidative damage could be reduced dramatically (Patil *et al*, 2011).

Table 2.5 Summary of mechanism of epileptogenesis and mechanism of anti-epileptic drugs (Engelborghs *et al*, 2000).

	Mechanism of epileptogenesis	Mechanism of anti epileptic drugs
GABA	Reduced GABA in microgyric cortex	Increased functional pool of GABA (vigabatrin, tiagabine)
	Reduced benzodiazepine receptor binding in medial thalamic nucleus (mesial temporal lobe epilepsy)	Enhanced GABA-ergic inhibition (benzodiazepines)
	Reduced benzodiazepine receptor density in CA1 region (hippocampal sclerosis)	GABA agonistic effects (progabide)
	Auto-antibodies to GAD (Stiff-man syndrome) Reduced GABA levels and GAD activity (epileptic foci)	Weaker GABA-ergic properties (phenobarbital, gabapentin, topiramate, valproate, zonisamide)
Glu	Up regulation of hippocampal ionotropic glutamate receptors (temporal lobe epilepsy)	Inhibition of glutamate release (lamotrigine)
	Anti-gluR3 antibodies (Rasmussen encephalitis)	Block of glycine site at NMDA receptor (felbamate)
	Increased plasma glutamate levels (absence seizures)	
Na^+	Mutation voltage-gated Na^+ channel (generalized epilepsy with febrile seizures)	Reduction of voltage-gated Na^+ currents (carbamazepine, felbamate, lamotrigine, oxcarbazepine, phenytoin, topiramate)
K^+	Mutation voltage-gated K^+ channel (benign familial neonatal convulsions)	Reduction of T-type Ca^{2+} currents (ethosuximide, valproate)
Ca^{2+}	Reduced ACh-mediated Ca flux (nocturnal frontal lobe epilepsy)	Decreased membrane excitability

2.16 Plants used in the treatment of epilepsy

In a study done by Stafford (2008) 43 African plants used in traditional medicine were screened for activity to treat epilepsy which included *Apiaceae* used in Malawi, *Araliaceae* used in Ghana, *Asteraceae* and *Crassulaceae* used in South Africa, *Combretaceae* used in Zimbabwe, *Mimosa* used in Madagascar and *Passifloraceae* and *Zingiberaceae* used by the Zulus.

Numerous plants indigenous to South Africa were screened for compounds with affinity for the flumazanil sensitive benzodiazepine sites on the $GABA_a$ receptor. The ethanolic extract from *Cotyledon orbiculata* showed anticonvulsant effects *in vivo*, but the ethanolic extract showed no *in vitro* effects against epilepsy. A different mechanism of action, other than that of inducing GABA, is suggested as the main mechanism of action for the plant's anti-convulsant effects (Stafford *et al*, 2008).

A possible explanation can be that *Cotyledon orbiculata* has a high concentration of molecules that can act as antioxidants. Antioxidants can prevent oxidative damage from reactive oxygen species that cause damage in lipids, proteins and DNA.

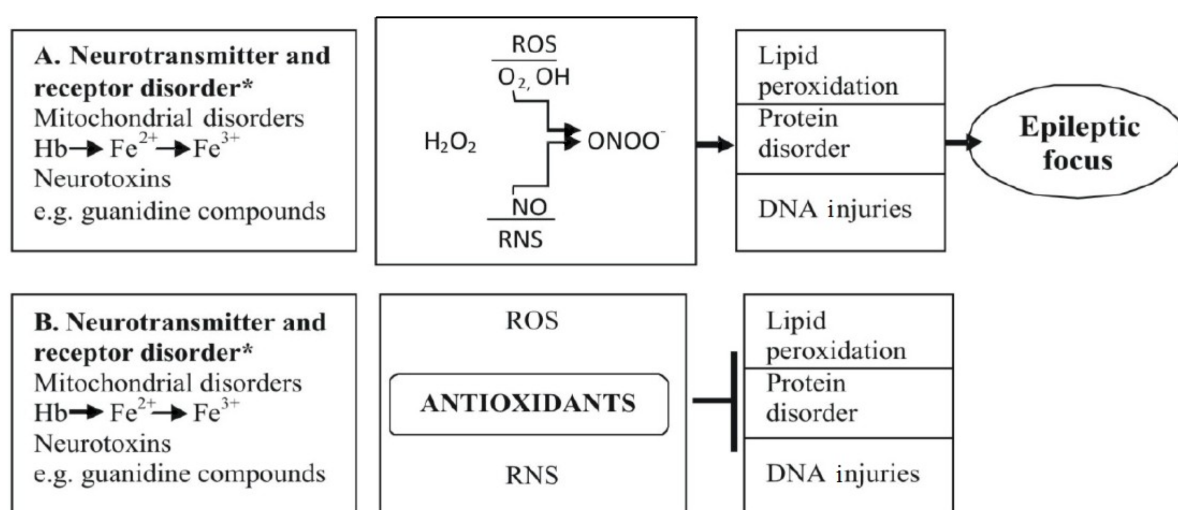


Figure 2.1 The possible anti-convulsant effects of antioxidants (Patil *et al*, 2011).

A – Involvement of ROS and RNS in the mechanism of epileptic focus formation.

B – Anticonvulsant effect of antioxidants, preventing epilepsy focus formation.

2.17 Traditional medicine in South Africa

2.17.1 Healthcare in South Africa

South Africa has a dual healthcare system. The first is traditional medicine, based on a traditional approach while the other is modern medicine based on western approaches. Traditional medicine is popular among a vast majority of people in South Africa. It is estimated that 80% of the black population and 60% of the total population of the country make use of this service. The major contributor to this fact

is that traditional medicine is cheap, individualised and more culturally acceptable (Stafford *et al*, 2008).

2.17.2 What is traditional medicine?

The World Health Organisation (WHO) defines traditional medicine as "diverse health practices, approaches, knowledge and beliefs incorporating plant, animal and/or mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness" (WHO, 2002).

The WHO also defines African traditional medicine as "The sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or societal imbalance, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing" (WHO, 2002).

2.18 The South African diversity

South Africa is a country of great diversity. The cultural, historic and floral diversity are just a few. In South Africa there are 11 official languages of which nine are indigenous to this country. South Africa is a very complex country, and is called "the rainbow nation" for a good reason. Different climate zones and a host of different habitat types make South Africa the perfect platform for the rich floral diversity (Thring & Weitz, 2005).

South Africa boasts with over 30 000 species of flowering plants which is estimated to be about one tenth of the floral population of the world. Of this collection there are 10 endemic families, where 80% of species and 29% of genera are indigenous to South Africa (Stafford *et al*, 2005). A tenth of all the species in South Africa is said to have medicinal properties. This tenth accounts for over 3 000 different plants (Thring & Weitz, 2005). It is said that about 500 species are traded in large volumes in rural markets, which contributes to a very large "hidden" economy in South Africa (Light *et al*, 2005).

A large number of plants are used in traditional medicine which has led to a great reliance by rural communities on this service to fulfil their daily medicinal needs (Amabeoku *et al*, 2007).

One of these plants used widely by traditional healers to treat multiple illnesses and conditions is *Cotyledon orbiculata*

2.19 *Cotyledon orbiculata*

2.19.1 Classification and background

Figure 2.2 *Cotyledon orbiculata* plant (South African National Biodiversity Institute).

Cotyledon orbiculata L. var *orbiculata* (Haw.) DC. is a member of the family *Crassulaceae. Cotyledon orbiculata* has been used in traditional medicine to treat epilepsy. Traditional healers in South Africa use a wide variety of plants which consists of over 60 families and 150 species to treat neurological disorders, one of which is epilepsy (Stafford *et al*, 2008).

The genus name *Cotyledon* is derived from the Greek word *kotyledon*, which translates as “cup shaped hollow”. This is in reference to the fleshy leaves of the plant. The species name *orbiculata* originates from the Latin word meaning “round or

circular". Another name, "pig's ear", comes from the oval shape of the leaf of some forms of the plant. Other common names include: "*plakkie*", "*platjies*", "*varkoorblaar*", "*varkoor*" and "*kouterie*" in Afrikaans. Two Zulu names for the plant are "*imphewula*" and "*intelezi*" (South African National Biodiversity Institute, 2000).

This succulent shrub has thick leaves that vary from green to light grey, occasionally with a red line on the rim of the leaf. Five varieties of *Cotyledon* appear in nature with distinguishing flower and leaf shapes. The shape, size and colour of the leaves are directly influenced by the type of soil, watering conditions, minerals in the soil etc.

Figure: 2.3a

Figure: 2.3b

Figure 2.3a - Leaf shape of *Cotyledon orbiculata* with red rim.

Figure 2.3b – Red bell shaped flowers of *Cotyledon orbiculata*.

Cotyledon mostly carries flowers in the South African winter from June to August, except in the winter rainfall areas, such as the Cape. The plant usually carries red to orange flowers. The flowers are bell shaped tubular hanging flowers and are in clusters on the main flowering stem. Yellow flowers were also reported for *Cotyledon*.

The leaves are coated with a powdery white substance that reflects most of the sun's heat. The bright flowers also attract birds and bees that feed of the sweet nectar of *Cotyledon orbiculata*.

The plant is widely spread in South Africa, but more commonly found in rocky outcrops, grassland, Karoo and fynbos areas. Black frost in winter will damage the flowers, but the plant is resistant to frost conditions.

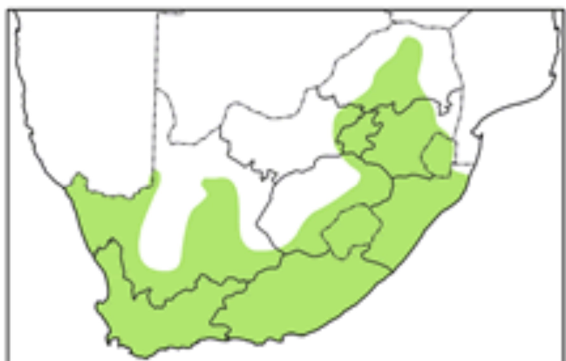


Figure 2.4 Distribution map of *Cotyledon orbiculata* in South Africa (South African National Biodiversity Institute).

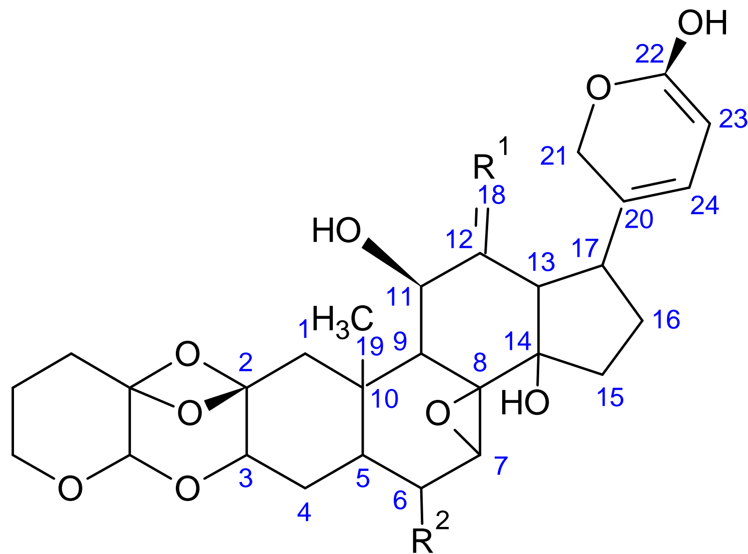
2.19.2 Chemical constituents

The most common active molecules in *Cotyledon* are the three bufadienolides named orbicuside A, -B and -C (South African National Biodiversity Institute, 2000).

Bufadienolides are polyhydroxy C_{24} steroids and their glycosides. A characterizing property of the bufadienolides is the presence of a six membered lactone ring. Many bufadienolides also have hydroxyl groups, a trans B/C ring junction and an aldehyde group. Most bufadienolides found in plants are glycosides, with up to three sugars linked in a chain.

The structure activity relationship for cardiac glycosides is summarized as follows:

The sugar moiety is only important in the distribution and transport of these molecules over the membrane and has no biological action. The OH group at position 14 has been found not to have an impact on activity. The unsaturated lactone connected at C-17 plays an important role in receptor binding, saturation of this ring leads to dramatically reduced biological activity (Desai, 2000).



Compound	R^1	R^2
Orbicuside A	-H	=O
Orbicuside B	-H	-β-OH, α-H
Orbicuside C	-OH	=O

Figure 2.5 Basic structure of bufadienolides from *Cotyledon orbiculata* (South African National Biodiversity Institute).

Kabatende (2005) used methods by Harborne and Ikhiri to do a phytochemical analysis of *Cotyledon orbiculata* leaves. His studies showed that there are four other major groups of molecules in *Cotyledon orbiculata* other than cardiac glycosides. These groups are triterpene steroids, tannins, reducing sugars and saponins.

2.19.3 Dangers of *Cotyledon orbiculata*

Plant poisoning in animals is usually accidental and more prominent in times with unfavourable conditions like drought, veld fires, overstocking and trampling of grazing areas. Plant poisoning in animals is diagnosed by the clinical syndrome, post mortem studies, evidence of ingestion and remains of plant in the gastrointestinal tract and history of illness in livestock.

Human poisoning occurs when poisonous plants are mistaken for edible plants, used as remedies or contamination of food products. This can also be intentional or unintentional. Plant poisoning can affect organs at various levels with deadly

consequences. The level of poisoning depends on the harvest season of the plant, the amount taken in, growth stage of the plant, the ingested part of the plant, species and susceptibility of the person.

Many plants used in ethnobotanical treatment of disease in animals and humans are a major concern in toxicity and death. The correlation between pharmacology and toxicology is an important tool as the therapeutic effect is reached at a low dose whereas toxicity usually occurs with overdosing (Botha & Penrith, 2008).

The major contributor to poisoning with *Cotyledon orbiculata* is the bufadienolide orbicusides. In animals grazing on *Cotyledon* it was found that, "*krimpsiekte*" or translated to shrinking disease, was a major concern. Animals affected with shrinking disease are usually found at the back of the pack, walking with their necks and heads hanging down, backs arched and feet close together. This is called the shrinking position. Mortality can be as high as 90% in affected animals (Botha & Penrith, 2008). *Cotyledon orbiculata* is also widely used as a traditional remedy and can cause digitalis-like poisoning in people consuming this product (South African National Biodiversity Institute, 2004).

According to Botha and Penrith (2008), poisoning with bufadienolides is categorised in acute poisoning with non-cumulative bufadienolides and chronic poisoning with cumulative bufadienolides.

Cardiotoxic effects are the main cause of death, Kapitanyan (2009) states that the pathophysiology that produces cardiotoxicity involves prolonging the refractory period in the arterioventricular node, shortening the refractory periods in the atria and ventricles, and decreasing the resting membrane potential. Dysrhythmias which increase automaticity and decrease conduction are clear symptoms of cardiac glycoside poisoning. The gastrointestinal tract, respiratory system and nervous system are the other organ systems affected by bufadienolides.

Gastrointestinal effects include bloating, colic and diarrhoea. Respiratory symptoms are dyspnoea, polypnoea and apnoea. Nervous system symptoms include posterior paresis.

