EVALUATION AND APPLICATION OF ELECTROANALYSIS FOR THE DETERMINATION OF ANTIOXIDANTS

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by
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ABSTRACT

The role of antioxidants in the prevention of neurodegenerative diseases has been well documented. The use of synthetic antioxidants has decreased due to the association of these compounds with certain cancers. Thus, the search for novel natural antioxidants has gained much focus in research. Most common methods of determining antioxidant capacity are the radical generated assays and biological assays such as lipid peroxidation and the nitroblue tetrazolium assay. Electrochemical methods have been proposed for the determination of bio-active compounds such as antioxidants. The electrochemical methods of cyclic voltammetry and square wave voltammetry were evaluated for the determination of antioxidant capacity initially examining known antioxidants and then using plant extracts of *Sutherlandia frutescens* as a case study. The antioxidant properties determined by electrochemical methods were validated utilising the non-biological methods of the DPPH, TEAC, ferrozine and FC assay and biological pharmacological methods. The results indicated that *Sutherlandia frutescens* contains potent antioxidant compounds that are able to reduce lipid peroxidation. The electrochemical techniques of square wave voltammetry and cyclic voltammetry were applied for the screening of a large number of extracts of various algae for the detection of antioxidant compounds. The results indicated that electrochemistry can be used as a preliminary method for the rapid screening of a large number of crude samples for antioxidant compounds. Electrochemical methods were also evaluated as a method for guiding the isolation and purification of antioxidant metabolites in *Sargassum elegans*. Solvent partitioning and fractionation of the marine alga allowed for the purification of antioxidant compounds. At each step of purification electrochemical methods were utilized to determine which fractions contained the more potent antioxidant compounds and thus guide further purification. The purified antioxidant compounds were elucidated using NMR to determine the structure of the antioxidant compounds.

Key words: electrochemistry, antioxidants, *Sutherlandia frutescens*, DPPH, TEAC, lipid peroxidation, screening, *Sargassum elegans*.

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^{1}H NMR	= hydrogen nuclear magnetic resonance
ABTS	= Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt radical assay
AdSV	= Adsorptive Stripping Voltammetry
Ag/AgCl	= silver/ silver chloride
ASV	= Anodic Stripping Voltammetry
AUC	= area under the curve
BDE	= bond dissociation energy
BHT	= butylated hydroxytoluene
CSV	= Cathodic Stripping Voltammetry
CV	= cyclic voltammetry
μC	= micro-Coulomb
DCM	= dichloromethane
DPPH	= α, α-diphenylpicrylhydrazyl
E	= potential
EDTA	= Ethylenediamine-tetraacetic acid
E_{pa}	= anodic potential
E_{pc}	= cathodic potential
ET	= electron transfer
EtOAc	= ethyl acetate
FC	= Folin-Ciocalteu assay
FRAP	= ferric reducing ability of plasma
GABA	= Gamma-aminobutyric acid
GAE	= Gallic acid equivalence
GCE	= glassy carbon electrode
H_2O_2	= hydrogen peroxide
HAT	= hydrogen atom transfer
HMWA	= high molecular weight antioxidants
HPLC	= high performance liquid chromatography
HW extract	= hot water extract of *Sutherlandia frutescens*
IP	= ionization potential
I_{pa}	= anodic current
I_{pc}	= cathodic current
$K_2S_2O_8$	= potassium persulfate
LDL	= low density lipoprotein
LMWA	= low molecular weight antioxidant
LP	= lipid peroxidation
MDA	= malondialdehyde
MeOH	= methanol
MeOH extract	= methanolic extract of *Sutherlandia frutescens*
NBD	= nitroblue diformazan
NBT	= nitroblue tetrazolium
NMR	= nuclear magnetic resonance
O	= oxidised species
$O_2^{\cdot-}$	= superoxide anion
$OH^{\cdot}$	= hydroxyl radical
OP	= oxidation potential
ORAC	= oxygen radical absorbance capacity
PUFA	= polyunsaturated fatty acids

QA	= Quinolinic acid
R	= reduced species
ROS	= reactive oxygen species
SWV	= square wave voltammetry
TBA	= Thiobarbituric acid
TCA	= Trichloroacetic acid
TEAC	= TROLOX equivalence antioxidant capacity

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CHAPTER 1: GENERAL INTRODUCTION

The use of antioxidants has become an essential part of everyday life, not only in the medical sector but also in the food, pharmaceutical, cosmetic and chemical industry. Antioxidants are important due to their ability to prevent or reduce the degradation of valuable compounds that are oxidized when exposed to the atmosphere [Halliwell, 1989]. Synthetic antioxidants, such as butylated hydroxytoluene and butylated hydroxytoluene, have been used in commercial products, though recent research has shown that these compounds may cause cancer in animal models. This has resulted in an increased focus on natural antioxidants for new sources of novel antioxidants.

Plants and other photosynthetic organisms are often the focus of the search for new antioxidant compounds as they are constantly exposed to oxygen and sunlight that cause the formation of reactive oxygen species [Hostettman, 1998]. Plants and photosynthesizing organisms have developed effective mechanisms to reduce and prevent damage caused by radical species [Li *et al.*, 2007]. *Sutherlandia frutescens* has a history of use among the Nama and Khoi people of South Africa, for the treatment of various ailments ranging from stomach ailments, internal cancers and diabetes [Tai *et al.*, 2004]. The leaf extracts of *Sutherlandia frutescens* have been shown to possess antioxidant properties but the antioxidant mechanisms have yet to be identified [Fernandes *et al.*, 2004].