2.19.4 Uses of *Cotyledon* in traditional medicine

It has been reported that the leaves of *Cotyledon orbiculata* have been used to treat corns and warts. The juice of the heated leaves is applied to the affected area every morning for a week to soften and remove corns and warts (Bhat & Jacobs, 1995). Peeled heated leaves are also applied to treat this condition (South African National Biodiversity Institute).

The juice of the leaves is also used to treat earache and toothache. (Amabeoku *et al*, 2007). An extract of leaves boiled in water is cooled and filtered and a drop of the mixture is then placed on the tooth or dropped into the ear to alleviate pain symptoms (Bhat & Jacobs, 1995).

To treat boils and inflammation the leaf is heated and applied to the affected area (Bhat & Jacobs, 1995). In the treatment of boils, abscesses or skin eruptions a peeled leaf is applied for a few days to treat the problem (South African National Biodiversity Institute).

Other uses include eating a single leave as a vermifuge, placing sap on a sprain or fracture and treatment of venereal disease and used per rectum as an enema. Southern Sotho healers used to dry leaves as a protective charm and plaything for orphaned children (Thring & Weitz, 2005).

Cotyledon orbiculata has also been used in the treatment of epilepsy. An infusion of the leaves is used to treat this condition. Another dosage form, half a cup of fresh leaf juice, is to be taken orally three times a day (Amabeoku *et al*, 2007).

CHAPTER 3: PREPARATION OF PLANT MATERIAL AND SELECTION OF FRACTIONS

3.1 Introduction

Cotyledon orbiculata L. var *orbiculata* (Haw.) DC. is a member of the family *Crassulaceae.* This succulent shrub has thick leaves that vary from green to light grey, occasionally with a red line on the rim of the leaf. *Cotyledon* mostly carries bell shaped flowers in the South African winter, from June to August, which range from red to orange. The leaves are coated with a powdery white substance that reflects most of the sun's heat. The plant is widely spread in South Africa, but more commonly found in rocky outcrops, grassland, karoo and fynbos areas.

Cotyledon orbiculata has been used widely in traditional medicine to treat patients suffering from epilepsy (Stafford *et al*, 2008).

Figure 3.1 The flowering plant of *Cotyledon orbiculata*.

3.2 Collection and storage of plant materials

Leaves of *Cotyledon orbiculata* was collected from the Botanical Gardens of the North-West University Potchefstroom campus. The plant was identified by Martin Smit, curator of the gardens, to be *Cotyledon orbiculata* L. var *orbiculata* (Haw.) DC*.* The plant is located at the back of the gardens in a rocky area next to a pond and a waterfall.

Figure 3.2 Map of the Botanical garden at the NWU and location of Cotyledon selected for experiments.

Samples of the plant were collected through the study period as the plant grows right through the year and is available in all seasons. Leaves were cut off close to the stem of the plant to maximize the size of the leaves and to make new space for the leaves to grow.

As *Cotyledon* is a succulent shrub, the leaves were firstly cut up into smaller pieces and stored in the freezer in the laboratory. The cut up leaves were then processed further into a pulp by using a kitchen blender. This pulp was then freeze dried and stored in air tight containers in a cupboard. The freeze dried plant material was then used for preparing extracts.

3.3 Preparation of extracts from *Cotyledon*

Soxhlet extraction was used to prepare crude extracts from freeze dried *C. orbiculata* leaves. Methanol was used as the solvent for extraction. The apparatus was set up in a laminar flow chamber where the plant material was extracted for 48 - 72 hours. The extract obtained was a very dark green colour with a slight white residue at the bottom of the flask. The extract was then filtered and concentrated using a rotary vacuum evaporator (BUCHI Rotavapor RII). The concentrated extract was then diluted with a small volume of ethanol, and decanted into a storage container that was stored in a cool dry place.

After collecting enough crude extract, the samples were bleached using UV irradiation to reduce the amount of chlorophyll in the samples (Scheepers *et al*, 2011). These samples were placed in the UV bath for 8-12 hours. After the UV treatment, the extracts were of a pale yellow colour.

3.4 Isolation and separation of compounds

Isolation and separation of the compounds of the extract was done on an Agilent 1100 series HPLC using a Phenomonex Synergi Fusion RP 4 µ (250mm x 10mm) column with security guard semi prep cartridges (Fusion RP 10x10) and security guard cartridge holders (10mm ID) connected to an Agilent 1200 Series fraction collector. The mobile phase was a gradient of 10% acetonitrlie (ACN) and a 0.1% phosphate buffer (H_3PO_4). Injection volume of the samples was 500 µl. The samples were visualized by a diode array detector at 210 nm.

A gradient system of 5% ACN was run for 1 minute where after the ACN was increased to 85% over 10 minutes and held stable for 5 minutes, resulted in the following HPLC chromatogram.

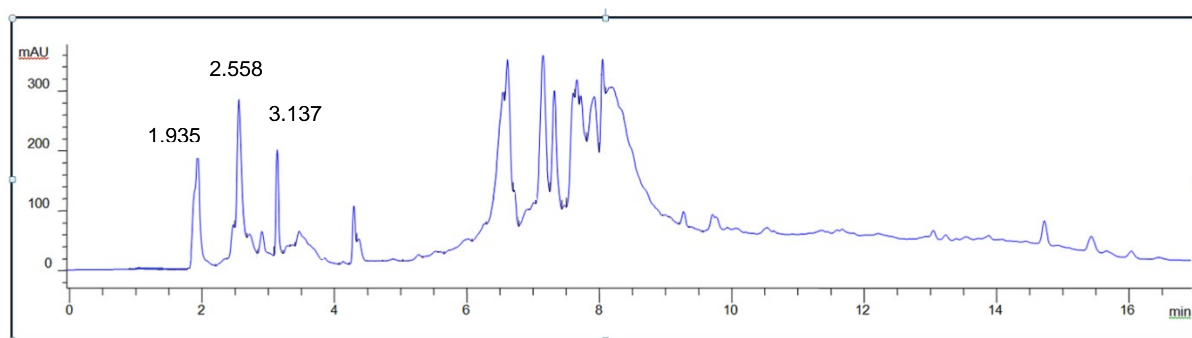


Figure 3.3 HPLC chromatogram of the crude UV-irradiated extract of *Cotyledon orbiculata.*

Three major fractions were selected for isolation. The peaks for these fractions were detected at 1.935 minutes, 2.558 minutes and 3.137 minutes respectively.

After collecting a large enough volume of the samples, the mobile phase was evaporated from the solution using a high vacuum evaporator until the sample was viscous. The samples were then places in a laminar flow chamber to dry further and freeze dried to remove any extra water in the solution. The samples were placed in containers and kept in a refrigerator between 2 ºC and 8 ºC.

The three fractions were also analysed by HPLC to determine the purity of the samples. The following chromatograms were obtained.

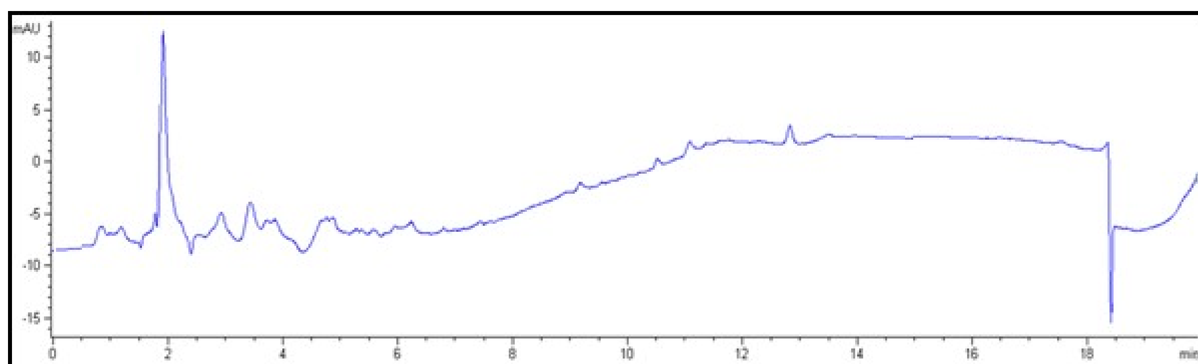


Figure 3.4 HPLC chromatogram of fraction one from *Cotyledon orbiculata*.

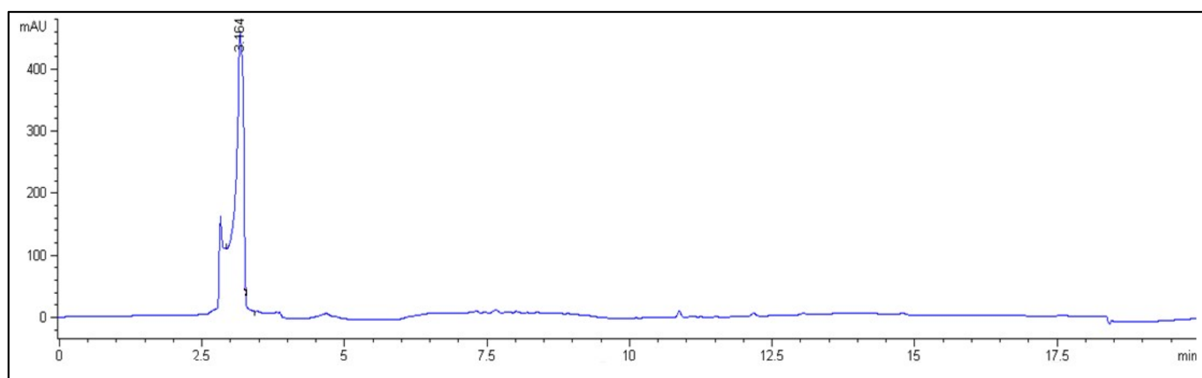


Figure 3.5 HPLC chromatogram of fraction two from *Cotyledon orbiculata*.

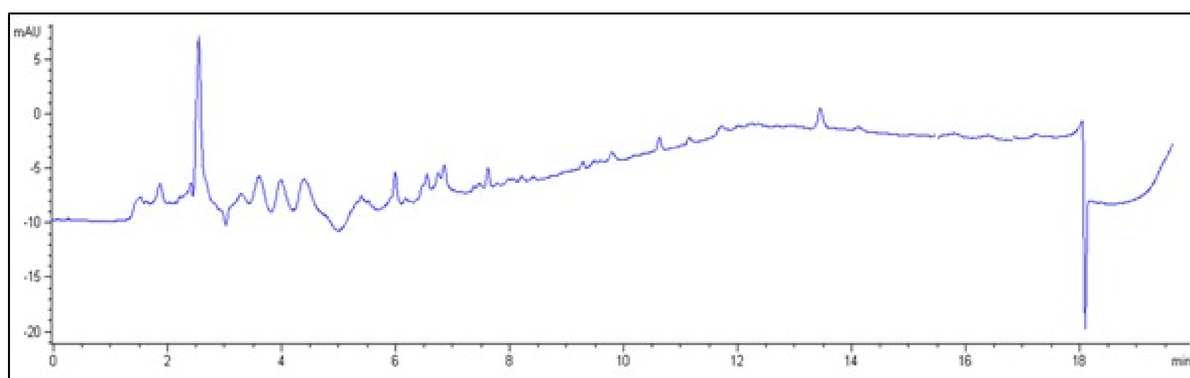


Figure 3.6 HPLC chromatogram of fraction three from *Cotyledon orbiculata*.

The three fractions varied in colour from light yellow (fraction two) to light brown (fraction one) and dark brown (fraction three).

These fractions were used “as is” in the following biological assays: the TBA (thiobarbituric acid) assay, NBT (nitroblue tetrazolium) assay and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. These extracts were also used in the toxicity assay.

CHAPTER 4: IN VITRO ASSAYS

4.1 Assays selected for experimentation

Three *in vitro* studies were selected to test multiple characteristics of the isolated compounds from *Cotyledon orbiculata*. These tests consisted of the TBA assay (thiobarbituric acid), which determines the extent of lipid peroxidation in biological samples, the NBT assay (nitroblue tetrazolium) which quantifies the ability of extracts to scavenge superoxide radicals and the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) which determines the toxicity of the extract towards mammalian cells.

4.2.1 TBA (thiobarbituric acid) assay

Oxidative stress has been identified to be a cause of a number of diseases, including certain neurodegenerative disorders. One target of oxidative stress is lipid peroxidation. These lipids and other polyunsaturated fatty acids are essential for the normal functioning of neural cell membranes. During lipid peroxidation many reactive aldehydes are generated, with malondialdehyde (MDA) being the most abundant form.

The TBA assay is currently one of the widely used assays to determine the lipid peroxidation of biological samples. According to Ottino and Duncan (1997) the principle of the assay is based on the reaction of TBA with MDA (malondialdehyde). MDA reacts with two molecules of TBA in an acid catalysed nucleophilic addition reaction resulting in a light pink chromagen. This molecule is then extracted by adding butanol and measured spectrophotometrically (Hodges *et al*, 1999).

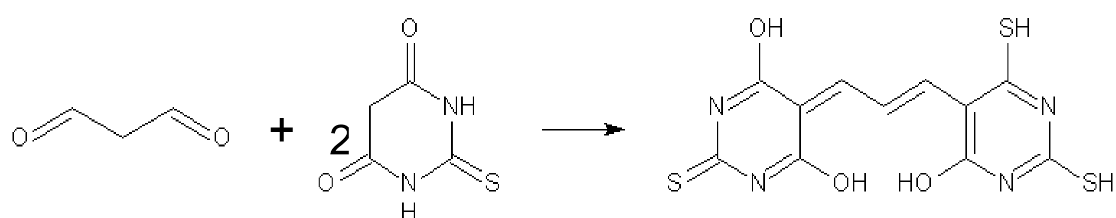


Figure 4.1 Reaction to form pink chromagen in the TBA assay (Hallwell & Chirico. 1993).

This assay has been proven to be sensitive to small changes in the amount of MDA and is recognised as a reliable method in the estimation of lipid peroxidation (Hodges *et al*, 1999).

A modified version of the TBA assay as used by Ottino and Duncan (1997a&b) was selected to do the screening of plant extracts. This assay uses the concentration of rat brain MDA as a guideline of induced oxidative damage.

4.2.2 Preparation of extracts

The dried extracts (fraction 1, fraction 2 and fraction 3) were prepared in concentrations of 0.3125 mg/ml, 0.625 mg/ml, 1.25 mg/ml and 2.5 mg/ml. The solvent used in the preparation of the extracts was methanol.

4.2.3 Animals

Whole rat brains were used for all the assays. Sprague-Dawley rats were used weighing 200 to 250 g. The animals were obtained from the experimental animal centre at the NWU Potchefstroom campus. They were housed in windowless, well ventilated environments with light cycles of 12 hours. The temperature was maintained at 21ºC and humidity at 55%. The animals received standard chow and water *ad lib*.

4.2.4 Chemicals and reagents

All chemicals used in the assay were of highest chemical purity and obtained from trusted chemical suppliers (Sigma Aldrich or Merck Pharmaceuticals).

Ascorbic acid (1.4 mM) – 0.00246 g was dissolved in 10 ml H_2O.