Current methods of antioxidant determination involve the generation of stable radical species that are quenched by potential antioxidant compounds. These assays are relatively quick and are based on a colour change that is represented as percentage inhibition of the radical species [Huang *et al.*, 2005]. One of the main disadvantages of these assays is that the radical species used are stable and not as reactive as those encountered in biological systems [Frankel and Meyer, 2000].

Pharmacological methods of determining the effectiveness of antioxidants utilises substrates that would be oxidized by the radical species in biological systems. These assays are considered more relevant than the radical assays but are often time consuming and require numerous reagents [Duthie *et al.*, 1999].

A major criticism of these methods, when determining antioxidant properties of plant samples, is the presence of numerous compounds in these samples that may react with the reagents resulting in an overestimation of antioxidant capacity [Hodges *et al.*, 1999]. These methods may also utilize live animals and this requires ethical approval to ensure the humane treatment of these animals. New methods and technologies are thus required for the rapid screening of antioxidants to provide information, with respect to their effectiveness.

Electrochemical methods have recently been shown to offer such a solution that may be used alone or in conjunction with other traditional methods. Electrochemical methods are based on the application of an anodic current which enables the 'removal' of an electron from an antioxidant compound, thus enabling the determination of the ability of an antioxidant to donate an electron.

The overall aim of this study was to critically examine electrochemistry as an analytical method for the determination of antioxidants. The objectives of the present study were to:

Evaluate the use of electrochemical methods for the determination of total antioxidant capacity of known antioxidants and crude plant samples using *Sutherlandia frutescens* as a case study (Chapter 3)

Utilise conventional biological and non-biological methods to validate the antioxidant properties of *Sutherlandia frutescens* (Chapters 4 and 5)

Apply electrochemical methods to screen batch samples of algal extracts for antioxidant properties (Chapter 6).

Evaluate electrochemical methods to direct the isolation of antioxidant compounds and then elucidate the structure of these compounds with NMR analysis (Chapter 7).

Chapter 2 presents a review of the literature of relevance of this study while chapter 8 provides a general conclusion to this study.

CHAPTER 2: LITERATURE REVIEW

2.1. Electrochemical Sensors

Electro-analytical sensors can be divided into three groups as described by Kissinger and Heinemann (1996):

1. Physical sensors for measuring distance, mass, temperature and pressure.
2. Chemical sensors which measure chemical substances by chemical or physical responses.
3. Biosensors which measure chemical substances by using a biological sensing element.

A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal. The chemical information, mentioned above, may be due to the chemical reaction of the analyte or from a physical property of the system (IUPAC, 1997). The signal observed in chemical sensors is usually electronic which is proportional to the concentration of the specific chemical or group of chemicals [Wang, 1994]. Electrochemical sensors are connected to transducers that allow for the response to be observed. A transducer is a device that converts a change (such as a chemical reaction) into a measurable signal [Wang, 1994].

2.2. Electrochemical transducers

2.2.1. Potentiometric transducers

These determine the potential when the current is set at zero and this potential is proportional to the log of the concentration of the specific analyte.

2.2.2. Voltammetric transducers

These transducers are used for the application of an increasing and/or decreasing potential to initiate the oxidation or reduction of the specific analyte so that a sharp rise/fall in the current is observed. The peak height is directly proportional to the concentration of the electro-active analyte.

2.2.3. Conductometric transducers

This type of transducer detects changes in the composition of the test solution. These transducers initiate a change in the electrical conductivity of the test solution and that change is determined in terms of electrical current.

2.2.4. Field effect transistor-based transducers

For the construction of field effect-based transducers, one of the aforementioned transducers is assembled onto a silicon-chip-based field effect transistor [Settle, 1997].

Electrochemical techniques are advantageous as they:

1. are sensitive to organic and inorganic species,
2. are useable in numerous solvents, electrolytes and temperatures,
3. allow for rapid analysis times,
4. enable the determination of multi-species solutions simultaneously,
5. enable the determination of reaction kinetics and mechanisms of the reaction, and
6. enable the ease of use of different wave-forms [Wang, 1994].

The mode of action of most voltammetric techniques requires the use of an applied potential (E) to an electrode and the measurement of the resultant current. The applied potential affects the concentration of the redox species at the electrode surface and the rate of reaction [Taylor and Schultz, 1996]. The current from this redox reaction is known as the Faradaic current and is related to the flux of material at the electrode-solution interface that is shown by Fick's Law [Taylor and Schultz, 1996].

2.3. Equipment

The basic instruments for electro-analysis are (i) a potentiostat, (ii) an electrochemical cell and (iii) a computer. The potentiostat is responsible for supplying the applied potential and measuring the current. The electrochemical cell consists of the sample in a conducting solution, an electrolyte and two or more electrodes [Taylor and Schultz, 1996]. An orthodox three electrode electrochemical cell (Figure 2.1) consists of a working, reference and a counter electrode.

The reduction or oxidation of a species at the working electrode surface causes new material to be transported to the surface to generate the current [Malhotra and Turner, 2003], and the working electrode enables charge delivery and/or the determination of the resulting current.

Working electrodes vary in shape and composition ranging from mercury (Hg) drop to gold, platinum and glassy carbon [Settle, 1997]. The glassy carbon electrode (GCE) works as a good transducer because of the small background current that is due to the charging current as the potential is scanned. It is also cost-effective and has a wide scanning range [Wang, 1994]. The GCE has a low chemical reactivity and the surface can be renewed by polishing.

Most often the counter electrode is a platinum (Pt) wire but graphite or gold may also be used. The reference electrode is easy to assemble and maintain and provides an electrochemically reversible half-reaction that is constant over time. There are various reference electrodes such as the Ag/AgCl, hydrogen and saturated calomel electrodes.

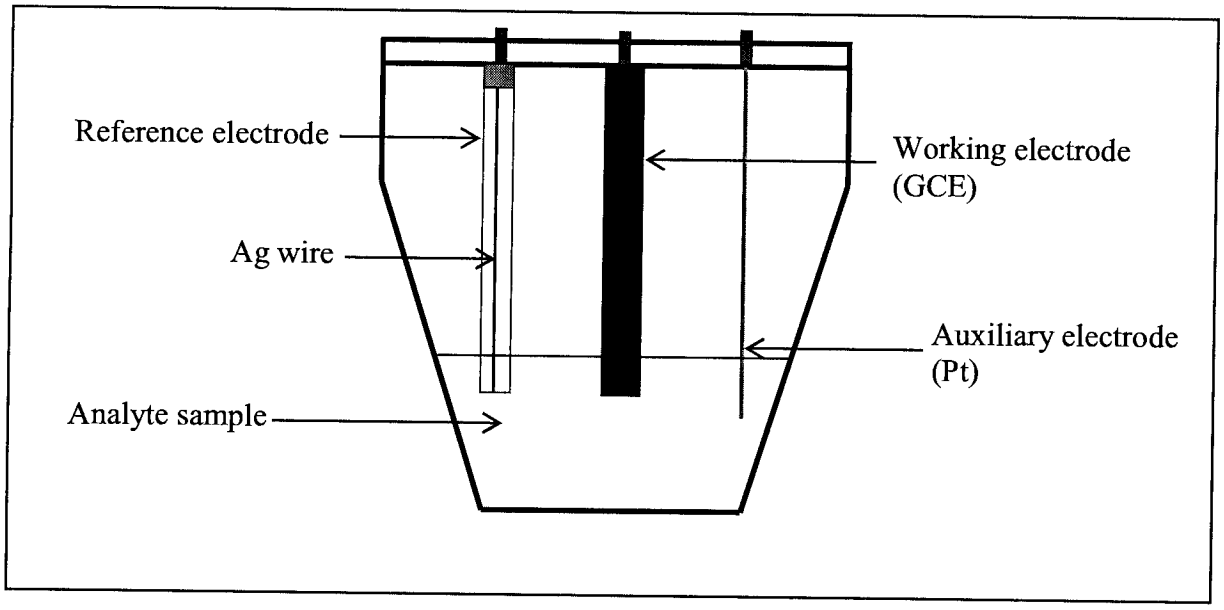

Figure 2.1: **Representation of a typical three electrode electrochemical system consisting of reference, working and auxiliary electrode. [Figure adapted from Malhotra and Turner, 2003].**

2.4. Electrochemical Techniques

2.4.1. Cyclic voltammetry (CV)

This is the most often used electro-analytical technique for obtaining qualitative electrochemical data. CV provides information about the thermodynamics of redox processes, kinetics of heterogeneous electron-transfer reactions, coupled chemical reactions and adsorption processes. CV allows for the location of redox potentials of electro-active species [Kissinger and Heinemann, 1996].

During the potential sweep, the potentiostat measures the current that results from the applied potential. As the applied potential approaches the oxidation potential of the electro-active species, in the forward scan, the current begins to increase until a peak is obtained [Wang, 1994]. For a reversible scan (Figure 2.2), the current is directly proportional to the concentration and the peak separation can be used to determine the number of electrons transferred [Wang, 1994]. A scan in which the potential becomes increasingly positive (forward direction) is called the positive scan during which oxidation occurs. During the negative (or return) scan the potential becomes increasingly negative to cause the reduction of the species (O) at the electrode surface [Kissinger and Heinemann, 1996].

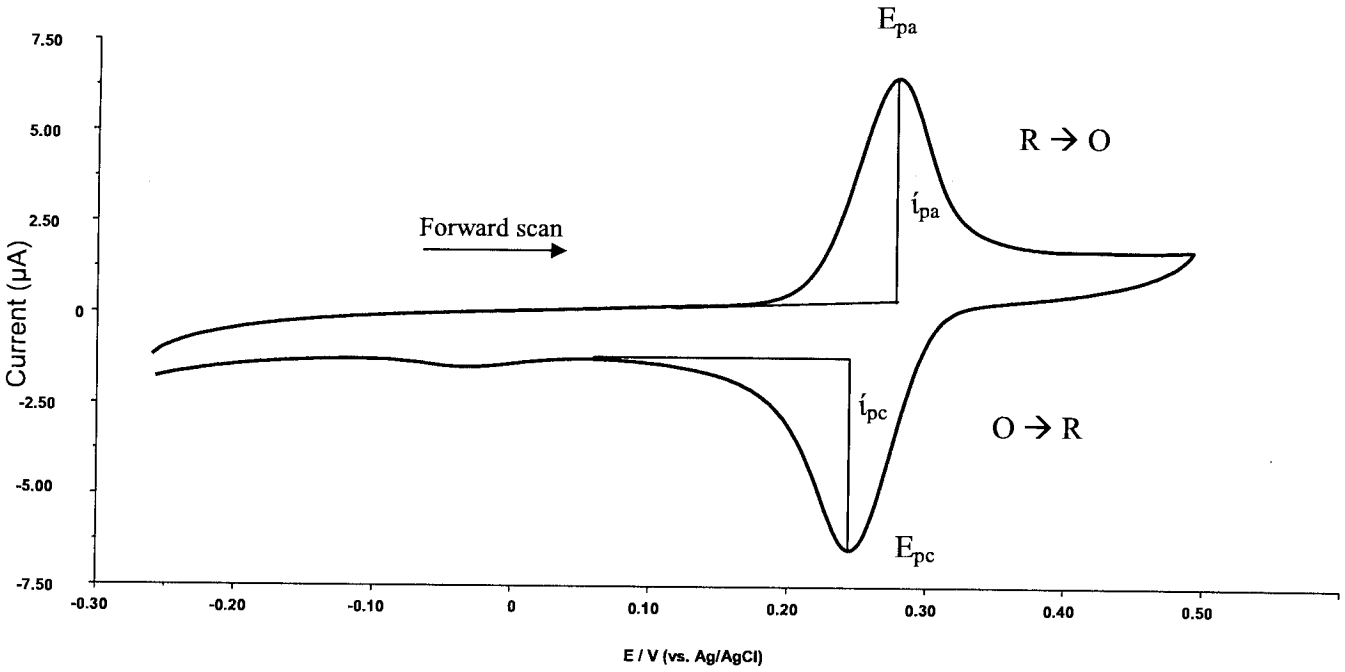

Figure 2.2: Illustration of a reversible cyclic voltammogram.