BHT – 0.05 g BHT (butylated hydroxytoluene) was dissolved in 100 ml H_2O. BHT can be stored in a fridge and can be reused.

$FeCl_3$ (4.88mM) – 0.013 g $FeCl_3$ was dissolved in 10 ml H_2O.

H_2O_2 (20 volume = 6 %) – the dilution started in a test tube containing 10 ml H_2O and 27.7 µl H_2O was removed from the test tube. 27.7 µl of H_2O_2 (hydrogen peroxide) was then added to the test tube containing the H_2O.

MDA (50 nM) – to prepare MDA (malondialdehyde) in a concentration of 50 nM, two test tubes with 10 ml phosphate buffer (PBS) in each were prepared. 82 µl was removed from the first test tube and 82 µl of MDA was added to this test tube. 10 µl of PBS from the second test tube was then removed and 10 µl from test tube 1 to test tube 2 was transferred. The final concentration of the MDA was 50 nM.

PBS (Phosphate buffer) 10% – 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4. PBS was prepared with 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4. These salts were dissolved in 800 ml of double distilled water and the pH was set to 7.4 using HCl to lower, or NaOH to increase the pH of the solution. The solution was then topped up to one litre to complete the buffer solution.

TBA (0.33 % in H_2O) – 0.132 g TBA (thiobarbituric acid) was added to 40 ml H_2O. Fresh TBA was prepared daily and covered in aluminium foil. TBA is light sensitive.

TCA (10% in H_2O) – 10 g of TCA (trichloroacetic acid) was dissolved in 100 ml H_2O.

Trolox (10mM) – 0.005 g trolox was dissolved in 2ml H_2O and sonicated.

Hydrogen peroxide was used as the toxin to generate hydroxyl radicals in the rat brain homogenate (Garcia *et al*, 2000). Ferric chloride and ascorbic acid was also added to generate hydroxyl radicals in the rat brain (Kang *et al*, 2003)

4.2.5 Preparation of standard curve

A series of reaction tubes were prepared with known quantities of MDA. The tubes were prepared as follows. See table 4.1.

Table 4.1 Preparation of MDA standard solutions.

Concentration (nmole/L)	Volume of MDA (µl)	Volume of PBS (µl)
0	0	100
5	20	80
10	40	60
15	60	40
20	80	20
25	100	0

These reaction tubes were mixed on a vortex mixer and 100 µl BHT, 200 µl TBA and 100 µl TCA were added to all the tubes. The tubes were again mixed and incubated for an hour at 60ºC. The tubes were removed from the incubator and cooled on ice until room temperature.

Once the tubes were cooled, 400 µl butanol was added to all the tubes. The tubes were then centrifuged at 2000 x g for 5 minutes. 200 µl of the top layer was removed and placed into a 96 well cell culture plate. This plate was then scanned at 530 nm and results were obtained. Butanol was used as blank.

A graph of absorbance was plotted against concentration of MDA to result in a straight line equation of $y = 0.017x + 0.012$ with a R-squared value of $R^2 = 0.999$.

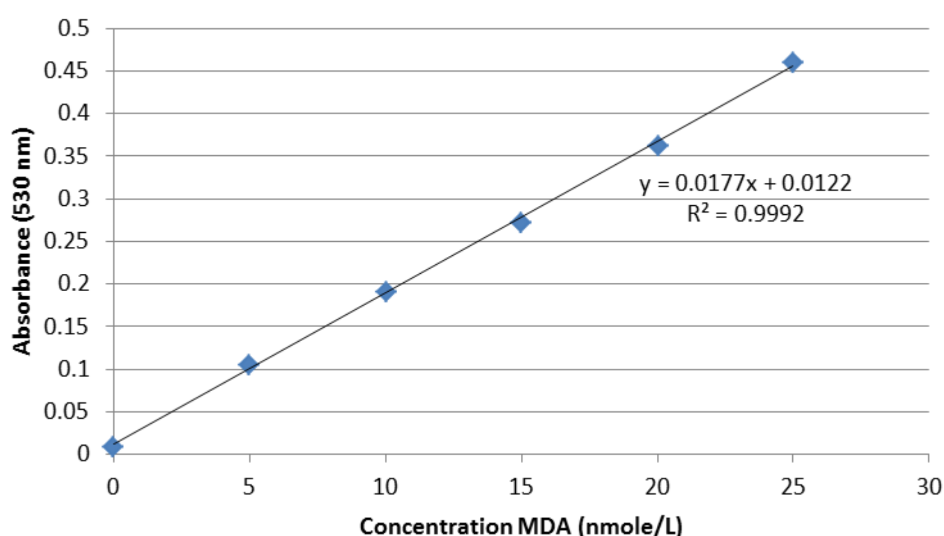


Figure 4.2 MDA standard curve obtained from TEP.

4.2.6 Brain homogenates

Adult male Sprague-Dawley rats were decapitated and the brains rapidly removed and stored in PBS. The brain was then homogenised in 10% (w/v) PBS using a homogeniser blender.

4.2.7 Assay

A series of tubes to be tested were prepared and kept on ice. The tubes were prepared as follows. See table 4.2.

Table 4.2 Preparation of tubes for assay.

	Homogenate (µl)	PBS (µl)	H_2O_2 (µl)	$FeCl_3$ (µl)	VitC (µl)	Trolox (µl)	Ethanol (µl)	DMSO (µl)	Drug (µl)
Control	160	40							
Toxin	160	20	10	5	5				
Trolox	160		10	5	5	20			
Ethanol	160		10	5	5		20		
DMSO	160		10	5	5			20	
Drug	160		10	5	5				20

After addition of all reagents, the tubes were mixed using a vortex mixer. These tubes were then incubated in an oscillating water bath at 37ºC for 60 minutes to induce lipid peroxidation. After the incubation period the tubes were centrifuged at 2000 x g for 20 minutes to remove all insoluble proteins. The supernatant was removed and added into new tubes.

100 µl of BHT was added to stop the reaction, 200 µl TCA was added to precipitate macromolecules (proteins and DNA) and 100 µl TBA was added to serve as the colouring reagent. All the tubes were mixed again and incubated at 60ºC for 60 minutes. After incubation, the tubes were cooled on ice until it reached room temperature.

400 µl of butanol was added to all tubes and mixed again. The tubes were centrifuged at 2000 x g for 10 minutes. 200 µl of the supernatant was removed and placed in a 96 well cell culture plate. The absorbance values were obtained spectrophotometrically at 530 nm. Butanol was used as blank.

4.3 NBT (nitroblue tetrazolium) assay

Oxygen is absorbed into living cells and changed to multiple reactive oxygen species and free radicals. These free radicals can furthermore partake in a cascading reaction to produce more free radicals. The superoxide radical (O_2^-) is one of the most potent reactive oxygen species generated. O_2^- can convert to other reactive oxygen species, such as hydrogen peroxide and the hydroxyl radical.

The principle of the NBT assay is based on the reducing ability of free radicals to change the yellow dye (NBT) to a water insoluble blue dye (NBD). NBD is then extracted using glacial acetic acid and the absorbance values were obtained spectrophotometrically at 560 nm. This assay is used to determine the ability of the extracts to scavenge superoxide radicals. A modified method of the NBD assay as used by Ottino and Duncan (1997) was used for completion of these tests.

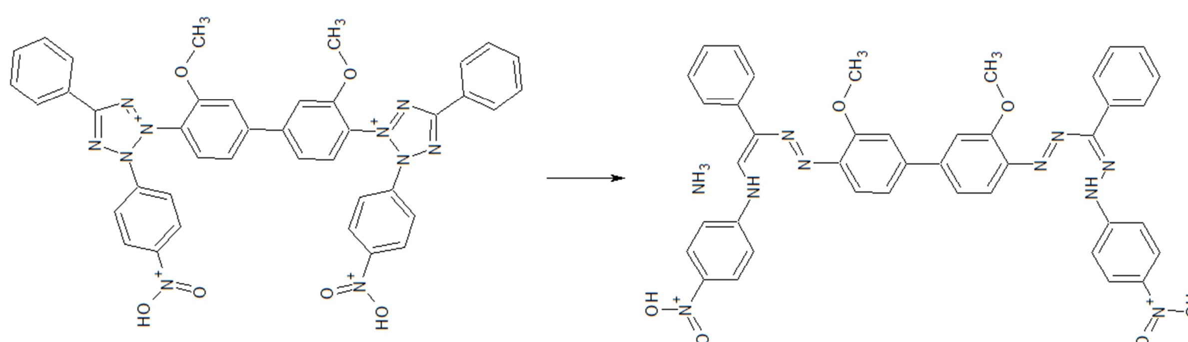


Figure 4.3 Reduction of NBT to NBD in the NBT assay (Kaur & Geetha, 2006).

4.3.1 Preparation of extracts

The solutions for this assay were prepared in the same manner as for the TBA assay, see 4.2.2.

4.3.2 Animals

The animals used in this assay were treated in the same manner as for the TBA assay, see 4.2.3.

4.3.3 Chemicals and reagents

PBS (Phosphate buffer) – the PBS was prepared in the same manner as in the NBT assay, see 4.2.4.

KCN (4 mM) – 0.026 g of KCN (potassium cyanide) was dissolved in 100 ml H_2O. This solution was prepared in the fume hood while wearing gloves to minimize the exposure to the lethal cyanide.

NBD (400 μM) – 0.0075 g was dissolved in 25 ml glacial acetic acid (GAA). NBD was prepared fresh daily and covered in foil.

Trolox (4 mM) – 0.001 g was dissolved in 1 ml H_2O.

BSA – 2 mg BSA (bovine serum albumin) was dissolved in 1 ml PBS.

Bradford reagent – a sufficient amount was withdrawn from the bottle for the completion of the experiment.

4.3.4 Preparation of standard curves

4.3.4.1 NBD Standard curve

A series of solutions was prepared directly into a 96 well cell culture plate. The plate was prepared as follow to determine the NBD standard, see table 4.3. The absorbance values were then obtained spectrophotometrically at 560 nm.

Table 4.3 Table of tubes prepared for NBD standard solutions.

Concentration (μM)	NBD (μl)	GAA (μl)
0	0	255
100	63.75	191.25
200	127.5	127.25
300	191.25	63.75
400	255	0

GAA - glacial acetic acid.

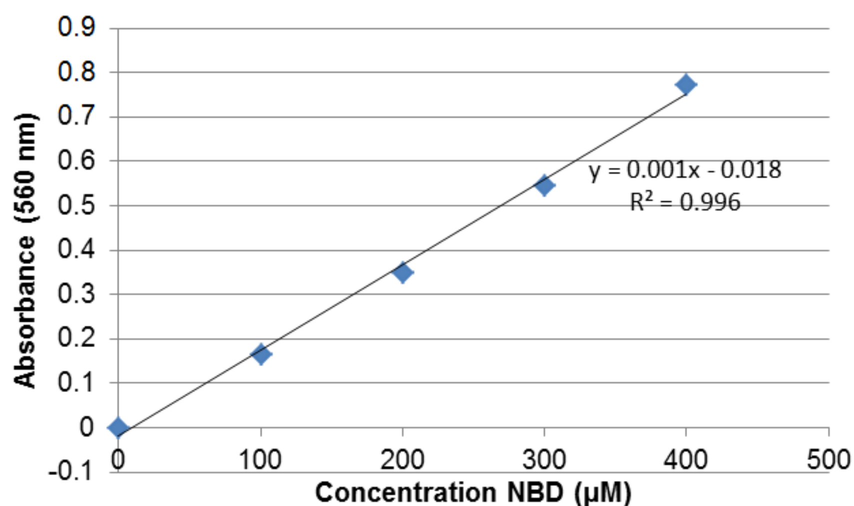


Figure 4.4 Standard curve generated for NBD.

4.3.4.2 BSA Standard curve

The BSA standard curve was prepared by preparing a series of solutions containing BSA and PBS. The solutions were prepared as follows, see table 4.4.

Table 4.4 Preparation of BSA standard solutions.

Protein concentration (mg/ml)	Volume BSA (µl)	Volume PBS (µl)
0	0	100
0.1	5	95
0.4	20	80
0.7	35	65
1	50	50
1.4	70	30

The Bradford protein assay was done simultaneously with the BSA assay. This assay was done to get accurate readings from the brain tissues, as concentrations of protein higher than 1.4 mg/ml yields unreliable data. A series of tubes were prepared containing rat brain homogenate and PBS. The tubes were prepared as follows, see table 4.5.

Table 4.5 Preparation of tubes for Bradford protein assay.

Volume homogenate (µl)	Volume PBS (µl)
100	0
10	90
5	96
2	98
0	100

From these tubes, 5 µl was carried over to the 96 well cell culture plate in triplicate. This was done with the Bradford protein assay and the BSA standard tubes. 250 µl of Bradford reagent was added to all the wells and then shaken for 30 seconds on the mixer of the plate reader. The samples were then incubated at room temperature for 15 minutes and the absorbance values were obtained spectrophotometrically at 560 nm.

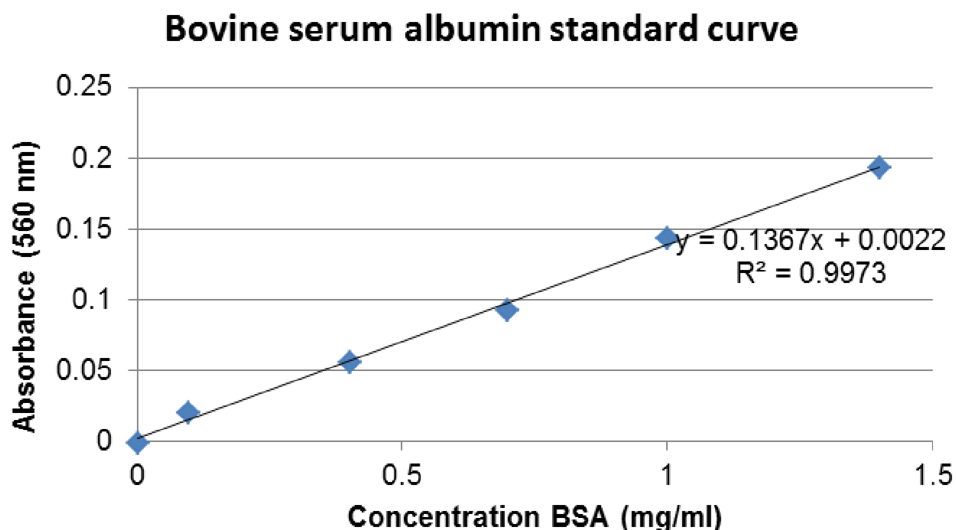


Figure 4.5 Standard curve generated for the BSA standard.

4.3.5 Brain homogenates

The brain homogenates were prepared in the same manner as for the TBA assay, see 4.2.6.

4.3.6 Assay

A series of tubes were prepared for testing. The tubes were prepared as follows, see table 4.6.

Table 4.6 Tubes prepared for the NBT assay.

	Homogenate (µl)	PBS (µl)	KCN (µl)	Trolox (µl)	Ethanol (µl)	DMSO (µl)	Drug (µl)
Control	100	100					
KCN	100	50	50				
Trolox	100		50	50			
Ethanol	100		50		50		
DMSO	100		50			50	
Drug	100		50				50

All tubes were mixed on a vortex mixer. 80 µl of NBT was then added to the solutions. The tubes were mixed again and incubated at 37ºC for 60 minutes. During this incubation time, the Bradford protein assay was done.