A reversible electrochemical reaction (Figure 2.2) is represented by:

$$O + ne^- \leftrightarrow R$$

Where: O is the oxidized species, and R is the reduced species.

A redox couple in which both R and O rapidly exchange electrons with the working electrode is called an electrochemically reversible couple [Kissinger and Heinemann, 1996]. The important information obtained from CV includes (i) the peak potentials (Ep_c and Ep_a) and (ii) the peak currents (ip_c and ip_a) of the cathodic and anodic peaks [Settle, 1997]. When O or R is consumed, the drop in concentration causes diffusion from the bulk solution to the electrode surface. Thus, current is the quantitative determination of how fast a species is oxidized or reduced at the electrode surface [Settle, 1997].

The applied potential determines the concentrations of R and O at the electrode and allows it to fit the Nernst equation:

$$E = E^o - {}^{RT}/_{nF} \ln {}^{[O]}/_{[R]}$$

Where: R is the molar gas constant (8.3144 $Jmol^{-1}K^{-1}$), T is the absolute temperature (K), n is the number of electrons transferred, F is Faradays constant (96 485 C) and E^o is the standard reduction potential for the redox couple [Wang, 1994].

2.4.2. Square Wave Voltammetry (SWV)

The excitation signal in SWV (Figure 2.3) consists of a symmetrical square wave pulse of amplitude (E_{sw}), superimposed onto a staircase wave-form of step height (ΔE) where the forward pulse of the square wave pulse coincides with the staircase step [Wang, 1994]. The net current, (i_{net}), is the difference between the forward and reverse current ($i_{for} - i_{rev}$) and is symmetrical around the redox potential. The peak height is directly proportional to the concentration of the electro-active species [Settle, 1997].

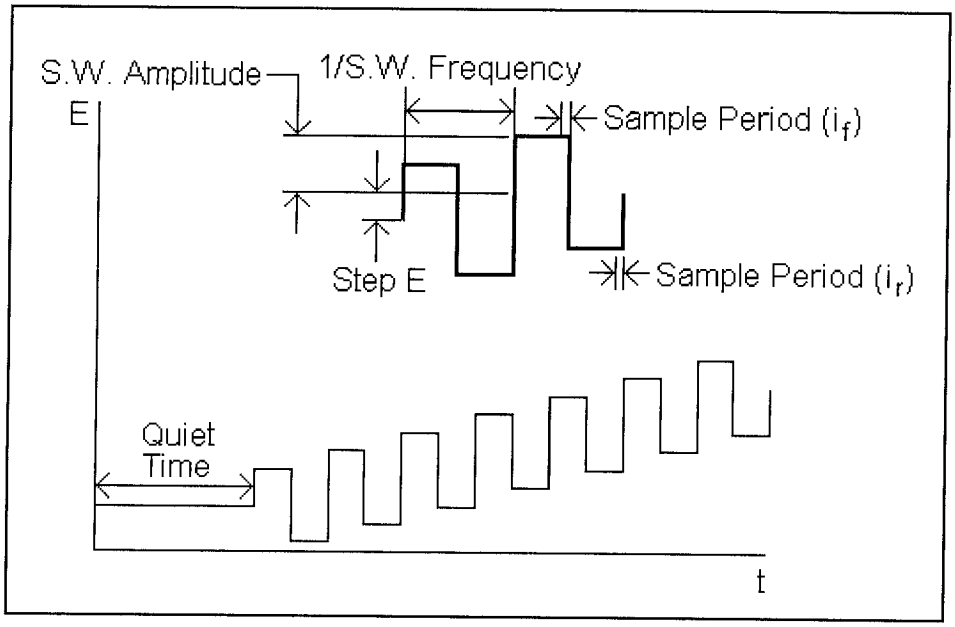

Figure 2.3: **Diagram depicting the mode of action of Square Wave Voltammetry [Internet 1].**

A high sensitivity can be obtained as the net current is larger than both the forward or reverse pulses and this is combined with the discrimination against the charging background current.

The major advantage is the speed of analysis [Wang, 1994] and when coupled with computer control and signal averaging, enables repetitive analysis and an increased signal-to-noise ratio. SWV has been used to determine electrode kinetics with regard to chemical reactions and trace levels of redox compounds [Settle, 1997].

2.4.3. Stripping Voltammetry

Stripping voltammetry is commonly used for the determination of metal ions. These techniques require minimal sample preparation and selectivity is very good. The three main methods of stripping voltammetry are: (i) anodic (ASV), (ii) cathodic (CSV), and (iii) adsorptive stripping voltammetry (AdSV), with all having the same basic two step principle. In the first step the analyte species is concentrated at the working electrode and in the second step, the measurement or stripping of the pre-concentrated analyte from the electrode by applying a potential scan is determined. [Settle, 1997].

2.4.3.1. Anodic Stripping Voltammetry (ASV)

Metal ions are concentrated to the electrode by applying a sufficiently negative potential. These metals are stripped (oxidized) from the electrode by scanning in the positive potential direction [Wang, 1994].

2.4.3.2. Cathodic Stripping Voltammetry (CSV)

An applied positive potential causes the analyte to form a layer on the electrode surface and a potential scan in the negative direction strips (reduces) the layer from the electrode [Settle, 1997].

2.4.3.3. Adsorptive Stripping Voltammetry (AdSV)

The chelation or binding of appropriate ligands to metals allows for the improved detection of the metal in the presence of an appropriate ligand. This technique allows for the formation of metal-ligand complexes which increases the pre-concentration of the metal at the electrode surface and thus increases the detection of the metal (Figure 2.4). AdSV involves the accumulation of the metal-ligand at the surface of the working electrode [Zhang *et al.*, 1991, Wang *et al.*, 1993].

The accumulation occurs in two steps *i.e.* the formation of the complex with a suitable ligand and its adsorption to the surface of the working electrode at an applied potential at a pre-defined time [Wang and Tian, 1992]. The potential is then scanned in the negative direction until the bond between the metal and ligand is broken to produce a current response. However, if the bond between the ligand and the metal is too strong the metal will not be released from the metal-ligand complex resulting in a decreased current response [Wang, 1994]. Thus, a ligand that has the ability to bind strongly to the metal may exhibit one of the qualities of antioxidants which is the ability to chelate transition metals.

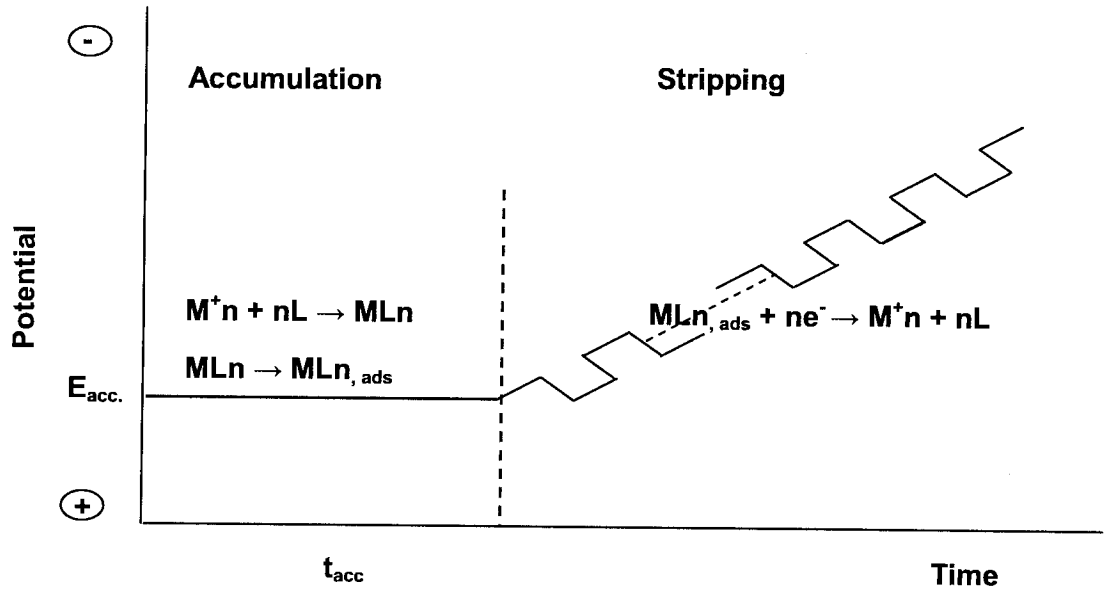

Figure 2.4: A scan of an AdSV of iron (III) [Adapted from Wang, 1994].

2.5. Free radicals

A free radical is defined as any species that can exist independently and contains one or more unpaired electrons. A radical can donate its unpaired electron to another molecule, or it may abstract an electron from another molecule to form a stable electron pair [Halliwell, 1989]. Metabolism produces free radicals that can damage the tissues in the vicinity of production. Thus, a metabolically active cell that is healthy requires an adequate intake of antioxidant nutrients. Free radical damage may increase the risk of infection or physical trauma and external factors such as pollution, drugs, cigarette smoke, illness, stress and strenuous exercise can increase free radical damage [Percival, 1998].

The superoxide anion radical ($O_2^{\cdot-}$) is produced when an electron enters a π^* 2p orbital of oxygen. Superoxide undergoes a dismutation reaction in aqueous solution to form H_2O_2. H_2O_2 is a weak reducing agent that is able to diffuse across biological membranes. The superoxide anion ($O_2^{\cdot-}$) can be formed *in vivo* by the mitochondria when electrons that pass through the respiratory chain 'leak' from electron carriers and pass to oxygen molecules to form the $O_2^{\cdot-}$ anion. H_2O_2 has no unpaired electrons and hence cannot be called a free radical, but it does fall under the reactive oxygen species (ROS) label [Halliwell, 1989].

The hydroxyl radical ($OH^{\cdot}$) is the most reactive radical species known to exist in biological systems. It has an extremely short half-life due to its high reactivity. When the $OH^{\cdot}$ radical is generated in the vicinity of lipid membranes a free radical chain reaction called lipid peroxidation is initiated [Halliwell, 1989]. The $OH^{\cdot}$ radical removes the H^{+} ion from the allylic carbon to form H_2O. This results in a radical being formed within the membrane [Halliwell *et al.*, 1995]. This radical generally combines with oxygen to form a peroxyl radical, which can then attack adjacent polyunsaturated fatty acid (PUFA) to form other radicals. Therefore, one $OH^{\cdot}$ can initiate a chain reaction that may lead to the damage of lipid membranes [Halliwell, 1989].