Once the incubation was done, the tubes were centrifuged at 3000 x g for 10 minutes. The supernatant was discarded and 400 µl of glacial acetic acid was added to the resting pellet. The tubes were mixed again and 255 µl of the top layer was removed and placed in a 96 well cell culture plate. The absorbance of the solutions was obtained spectorphotometrically using UV light at 560 nm. Glacial acetic acid was used as blank.

4.4 Toxicity testing

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used. This assay was first described by Mossman (1983) and is still one of the most popular assays used to test toxicity towards mammalian cells (Freimoser *et al*, 1999).

The assay is based on the same principles as the NBT assay. Yellow MTT crystals are reduced to purple formazan crystals. The purple formazan crystals are then extracted by using isopropanol and analysed spectrophotometrically. The colourimetric signal that is obtained is proportional to the amount of viable cells and conversion can be done with a simple formula, see equation 4.1.

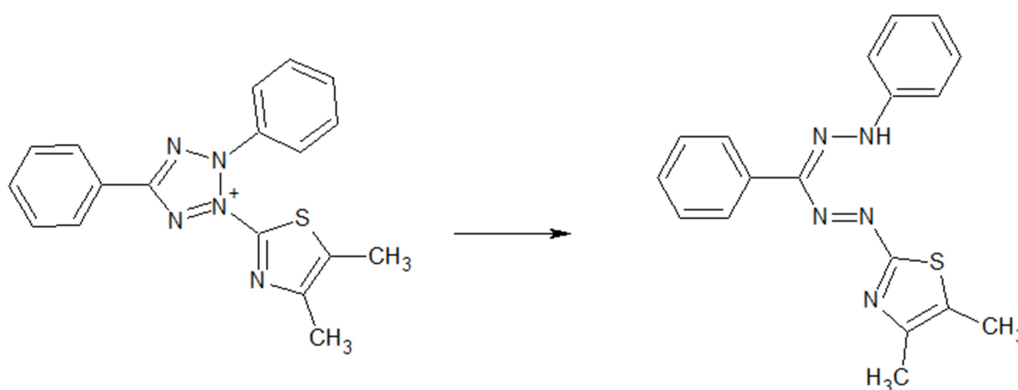


Figure 4.6 Formation of the purple formazan crystals from MTT in living cells (Mossman, 1983).

4.4.1 Chemicals and reagents

Cell growth medium – 40 ml DMEM (Dulbecco's modified eagle medium) was placed in a 60 ml falcon tube. 10 ml FBS (foetal bovine serum) was added to the DMEM as well as 500 µl of PenStrep (penicillin and streptomycin) and 500 µl of Fungizone to combat infection of the cells. These falcon tubes were marked with its contents and stored in the fridge between 2 and 8ºC.

Isopropanol – the required volume of isopropanol was used on the day of the assay.

MTT – 500 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was diluted to 10 ml on the day of the assay with PBS (Phosphate buffer).

PBS (Phosphate buffer) – the PBS was prepared in the same manner as in the NBT assay, see 4.2.4. The PBS was filtered in the laminar flow chamber prior to use to prevent infection of the cells.

Tryptan EDTA – one bottle of tryptan EDTA was defrosted and split into 15 ml Falcon tubes to prevent the original packaging from being frozen and thawed repeatedly.

4.4.2 Cell preparation

The neuroblastoma cells used in the assay were prepared beforehand. The cells were kept in a chest freezer at -150ºC. The cells were thawed and placed in a 15 ml falcon tube with 10 ml of cell growth medium (DMEM). The tube was centrifuged at 1000 x g for 10 minutes and the supernatant was removed. 10 ml of new growth medium was added to the tube and shaken until the cells were in suspension. The cell suspension was then added to a 150 ml cell culture flask (with vented cap) with 20 ml of the growth medium. The cells were then placed in the incubator at 37ºC, with 5% CO_2 and relative humidity of 100%.

4.4.2.1 Change of growth medium

The cells were inspected daily and the growth medium was changed when necessary (2-3 times a week) to ensure cells had enough growth medium to multiply successfully.

The growth medium of the cells was changed by removing the cells from the incubator and spraying the culture flasks with 70% ethanol solution to disinfect the exterior of the flasks and placed in the laminar flow chamber. The old medium was decanted in a large falcon tube and 30 ml of new growth medium was added to the culture flask. The culture flasks were sprayed with a 70% ethanol solution and placed back in the incubator.

The old medium was aspirated into a large vacuum container for disposal at a later stage.

4.4.2.2 Splitting of cells

Cells were split when the culture flasks reached a total confluence of about 80%. Splitting of the cells was done by decanting the growth medium into waste containers and adding 2 ml of tryptan EDTA to the flask to rinse the remaining medium from the flasks. 3 ml of tryptan EDTA was then added to the flask and placed in the incubator for 5 minutes. After 5 minutes the flasks were shaken and placed back into the incubator for 5 more minutes.

After a 10 minute period the flask was inspected under the microscope to ensure the cells are in suspension and ready for transfer to new cell culture flasks.

To split one flask into two, 5 ml of the growth medium was added to the tryptan EDTA cell suspension and the solution was mixed with a pipette to ensure an even spread of the cells throughout the suspension. This suspension was then divided in two portions of 4 ml (total of 3 ml tryptan EDTA and 5 ml of growth medium) and placed into new cell culture flasks. 26 ml of the growth medium was added to each of the new flasks to reach a total volume of 30 ml of cell suspension in each flask (this formula can be adjusted to split the cells in more than two fractions). The flasks were sprayed with 70% ethanol solution and placed in the incubator.

The tubes used for disposal were aspirated into a vacuum container for disposal at a later time.

4.4.3 Method

The assay was conducted over three days with certain preparation needed on each day. Day one was the seeding of the cells, day two the pre-treatment of the wells and day three was the conclusion of the assay.

4.4.3.1 Day one

Two confluent (80%) cell culture flasks were used to prepare the stock solution of cells. This was done by adding 3 ml of tryptan EDTA to the flasks and incubating the flasks at 37ºC for 10 minutes. At 5 minutes the flasks were removed and shaken to loosen the cells. After 10 minutes of incubation the flasks were inspected to ensure the cells were in suspension. 5 ml of growth medium was added to the tryptan EDTA

cell suspension and carried over to a falcon tube using a pipette. This suspension is known as the stock suspension.

A ten times dilution was then prepared to determine the number of cells present in the stock suspension. 50 µl of the cell suspension was withdrawn from the stock suspension and added to 450 µl of growth medium. The stock suspension was kept on a rotating stand to prevent cells adhering to the sides of the falcon tube.

20 µl of the diluted cell suspension was placed on a heamocytometer to count the cells under the microscope. All nine squares of the haemocytometer were tallied and an average was derived from the total. The dilution factor was corrected and the amount of cells needed per well was calculated with the following equation, see equation 4.1.

Equation 4.1

$$VCR = (TC / NCC) \times FVC$$

Where VCR is the volume of cells required, TC is the total number of cells per well, NCC is the number of counted cells, and FVC is the final volume of cells and DMEM required to seed.

By following this equation the total volume of cells required can be determined and the required amount of cells can then be adjusted accordingly.

The new suspension of cells (volume of cells required for seeding) was prepared and 1 ml of the suspension was added to all the wells (24 well cell culture plates were used) required in the assay. This was done in a laminar flow chamber and care was taken to prevent unnecessary contamination of the cells. The plate was then sprayed with a 70% ethanol solution to decontaminate the external areas of the plate and placed in the incubator for 24 hours.

4.4.3.2 Day two

Preparation of the methanol extract and fractions to be tested was done prior to day 2; the solutions were all prepared in the laminar flow chamber and filtered before use. Stock solutions of 10 mg/ml, 2 mg/ml, 0.4 mg/ml and 0.08 mg/ml were prepared by using PBS as solvent.

The cell culture plates were prepared by aspirating the growth medium after a 24 hour incubation period and preparing the wells as follows, see table 4.7.

Table 4.7 Preparation of 24 well plates for MTT assay, where extract 1 is 10 mg/ml, extract 2 is 2 mg/ml, extract 3 is 0.4 mg/ml and extract 4 is 0.08 mg/ml.

0% growth	100% growth	Methanol Extract	Fraction 1	Fraction 2	Fraction 3
1 ml cells, 500 µl DMEM	1 ml cells, 400 µl DMEM, 100 µl Solvent	1 ml cells, 400 µl DMEM, 100 µl Extract	1 ml cells, 400 µl DMEM, 100 µl Fraction	1 ml cells, 400 µl DMEM, 100 µl Fraction	1 ml cells, 400 µl DMEM, 100 µl Fraction
1 ml cells, 500 µl DMEM	1 ml cells, 400 µl DMEM, 100 µl Solvent	1 ml cells, 400 µl DMEM, 100 µl Extract	1 ml cells, 400 µl DMEM, 100 µl Fraction	1 ml cells, 400 µl DMEM, 100 µl Fraction	1 ml cells, 400 µl DMEM, 100 µl Fraction
1 ml cells, 500 µl DMEM	1 ml cells, 400 µl DMEM, 100 µl Solvent	1 ml cells, 400 µl DMEM, 100 µl Extract	1 ml cells, 400 µl DMEM, 100 µl Fraction	1 ml cells, 400 µl DMEM, 100 µl Fraction	1 ml cells, 400 µl DMEM, 100 µl Fraction
Blank	Blank	Blank	Blank	Blank	Blank

The plates were then sprayed with a 70% ethanol solution to prevent contamination and placed in an incubator for 24 hours.

4.4.3.3 Day three

The stock solution of MTT was prepared after the 24 hour incubation period and 200 µl of the MTT was added to all the wells and plates. The addition of MTT terminates cell growth and the cleavage of the yellow MTT crystals. The plates were then placed back in the incubator for a further 2 hours at 37ºC.

The supernatant was aspirated from the wells and 250 µl of isopropanol was added to the wells to dissolve the formazan crystals. The plates were shaken lightly to help with this process. 100 µl of this solution from all the wells were transferred to a 96 well cell culture plate and the absorbance values were obtained spectrophotometrically at 560 nm and a reference wavelength of 650 nm.

Results were expressed as a percentage of viable cells by using the following equation, see equation 4.2.

Equation 4.2

$$\% \text{ cellular viability} = (\Delta \text{ absorbance} - \Delta \text{ blank}) / (\Delta \text{ control} - \Delta \text{ blank}) \times 100$$

Where:

Δ absorbance = absorbance at 650 nm – absorbance at 560 nm,

Δ blank = mean of blank at 650 nm – mean of blank at 560 nm, and

Δ control = cell control at 650 nm – cell control at 560 nm.

CHAPTER 5: RESULTS AND DISCUSSIONS OF ASSAYS

5.1 Results of lipid peroxidation assay

The results for the lipid peroxidation assay are summarized in table 5.1 and figures 5.1, 5.2, 5.3 and 5.4.

Table 5.1 The effect of the methanolic extract and 3 fractions of *Cotyledon orbiculata* on H_2O_2 toxin induced lipid peroxidation in rat brain homogenate.

Test Compounds	Concentration	Lipid Peroxidation	± S.E.M.
Control		0.302	0.095
Toxin	5 mM H_2O_2 4.44 mM Vitamin C 1.68 mM Fe_3Cl	1.307	0.033
Trolox		0.127	0.166
Ethanol		1.304	0.101
Methanol extract	0.3125 mg/ml	0.693***	0.056
	0.625 mg/ml	0.453***	0.053
	1.25 mg/ml	0.241***	0.022
	2.5 mg/ml	0.146***	0.011
Fraction 1	0.3125 mg/ml	1.154	0.031
	0.625 mg/ml	1.035***	0.034
	1.25 mg/ml	0.943***	0.023
	2.5 mg/ml	1.110*	0.033
Fraction 2	0.3125 mg/ml	1.017**	0.113
	0.625 mg/ml	0.988***	0.067
	1.25 mg/ml	0.779***	0.122
	2.5 mg/ml	1.162	0.119
Fraction 3	0.3125 mg/ml	1.313	0.152
	0.625 mg/ml	1.016**	0.149
	1.25 mg/ml	1.385	0.125
	2.5 mg/ml	1.491	0.115

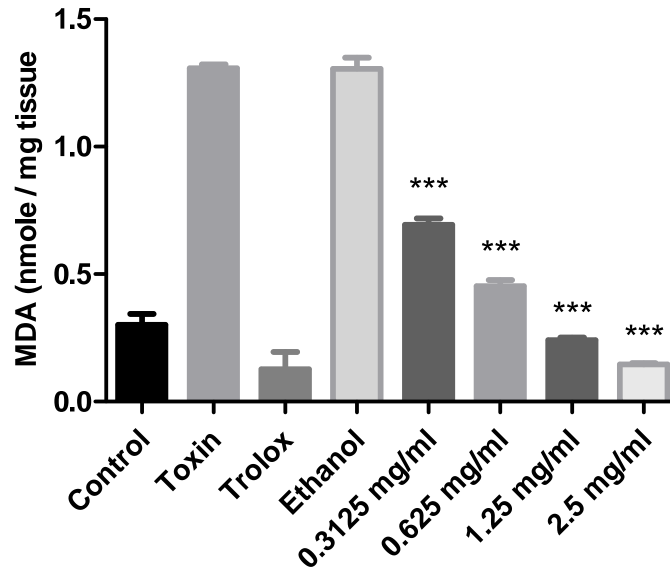


Figure 5.1 The effect of lipid peroxidation by different concentrations of a methanol extract of *Cotyledon orbiculata* extracts in whole rat brain homogenate. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

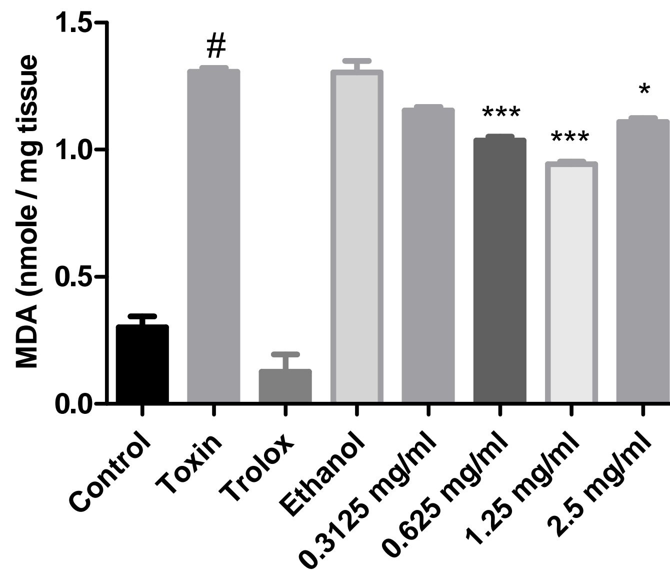


Figure 5.2 The effect of lipid peroxidation by different concentrations of fraction 1 of *Cotyledon orbiculata* extracts in whole brain homogenate. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