2.6. Transitional Metals

Transition metals have variable valencies such as iron that can exist as Fe^{2+} or Fe^{3+} ions and the change in valencies involves the acceptance or donation of single electrons. This makes transitional metal ions good catalysts of free radical reactions. Iron and copper can convert $O_2^{\cdot-}$ and H_2O_2 into an $OH^{\cdot}$ radical *via* the Haber Weiss reaction [Halliwell *et al.*, 1995] and H_2O_2 can also form the $OH^{\cdot}$ radical during contact with a transition metal *via* the Fenton reaction.

Metals have been shown to play an important role in neural toxicity as haemo-proteins and ferrous irons have been implicated as the primary catalysts of lipid peroxidation. Ferritin, a soluble iron containing storage protein found in high concentrations in the liver, spleen and skeletal muscles, releases iron in the presence of reducing agents, such as $O_2^{\cdot-}$, ascorbate and thiols that has the potential to initiate lipid peroxidation [Decker and Welsh, 1990].

2.7. Antioxidants

There has been growing interest in bioactive compounds such as antioxidants. These compounds play numerous roles in maintaining the normal functioning of biological systems and in the case of antioxidants, they are essential for the limitation of damage due to oxidative stress [Ziyatdinova *et al.*, 2005].

An antioxidant can be defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" [Halliwell *et al.*, 1995]. The 'oxidizable substrate' may include proteins, lipids, carbohydrates and DNA.

Oxidative stress has been shown to be involved in the etiology of cancer, arteriosclerosis, malaria, rheumatoid arthritis and numerous other ailments including neurodegenerative diseases. In living systems antioxidant and endogenous antioxidant enzymes protect against oxidative stress [Moure *et al.*, 2001]. This has resulted in an increasing interest in the mode of action and identification of antioxidants in biomedical research. ROS are constantly produced during normal aerobic metabolism and are removed by antioxidants occurring in the system; however this process is never 100% efficient, therefore repair systems are essential [Gutteridge, 1995].

When characterizing an antioxidant, the target oxidizable substrate and the source of oxidative damage are important as antioxidants that may protect one system may not protect others. Some antioxidants may raise the concentration of endogenous antioxidants [Halliwell *et al.*, 1995]. Antioxidants function by reducing the oxygen concentration; scavenging free radicals to prevent hydrogen abstraction from molecules; binding to transition metals that are able to produce free radicals; preventing the conversion of peroxides to more reactive species; and reacting with chain propagating radical to reduce continued H^+ extraction from fatty acid side chains [Duthie, 1999]. Antioxidants that scavenge ROS act as reducing agents by donating electrons to the ROS and oxidizing the ROS. The antioxidants themselves become radical species but are more stable [Kohen *et al.*, 2000].

In the chemical industry, antioxidants are referred to as compounds that retard auto-oxidation of chemical products. The auto-oxidation is usually caused by radical chain reactions due to atmospheric oxygen. In these cases, effective antioxidants are radical scavengers that break down the radical chain reactions. In the food industry, antioxidants are substances in the food that decrease the adverse effects of ROS on the quality of the food [Huang *et al.*, 2005]. Thus, the protection provided by antioxidants is not only important to biological systems but also in the food, chemical and pharmaceutical industries [Geletii *et al.*, 2002].

2.7.1. Antioxidant defence systems

The human body has evolved a complicated antioxidant protection system that involves numerous components that may be exogenous or endogenous and function in an interactive and synergistic manner to quench free radicals [Percival, 1998].

These components include [Duthie, 1999]:

(a) nutrient-derived antioxidants such as ascorbic acid (Vitamin C), tocopherols and tocotriends (Vitamin E), carotenoids and other Low Molecular Weight Antioxidants (LMWA) such as glutathione and lipoic acid;

(b) antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase/reductase which catalyze free radical quenching reactions;

(c) metal-chelating proteins which bind to free iron and copper ions that are capable of catalyzing oxidative reactions, and include ferritin, lactoferritin, albumen and ceruloplasmin and

(d) a variety of other antioxidant phytonutrients found in numerous plants [Duthie, 1999].

2.7.1.1. Antioxidant enzymes

a) *Superoxide dismutase (SOD)* causes the dismutation of superoxide to hydrogen peroxide (H_2O_2) which can then be removed by catalase or glutathione peroxidise [Fang *et al*, 2002].

b) *Catalase* is capable of generating water from hydrogen peroxide. The enzyme contains iron (III) which facilitates the conversion of H_2O_2 to water [Young and Woodside, 2001].

c) *Glutathione peroxidase and reductase* catalyses the oxidation of glutathione by degrading a hydroperoxide species and glutathione peroxidase is the main scavenger of H_2O_2 in sub-cellular components [Kohen and Nyska, 2002].

2.7.1.2. Chain breaking antioxidants

Free radicals may react with certain molecules that then become secondary radicals and react with other target molecules. Chain breaking antioxidants are usually small molecules that have the ability to either receive or donate an electron(s) from a free radical species to form more stable by-products [Vaya and Aviram, 2001].

These antioxidants function by delocalizing the electron charge over the antioxidant from a stable structure that does not accept or donate electrons [Young and Woodside, 2001].

Chain breaking antioxidants can be divided into lipid-phase and aqueous-phase antioxidants. Lipid-phase chain breaking antioxidants scavenge radicals in membranes and lipoproteins and are essential for the prevention of lipid peroxidation. The most important of this class of antioxidants is vitamin E. These antioxidants react faster with free radicals when compared to poly-unsaturated fatty acids (PUFA) and thus prevent the oxidation of PUFA. Carotenoids are also lipid soluble antioxidants that can effectively scavenge singlet oxygen [Duthie, 1999].

Aqueous-phase chain breaking antioxidants scavenge free radicals in the aqueous component, the most important being ascorbic acid. L-ascorbic acid (Vitamin C) is obtained by humans from their dietary sources. Ascorbic acid (vitamin C) can be synthesized by some plants and animals and is an effective radical scavenger that is capable of donating electrons to ROS to neutralize them [Kohen and Nyska, 2002]. Vitamin C can scavenge the superoxide anions, (H_2O_2, $OH^{\cdot}$), hypochlorous acid and singlet oxygen, losing two electrons during this process. Vitamin C also has the ability to chelate transitional metals and prevent the generation of free radicals [Percival, 1998]. Other antioxidants in this group include uric acid and glutathione [Young and Woodside, 2001].

2.7.1.3. Transitional metal-chelating proteins

Transitional metal-chelating proteins include ferritin, transferrin, lactoferrin, metallothionein and ceruloplasmin. They act by binding iron and copper and thus prevent these ions from forming the hydroxyl radical [Percival, 1998]. Iron (II) catalyses the Fenton reaction which produces the highly reactive hydroxyl free radical [Young and Woodside, 2001]. The indirect method of preventing oxidation involves chelating of transitional metals and preventing the metal mediated Haber-Weiss reaction.

2.7.1.4. Exogenous antioxidants

In some instances, the endogenous antioxidant system may not be efficient due to the effect of external influences. Numerous reports have linked plant polyphenols to a major role in the antioxidant defence system [Pietta, 2000].

i) Plant polyphenols

Plant polyphenols include ellagic acids, chalcones and flavonoids. They have the chemical ability to react with free radicals as they readily donate electrons or hydrogen ions from their hydroxyl substituents. Flavonoids are antioxidant compounds found in foodstuffs originating from plant sources. These compounds are polyphenolic in nature and have a basic chemical structure of $C_6C_3C_6$ [Duthie, 1999].

ii) Prominent exogenous antioxidants

D-α-Tocopherol (Vitamin E) is a phenolic compound synthesized by plants, which readily donates the hydrogen ion from the OH group on the ring structure to the free radicals. After donating the hydrogen ion, the phenolic group becomes an unreactive free radical because the unpaired electron can be delocalized over the aromatic ring, increasing stability. It mainly protects PUFA and other components of cell membranes from ROS damage [Percival, 1998] and thus is the most important fat soluble antioxidant that functions as a chain-breaking antioxidant and protects lipid membranes from lipid peroxidation. Beta-carotene and other carotenoids also provide protection to lipid membranes [Duthie, 1999].

Carotenoids can only be synthesized by photosynthetic microbes and plants. Due to their structure, they interact strongly with ROS and have been shown to exert their antioxidant effect over a range of oxygen related stresses [Duthie, 1999].

Naturally occurring antioxidants of high and low molecular weight, can differ in composition. They may be grouped into enzymes, high molecular weight antioxidants (HMWA) and LMWA.

iii) High molecular weight antioxidants (HMWA)

HMWA are able to bind to transitional metals and reduce the production of free radicals [Vaya and Aviram, 2001].

iv) Low molecular weight antioxidants (LMWA)

LMWA are a group of antioxidants that can directly or indirectly scavenge free radicals. The cell is capable of producing a limited amount of LMWA that are often small molecules able to enter cells where they effect their function in areas of highest risk of oxidative damage.

Diet plays a major role when it comes to the levels of LMWA [Chevion *et al.*, 2000]. LMWA is a larger group when compared to other antioxidant molecules and possess qualities that allow them to perform unique antioxidant functions. They are able to accumulate at specific locations of high ROS activity in cells and tissue [Duthie, 1999]. This enables direct protection against free radicals as they are generated and also allows the antioxidant effect to be exerted at areas that antioxidant enzymes and HMWA cannot access. The LMWA are able to act on ROS in various ways due to their structure [Vaya and Aviram, 2001].

v) Phytonutrients

A variety of plant compounds known as 'phytonutrients' have been cited for their antioxidant capability. These compounds act to protect the plant against stress, and in humans they aid in the biological response. Foods and beverages from plants are chemically complex and their effect may be due to many compounds that are present. Plants possess effective ROS scavenging systems that provide protection from oxidative reactions. Polyphenols possess ideal chemical structures for free-radical scavenging activities and metal chelation. Because of their oxidation, low density lipoproteins are important with regard to atherosclerosis. Hence, dietary antioxidants that are active in lipophilic mediums are of importance [Rice-Evens *et al.*, 1997].

Phenolic type compounds include numerous phytochemicals that possess antioxidant activity. Quinones are aromatic rings with two ketone substituents. The number of OH groups also determines the scavenging ability, *i.e.* more OH groups impart greater ability to scavenge [Heim *et al.*, 2002]. They have a high reactivity and can be oxidized and reduced very easily between hydroquinone and quinone. Quinones are a source of stable free radicals and complex irreversibly with nucleophilic amino acids in proteins [Cowan, 1999]. Phenolic compounds include substrates that possess an aromatic ring that contains one or more hydroxyl substituents.

The arrangement of the functional moieties on the aromatic ring is a major determining factor in antioxidant function. The ability to quench free radicals can be associated with the reactive OH group on the aromatic ring. The location of the OH is also very important regarding the rate of the reaction. OH groups on the β-ring of polyphenols donate an H^+ and an electron to a free radical and become a stable radical [Vaya and Aviram, 2001].

Amino acids and peptides have been shown to possess the ability to reduce lipid peroxidation. However, water is vital for the antioxidative activity of amino acids as it may allow for greater mobility of the amino acids to the radicals. The antioxidant mechanism for arginine is not established, but the guanizyl group may possess anti-radical activity [Park *et al.*, 2005].