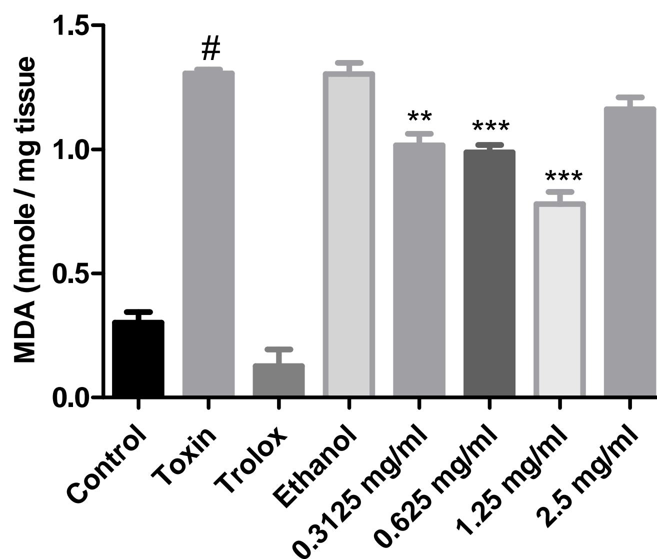


Figure 5.3 The effect of lipid peroxidation by different concentrations of fraction 2 of *Cotyledon orbiculata* extracts in whole brain homogenate. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

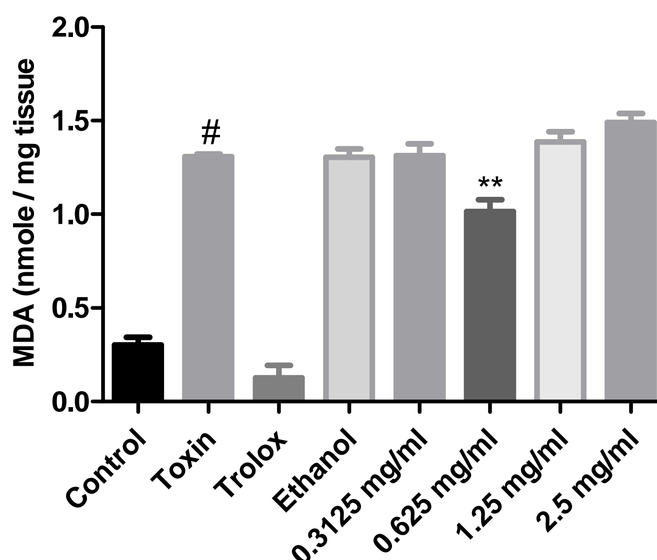


Figure 5.4 The effect of lipid peroxidation by different concentrations of fraction 3 of *Cotyledon orbiculata* extracts in whole brain homogenate. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

5.1.1 Statistical analysis

GraphPad Prism was selected as software to complete the statistical analysis. Results were given as the mean and ± S.E.M. of 5 repeats. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett test where all columns of data are compared to a selected column (Toxin). Differences between groups were considered to be significant if $p < 0.001$.

5.1.2 Discussion

The methanol extract of *Cotyledon orbiculata* showed very good antioxidant properties over the whole concentration range tested. All fractions and the methanol extract from *C. orbiculata* showed antioxidant activity *in vitro* with the TBA-assay.

Fraction 1 (at concentrations of 0.625 mg/ml and 1.25 mg/ml) and fraction 2 (also at concentrations of 0.625 mg/ml and 1.25 mg/ml) were the only fractions which showed activity significantly different from the toxin, fraction 3 (at a concentration of 0.625 mg/ml) showed activity slightly significant in difference over the toxin. All the fractions and the methanol extract had the same pattern with antioxidant activity increasing with higher concentrations of extract up to 1.25 mg/ml. The higher concentrations (fraction 1 and 2 at 2.5 mg/ml and fraction 3 at 1.25 mg/ml and 2.5 mg/ml) showed diminished antioxidant activity.

In comparison with the toxin, 1.307 ± 0.033 nmole MDA/mg tissue only 4 concentrations showed activity significantly different from the toxin, these were fraction 1 with concentrations of 0.625 mg/ml (1.035 ± 0.034 nmole MDA/mg tissue), fraction 1 with a concentration of 1.25 mg/ml (0.943 ± 0.023 nmole MDA/mg tissue), fraction 2 with a concentration of 0.625 mg/ml (1.017 ± 0.067 nmole MDA/mg tissue) and fraction 2 at a concentration of 1.25 mg/ml (0.779 ± 0.122 nmole MDA/mg tissue).

5.2 Results of the NBT assay

The results for the lipid peroxidation assay are summarized in table 5.2 and figures 5.5, 5.6, 5.7 and 5.8.

Table 5.2 The effect of the methanol extract and 3 fractions of *Cotyledon orbiculata* on KCN-induced superoxide anion formation in rat brain homogenate.

Test Compounds	Concentration	Diformazan	± S.E.M.
Control		22.032	3.229
Toxin	1 mM KCN	36.561	1.403
Trolox		21.850	0.741
Methanol extract	0.3125 mg/ml	25.552***	2.706
	0.625 mg/ml	24.768***	2.474
	1.25 mg/ml	23.720***	2.274
	2.5 mg/ml	22.968***	1.806
Fraction 1	0.3125 mg/ml	11.039***	1.634
	0.625 mg/ml	12.149***	1.058
	1.25 mg/ml	14.144***	1.448
	2.5 mg/ml	9.18***	0.791
Fraction 2	0.3125 mg/ml	31.053**	1.175
	0.625 mg/ml	30.351***	2.128
	1.25 mg/ml	21.919***	2.515
	2.5 mg/ml	32.323*	2.007
Fraction 3	0.3125 mg/ml	37.445	3.876
	0.625 mg/ml	27.699***	1.977
	1.25 mg/ml	41.684	3.825
	2.5 mg/ml	46.671	1.960

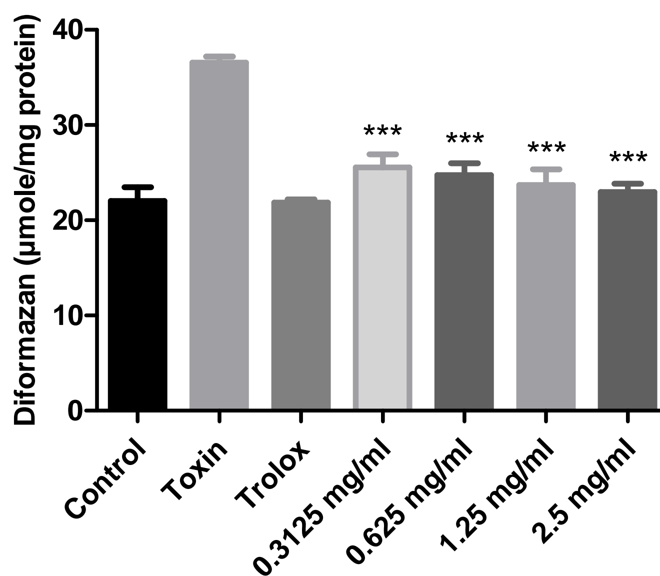


Figure 5.5 Superoxide scavenging ability of the methanol extract in the presence of KCN in rat brain homogenate. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

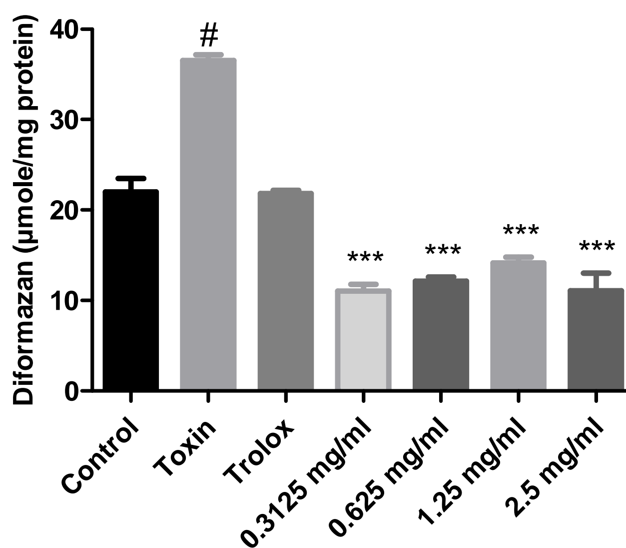


Figure 5.6 Superoxide scavenging ability of fraction 1 in the presence of KCN in rat brain homogenate. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

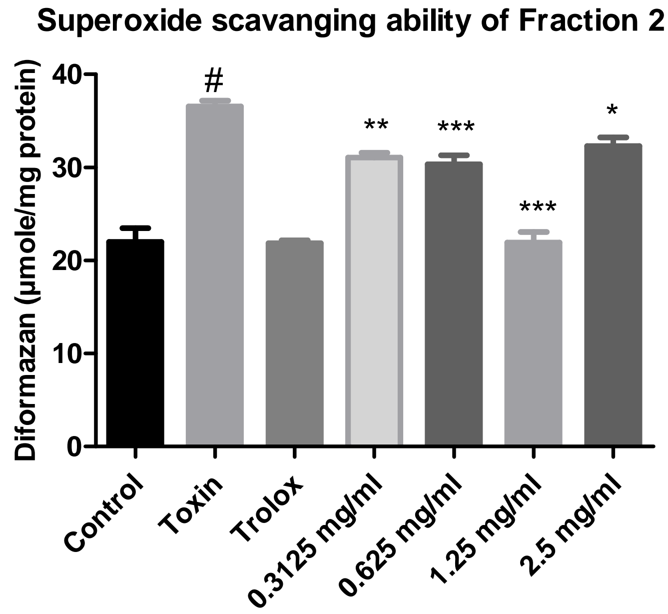


Figure 5.7 Superoxide scavenging ability of fraction 2 in the presence of KCN in rat brain homogenate. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

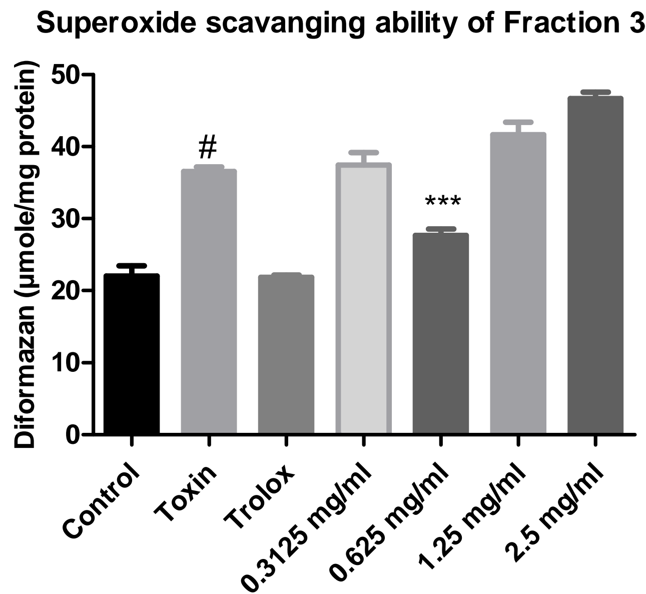


Figure 5.8 Superoxide scavenging ability of fraction 3 in the presence of KCN in rat brain homogenate. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

5.2.1 Statistical analysis

The statistical analysis was performed on the same way as for the TBA assay, see 5.1.1.

5.2.2 Discussion

The methanol extract of *Cotyledon orbiculata* had high antioxidant effects throughout the whole concentration range. All of the fractions from *C. orbiculata* showed antioxidant effects in the NBT-assay.

All fractions of *Cotyledon orbiculata* showed activity significantly different when compared to the toxin (36.561 ± 1.403 diformazan µmole/mg). Fraction 1 showed activity significantly different when compared to the toxin through all the concentrations, ranging from 0.3125 mg/ml extract to 2.5 mg/ml extract. Fraction 2 showed activity significantly different when compared to the toxin at concentrations of 0.625 mg/ml and 1.25 mg/ml. Fraction 3 only showed activity significantly different when compared to the toxin at a concentration of 0.625 mg/ml.

In comparison with the toxin there were eleven data sets that showed activity significantly different when compared to the toxin, these were the methanol extract and fraction 1 at all concentrations, fraction 2 at concentrations of 0.625 mg/ml (30.351 ± 2.128) and 1.25 mg/ml (21.919 ± 2.515) and fraction 3 at a concentration of 0.625 mg/ml (27.699 ± 1.977).

Fraction 1 showed the most promise in antioxidant activity.

5.3 Results of toxicity testing

The results for the toxicity testing assay are summarized in table 5.3 and figures 5.9, 5.10, 5.11 and 5.12.

Table 5.3 The effect of the methanol extract and 3 fractions of *Cotyledon orbiculata* on the cellular growth of neuroblastoma cells.

Test Compounds	Concentration	% Viable Cells	± S.E.M.
0 % Growth		0	0
100% Growth		100.052	8.576
Methanol extract	10 mg/ml	70.257***	7.417
	2 mg/ml	82.721*	5.471
	0.4 mg/ml	98.188	5.930
	0.08 mg/ml	97.469	3.597
Fraction 1	10 mg/ml	77.900	6.827
	2 mg/ml	86.991	2.426
	0.4 mg/ml	96.429	6.494
	0.08 mg/ml	95.844	3.609
Fraction 2	10 mg/ml	43.896***	4.829
	2 mg/ml	92.779	8.611
	0.4 mg/ml	100.052	3.293
	0.08 mg/ml	104.753	6.061
Fraction 3	10 mg/ml	74.779	1.928
	2 mg/ml	90.623	5.717
	0.4 mg/ml	92.909	2.469
	0.08 mg/ml	96.260	2.02

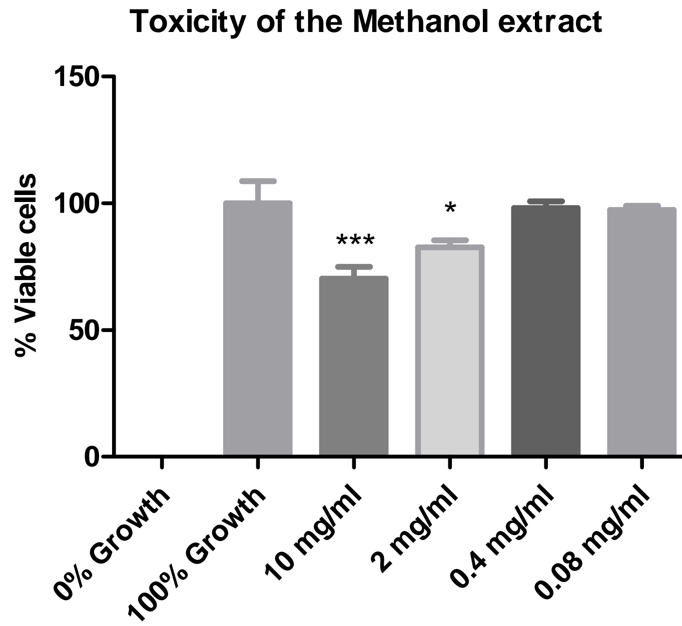


Figure 5.9 Results obtained by exposing neuroblastoma cells to the methanol extract of *Cotyledon orbiculata*. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

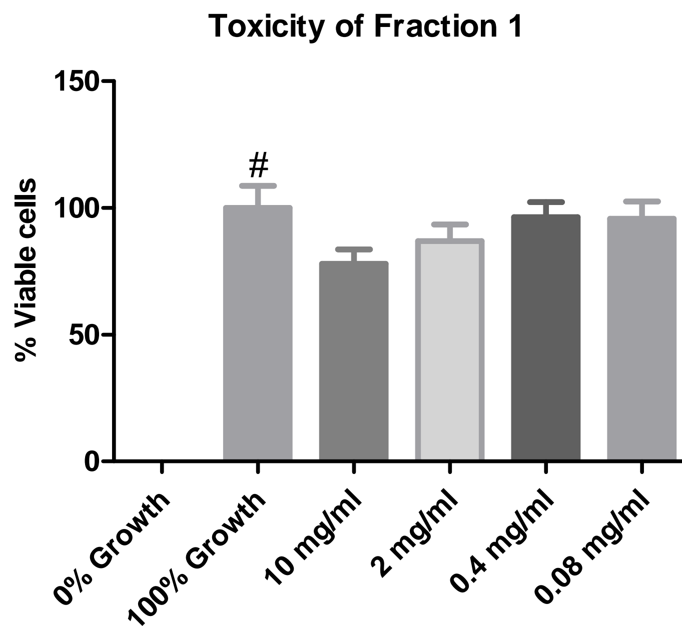


Figure 5.10 Results obtained by exposing neuroblastoma cells to fraction 1 of *Cotyledon orbiculata*. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

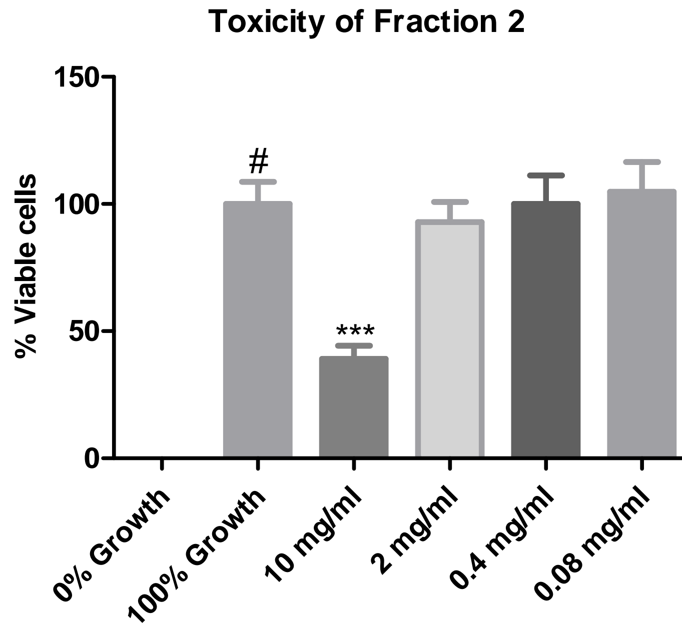


Figure 5.11 Results obtained by exposing neuroblastoma cells to fraction 2 of *Cotyledon orbiculata*. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

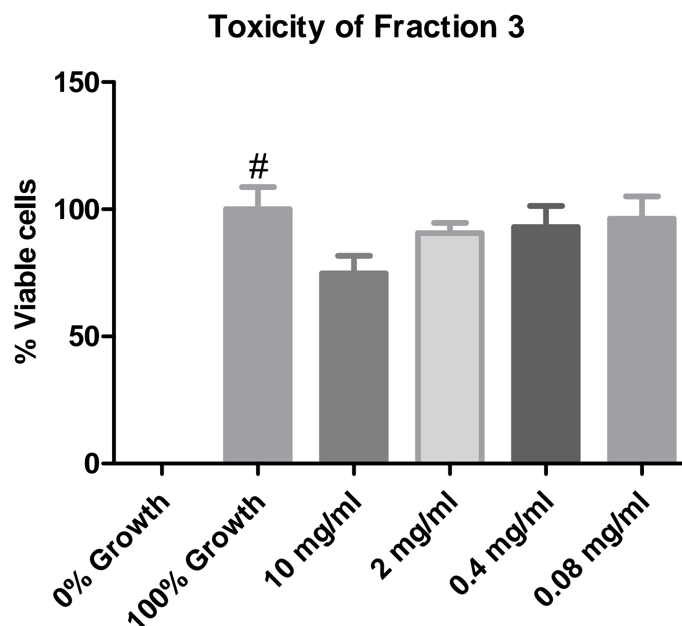


Figure 5.12 Results obtained by exposing neuroblastoma cells to fraction 3 of *Cotyledon orbiculata*. Each bar represents the mean ±S.E.M. (n=5). ***p<0.001 vs. toxin (#).

5.3.1 Statistical analysis

The statistical analysis was performed on the same way as for the TBA assay, see 5.1.1.

5.3.2 Discussion

All results obtained from the toxicity assay were compared to the control group, 100% growth (100.052 ± 8.576 % viable cells).

The methanol extract of *Cotyledon orbiculata* showed toxicity in the highest concentration of 10 mg/ml and was also slightly toxic at a concentration of 2 mg/ml.

All of the fractions obtained from *C. orbiculata*, except fraction 2 at a concentration of 10 mg/ml (43.896 ± 4.829 % viable cells), did not affect the cellular growth significantly.

The non-toxic fractions and the non-toxic methanol extract of *C. orbicuata*, with the concentrations ranging from 10 mg/ml to 0.08 mg/ml, showed a spread in cellular growth from 74.779 ± 1.928 % to 104.753 ± 6.061 %.

Only fraction 2 and the methanol extract at a concentration of 10 mg/ml showed toxicity significantly different from the control with 43.896 ± 4.829 % and 70.257 ± 5.096 % viable cells. Fraction 2 at a concentration of 10 mg/ml was the most toxic of all the extracts and fractions.

The effect of the extracts on the neuroblastoma cells is correlated to toxicity and therefore relate to the safe use of the extracts.

CHAPTER 6: STRUCTURE DETERMINATION\

6.1 Instrumentation

6.1.1 Nuclear magnetic resonance spectroscopy

^{1}H, ^{13}C, COSY, HSQC, HMQC and depth spectra were obtained using a Bruker Advance III 500; 5 mm BBO-Z probe; all spectra were recorded at 30 °C unless specified otherwise. Chemical shifts are in units of ppm, referenced to the residual protonated solvent in the deuterated solvent.

The software used for recording the data was Topspin version 2.1, patch level 6.

6.1.2 Mass spectroscopy

Analysis was performed using an Agilent 1200 Series Rapid Resolution LC System coupled to an Agilent 6510 Accurate-Mass Time-of-Flight spectrometer (LC/QTOF). The accurate mass measurements were performed by flow injection of 0.1 µl of the sample with a mobile phase flow rate 0.1 ml/min. The mobile phase consisted of a 50:50 (v/v) mixture of acetonitrile:water containing 0.1% formic acid.

The mass spectrometer parameters were:

Electrospray ionization in the positive mode, drying gas flow 8 l/min, nebulizer pressure 35 psi, dry gas temperature 300°C with the scanning range from 100 to 1000 *m/z* (4 GHz), an ion spray voltage of 4000 V, and fragmentor voltage of 130 V.

Data collection was performed using Agilent MassHunter Work-Station software

6.1.3 Infrared spectroscopy

The infrared spectra were recorded on a Nicolet Nexus 470-FT-IR spectrometer (Madison, Wisconsin, USA), over a range of 400-4000 cm^{-1}, using the diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) method.

Samples were prepared for DRIFT spectrometry by dispersing the sample in KBr.

6.2 Characterisation of compound

By comparing data obtained for the compound (WR1), which is the main chemical constituent present in fraction 2, to data found in literature, this compound can possibly be an organic acid ester. This compound was compared to diethyl malate (diethyl 2-hydroxysuccinate) found in the spectral database for organic compounds (SDBS) with an SDBS number of 10350. Comparison of the ^{1}H and ^{13}C spectra of diethyl malate to the spectra obtained from WR1, shared spectral data correlations. The comparison of the compound and diethyl malate is summarized in table 6.1 and table 6.2.

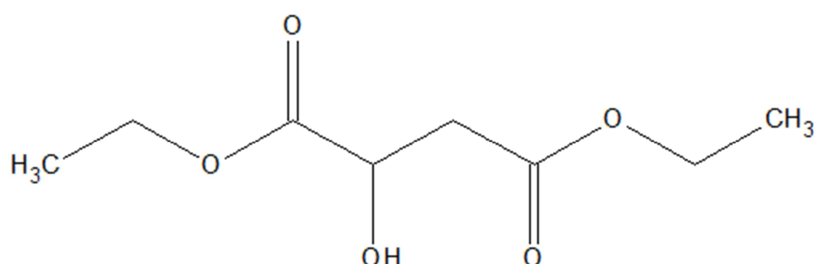


Figure 6.1 The chemical structure of diethyl malate.

Table 6.1 Comparison of ^{1}H NMR data, diethyl malate and WR1, (Appendix 1).

^{1}H NMR Spectral Data		
	Diethyl malate	WR1
C-H	4.49	4.48
O-CH_2	4.258	4.20
O-CH_2	4.181	4.13
OH	3.38	3.35
Ethanol		3.31
CH	2.86	2.76
CH	2.76	2.69
CH_3	1.308	1.27
CH_3	1.271	1.25
Ethanol		1.17

Table 6.2 Comparison of ^{13}H NMR data, diethyl malate and WR1 (Appendix 2).

^{13}C NMR Spectral Data		
	Diethyl malate	WR1
C=O	173.45	174.50
C=O	170.61	172.04
C-OH	67.44	68.57
CH_2	61.89	62.32
CH_2	60.94	61.95
Ethanol		58.31
CH_2	38.91	40.15
CH_3		18.44
Ethanol	14.14	14.46

The infrared spectrum that was obtained for WR1 showed broad peaks that couldn't accurately be identified. This could be due to the fact that there was still a high concentration of water in the sample, (Appendix 7).

The accurate mass of diethyl malate, found in literature and in the spectral database, was noted as 191.11 g/mole. The mass of the fraction WR1, containing the active compound, was determined as 191.1106 g/mole. It is thus possible to reason that WR1 could be diethyl malate, (Appendix 8).

A sample of WR1 was then compared to the methanol extract of *Cotyledon* to determine the presence thereof in the methanol extract of the plant. The dominant peak of WR1 was compared to the crude extract and was identified as an artefact that was probably generated during experimental or storage conditions.

Diethyl malate is an organic acid ester that can be generated from malic acid, which occurs in large quantities in the vacuoles of *Cotyledon* leaves (Barker *et al*, 1997). Ethanol was used to prepare the sample for HPLC separation. Diethyl malate, which is an analytical artefact sometimes obtained by HLPC separation of plant extracts, could be derived from a reaction that occurs between malic acid and ethanol (Middleditch, 1989).

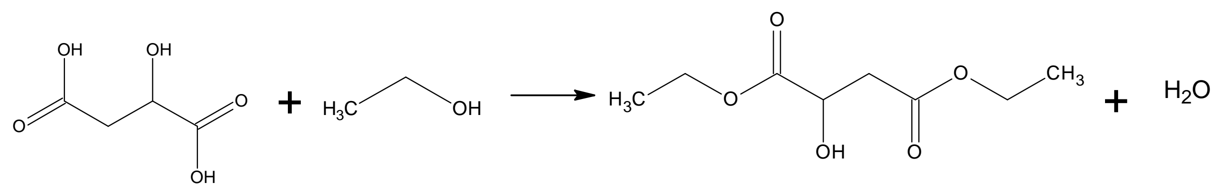


Figure 6.2 Synthesis of diethyl malate from malic acid and methanol.

CHAPTER 7: CONCLUSION

Free radicals and oxidants are produced in the body by the utilization of oxygen in normal physiological processes. Other sources of oxidants come from exogenous contributors such as diet and lifestyle (Martinez-Cayuela, 1994). These oxidants contribute to increased levels of free radicals and excessive oxidative stress. Oxidative stress is defined as the state where the complex antioxidant defence system of the human body is no longer able to process and eliminate all of the oxidants from the system. This stress leads to oxidative damage, early ageing and can ultimately cause neurodegenerative diseases (Sorg, 2004).

Oxidative damage and oxidative stress is implicated in the progression of many neurodegenerative diseases like Parkinson’s disease, Alzheimer’s disease and epilepsy. Antioxidants could play a major role as neuroprotective agents and could alter the progression of these diseases.

Antioxidants can alleviate some of these factors by reducing the reactive oxygen species. This is achieved by limiting free radical production, eliminating reactive oxygen species and up-regulating and supporting the natural antioxidant defences in the body. Therefore, the therapeutic effects of exogenous antioxidants are important in the regulation of free radicals and oxidants to prevent oxidative stress, neurodegeneration and premature ageing (Halliwell, 1995).

A large source of exogenous antioxidants comes from natural products such as plants. Many plants contain molecules (flavonoids, anthocyanins and carotenoids) with proposed antioxidant properties (Gilgun-Sherki, 2001).

Free radicals are produced in the brain by multiple pathways. The deposition of iron and copper ions after head injury can cause the accumulation of hydroxyl radicals and can initiate lipid peroxidation (Sharma *et al*, 2005). Neurotransmitters which are extensively used by the brain can also produce free radicals, including superoxide and nitric oxide (Volterra *et al*, 1994). The increased intercellular concentration of calcium can lead to depolarisation of neuronal cells and can cause the formation of epileptic focus development (Strijbos *et al*, 1994).

Cotyledon orbiculata is widely used to treat epilepsy in third world countries. The efficacy of this treatment could rest on the fact that Cotyledon orbiculata contains antioxidants that can improve the pathogenesis of the disease.

Amebeoku (2007) stated that the methanol extract of *Cotyledon orbiculata* had higher anti-convulsant effects when compared to the aqueous extract, and according to Mori *et al* (1999), many natural occurring antioxidants prevent epileptogenic focus formation and post traumatic induced seizures in the iron injected rat brain. With this data it could be deducted that the methanol extract may contain more potent antioxidants than the aqueous extract.

Cotyledon orbiculata was used in this study to determine the antioxidant properties of the methanol extract and its active components.

The methanol extract was prepared by extracting the freeze dried leaves of *Cotyledon orbiculata* with methanol in a Soxhlet apparatus.

The methanol extract was then analysed using HPLC and three major peaks were selected for isolation to determine the active compound. Isolation and separation of the extract was done on an Agilent 1100 series HPLC using a Phenomonex Synergi Fusion column connected to an Agilent 1200 Series fraction collector. A gradient system of 5% ACN was run for 1 minute where after the ACN was increased to 85% over 10 minutes and held stable for 5 minutes. Three peaks were selected for isolation, which were at 1.935 minutes, 2.558 minutes and 3.137 minutes.

The methanol extract and the three fractions from *Cotyledon orbiculata* were tested for antioxidant effects using the nitroblue tetrazolium assay and the thiobarbituric acid assay, both some of the most popular assays to assess anti-oxidant effects (Ottino & Duncan, 1997).

The methanol extract of *Cotyledon orbiculata* showed great antioxidant activity in both assays compared to those of the fractions collected. This antioxidant effect can be due to the fact that *Cotyledon orbiculata* has a high concentration of malic acid in the vacuoles of the cells, which is a known antioxidant (Kayashima & Katayama,

2002). The three fractions of *Cotyledon orbiculata* had less activity than the methanol extract, but still showed some potential as good antioxidants.

These fractions and the methanol extract were also tested for toxicity using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Only the methanol extract and fraction 2 at its highest concentration of 10 mg/ml showed increased toxic effects towards neuroblastoma cells that were used in the assay. The remaining concentration ranges were non-toxic.

In the characterization (recording of MS, IR and NMR spectroscopy) of the compound (WR1), an artefact was obtained or produced by experimental processes.

This artefact was identified as diethyl malate, which is an analytical artefact sometimes obtained by HLPC separation of plant extracts in the presence of diethyl malate and ethanol (Middleditch, 1989).

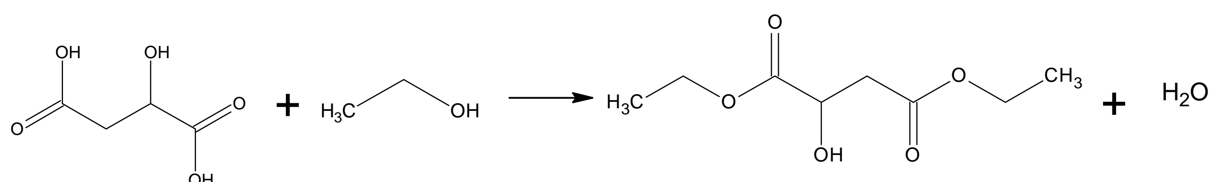


Figure 7.2 Synthesis of diethyl malate from malic acid and methanol.

A malic acid standard was injected into the HPLC and corresponded with the peaks that were selected for isolation. This artefact was then compared to a fresh methanol extract of the plant injected on HPLC and was found that the diethyl malate was not a molecule that normally occurs in the methanol extract of *Cotyledon orbiculata*.

The characterization of the active compounds of fraction 1 and 3 proved to be problematic. The spectroscopic data (NMR, IR and MS) that were obtained from these fractions were of low quality and showed possible contamination, or a mixture of molecules in the fractions and were not characterized.

In conclusion, the aim of the study was met. A methanol extract of *Cotyledon orbiculata* was tested for antioxidant properties and toxicity. Diethyl malate (WR1) was found as the main ingredient of fraction 2 identified from the methanol extract,

although not naturally occurring in *Cotyledon*, diethyl malate showed some promise in antioxidant effects.

The results obtained by this study further demonstrate the potential of natural antioxidants from medicinal plants as a natural exogenous source of antioxidants required on a daily basis.

The methanol extract of *Cotyledon orbiculata* has high antioxidant activity and could be due to the presence of malic acid in the leaves of the plant. The rationale in the use of *Cotyledon orbiculata* in the treatment of epilepsy could not be determined due to the isolation of an artefact, diethyl malate, obtained from the fraction.

Further research should include methods to prevent artefact formation and purification of the samples that are obtained.

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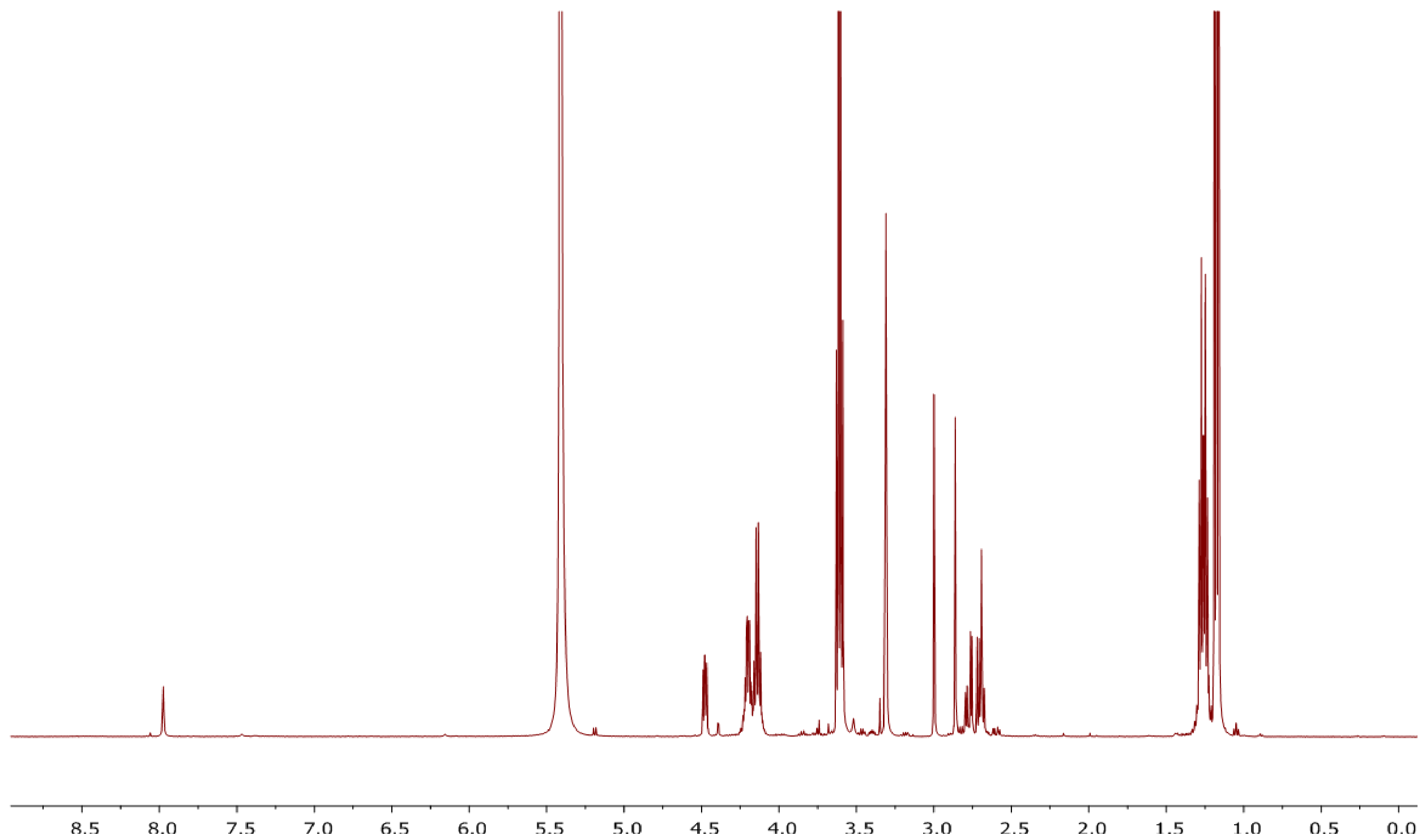
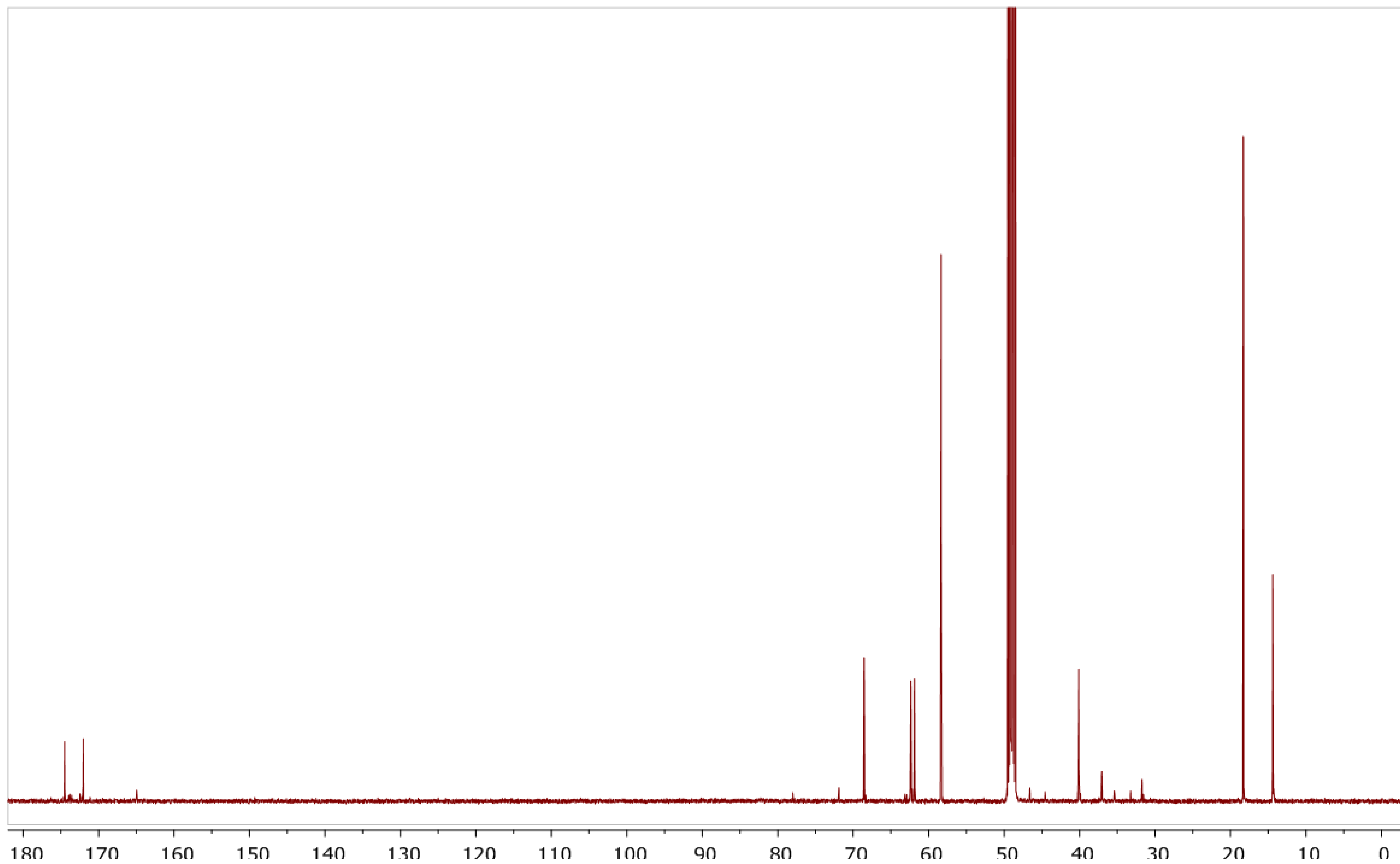
APPENDIX

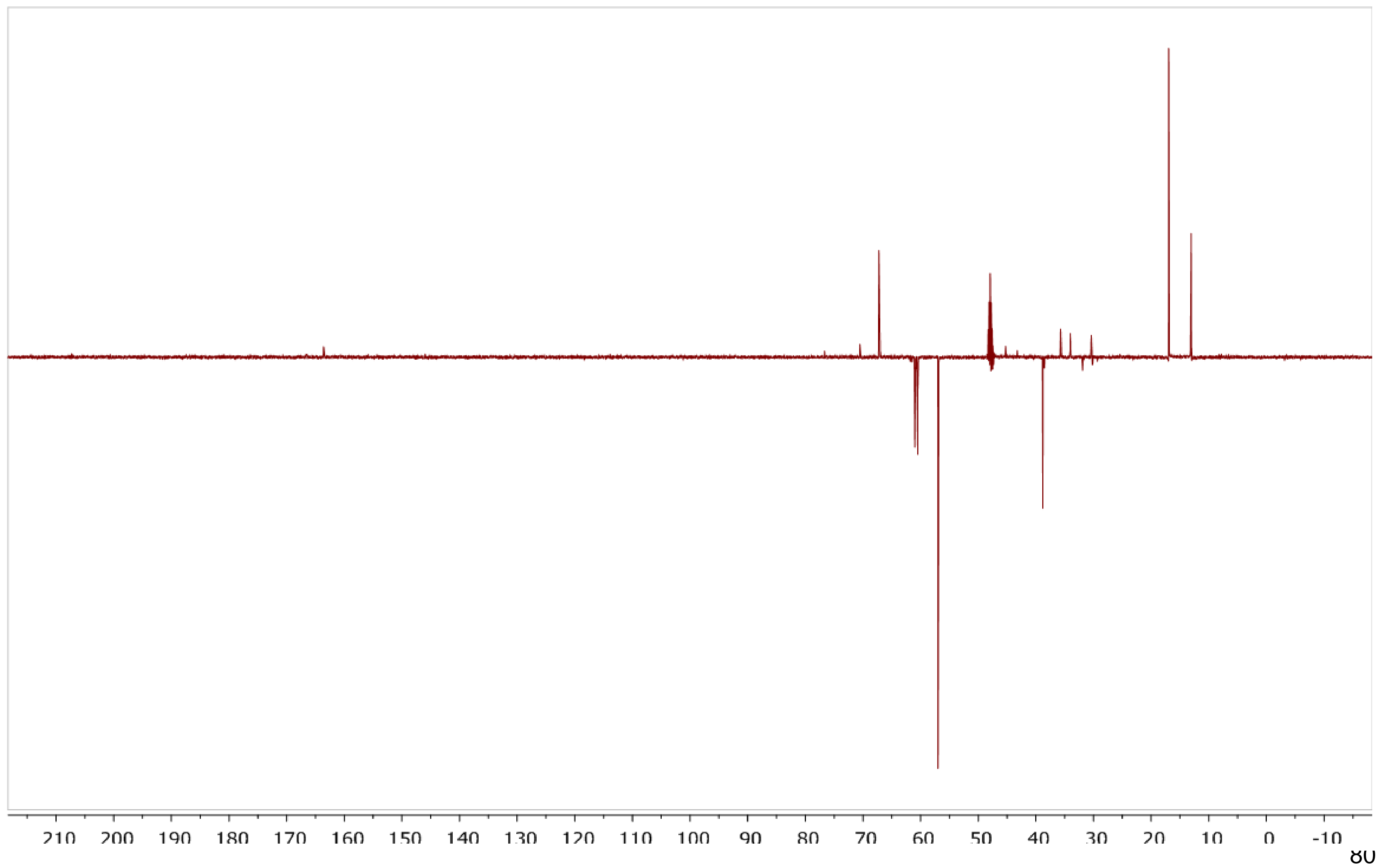
Appendix 1 – ^{1}H nmr spectra of WR2



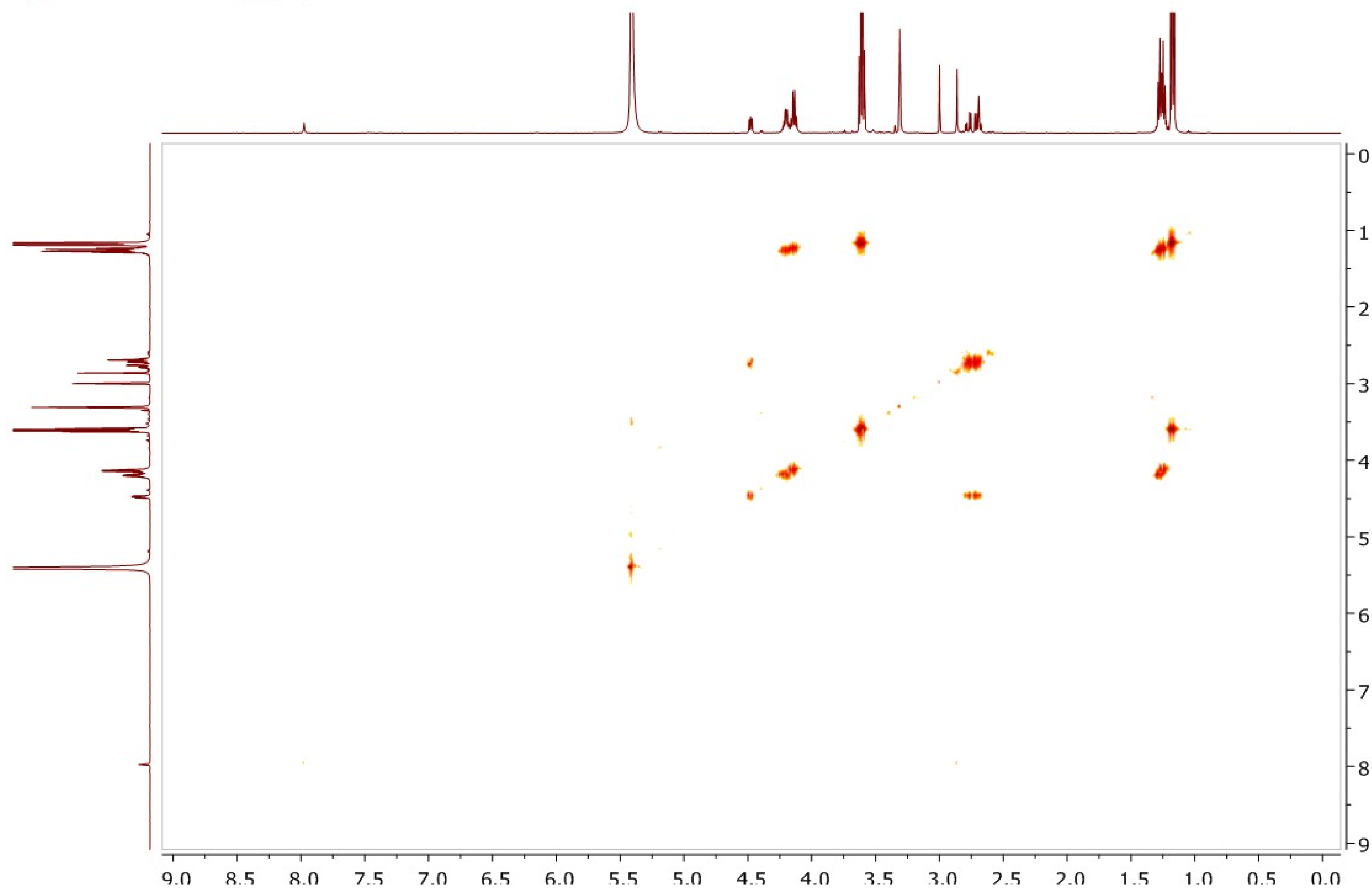
Appendix 2 – ^{13}C nmr spectra of WR2



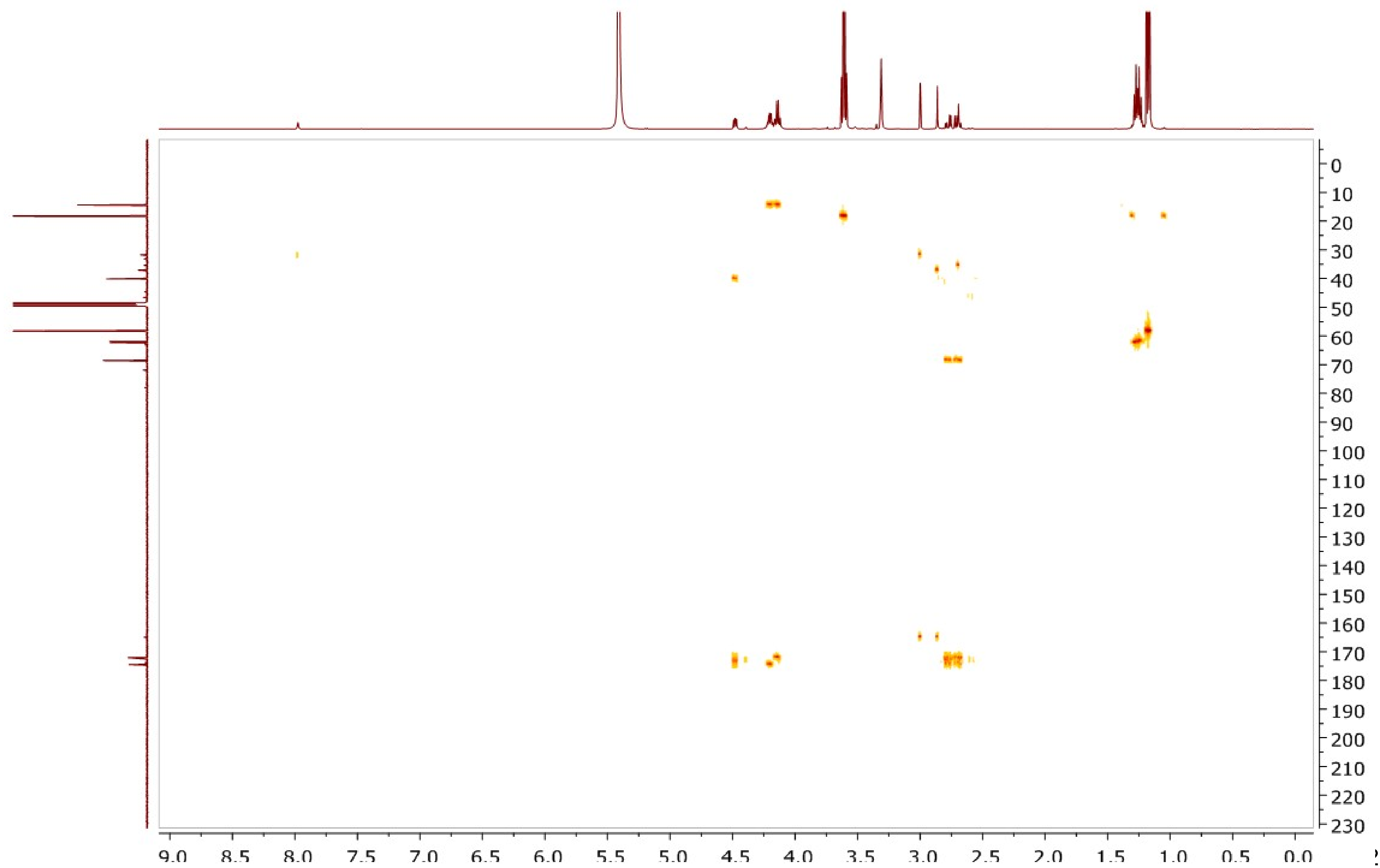
Appendix 3 – DEPT nmr spectra of WR2



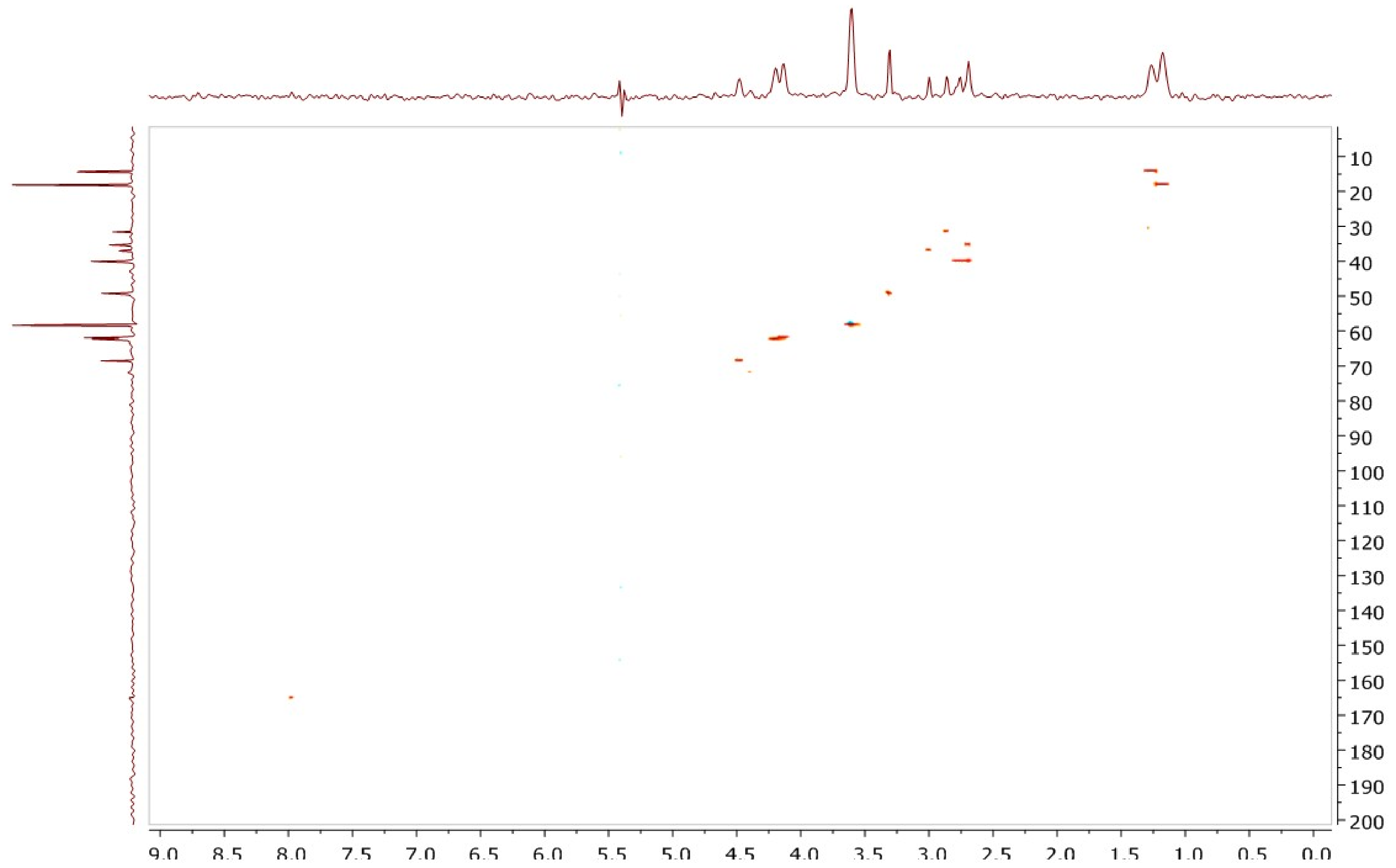
Appendix 4 – COSY nmr spectra of WR2



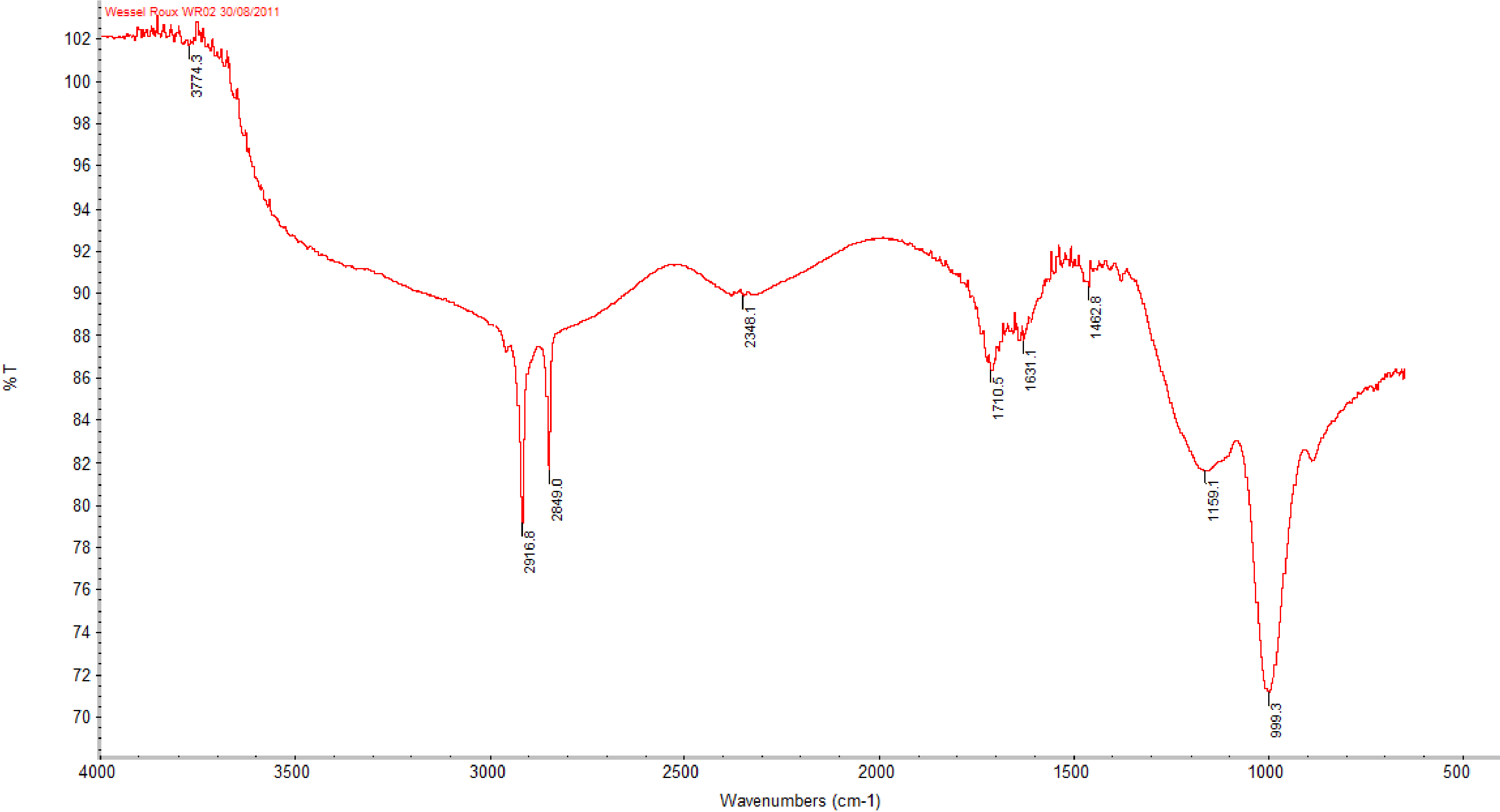
Appendix 5 – HMBC nmr spectra of WR2



Appendix 6 – HSQC nmr spectra of WR2



Appendix 7 – Infrared spectra of WR2



Appendix 8 – Mass spectra of WR2