Plant extracts generally contain antioxidant mixtures which allows for a synergistic mode of action [Becker *et al.*, 2004]. An antioxidant that possesses antioxidant properties *in vitro* may have additional properties in a complex system due to interactions with other antioxidants [Becker *et al.*, 2004]. There have been numerous reports of the uses of *Sutherlandia frutescens* for medicinal purposes. The Nama and Khoi people of South Africa have used the extracts of the plant to treat various ailments ranging from cancers, inflammation and viral diseases [Tai *et al.*, 2004].

Limited information is available in literature regarding the mode of action and pharmacological data of the extracts of *Sutherlandia frutescens* [Mills *et al.*, 2005]. The HW extracts of the leaves of *Sutherlandia frutescens* have been shown to possess antioxidant properties [Fernandes *et al.*, 2004]. Further evaluation of the antioxidant properties of *Sutherlandia frutescens* is provided in Chapter 3-5.

Seaweeds are rich in pigments (fucoxanthin), enzymes (catalase) and various functional polysaccharides that may impart antioxidant properties. Polyphenols are effective at protecting PUFA as they donate a H^+ ion to lipid peroxyl radicals and form the aryloxyl, which cannot act as a chain carrier, or couples with another radical and quenches the radical process [Karawita *et al.*, 2005]. Further evaluation of the antioxidant properties of marine algae is provided in Chapter 6-7.

2.8. Antioxidant Evaluation

Antioxidant capacity is the commonly used term for the characterization of crude extracts of plant materials, as they may contain numerous compounds that possess antioxidant properties [Arnao, 2000]. There are numerous assays available for the determination of antioxidant properties of complex mixtures. These assays may utilise biological or non-biological mechanisms to determine antioxidant capacity. The Folin-Ciocalteu (FC) assay is usually used to determine antioxidants in plants because most plant-derived antioxidants are polyphenols. The α,α-diphenylpicrylhydrazyl (DPPH) assay is commonly used due to its stability, simplicity and reproducibility. It has been shown that a combination of lipid peroxidation (LP) and DPPH assay is efficient for assessing the antioxidant potential of edible plants [Katsube *et al.*, 2004]. A standard three step procedure has been proposed for the evaluation of antioxidative effects [Becker *et al.*, 2004].

Step 1 involves the quantification and identification of total phenolic compounds in the crude extract. This can be determined by the FC assay which is based on the number of phenolic groups or other oxidizable groups present. The FC reagent is however, non-specific and can be reduced by non-phenolic compounds.

Step 2 involves the radical scavenging ability of the antioxidant. The TROLOX Equivalent Antioxidant Capacity (TEAC), α,α-diphenylpicrylhydrazyl (DPPH), ferric reducing ability of plasma (FRAP) and oxygen radical absorbance capacity (ORAC) assays are the most commonly used. Methods based on the use of stable radicals are often criticized as there are no stable radicals in biological systems.

Step 3 involves the ability of an antioxidant to inhibit or halt lipid peroxidation. The evaluation of antioxidants in these systems is based on the depletion of oxygen, or the change in the concentration of compounds being oxidized or the formation of oxidation products.

However, during the lipid peroxidation of crude plant samples, a variety of plant species contain compounds that may interfere with the absorbance determination of the MDA-TBA complex at 532 nm as they may bind to TBA and result in increased absorbance values.

2.8.1. Non-Biological Radical Assays

There are two main types of generated radical assays that are used to determine antioxidant activity. The first involves generating the radical species and observing the inhibition of that radical by either a hydrogen or electron-donating effect of the antioxidant. The second involves the antioxidant already being present during the generation of the radical.

Antioxidant assays are generally based on two aspects which define the reaction that occurs. They can either be a hydrogen ion transfer (HAT) reaction or an electron transfer (ET) reaction. HAT assays are based on a competitive mechanism where the antioxidant and substrate compete for a thermally generated peroxyl radical through the decomposition of azo compounds [Huang *et al.*, 2005]. ET assays are based on the transfer of electrons to the radical chromophore from the antioxidant compound(s) [Huang *et al.*, 2005]. Bond dissociation energy (BDE) and ionization potential (IP) are the two major factors that determine the mechanism and efficiency of antioxidants. HAT-based assays determine the ability of an antioxidant to donate hydrogen ion(s) to a radical species to quench it. The reactivity of the HAT assays is based on the BDE of the H-donating substituent(s). HAT assays are largely reliant on the solvent and pH [Tanko, 2003]. Electron-withdrawing groups such as CN, NO_2 and C=O have been shown to increase the OH bond dissociation energy, while Cl, Ph and CH=CHPh have the opposite effect. Electron donor groups (OR, alkyl) decrease BDE and donor substituents stabilise the resulting phenoxyl radical by resonance and destabilize the phenol [Tanko, 2003].

Electron acceptor groups stabilize the phenol *via* resonance and depending on their structure, they may stabilise (phenol and stryl) or destabilise (CN, NO_2 and C=O) the electron deficient phenoxyl radical [Tanko, 2003]. ET assays are based on the ability of an antioxidant to donate an electron to reduce a ROS. ET reactions rely on deprotonation and IP of the reactive substituents. ET reactions are pH dependant and IP values decrease with increasing pH which indicates an increased electron-donating ability with deprotonation [Prior *et al.*, 2005]. ET reactions are generally slow, which results in the antioxidant capacity being determined by percentage decrease rather than kinetics [Prior *et al.*, 2005].

The compound that acts as the free radical, accepts an electron from the antioxidant in ET assays, which causes a colour change, the degree of which is proportional to the concentration of the antioxidant [Huang *et al.*, 2005]. For ET assays, it is assumed that antioxidant capacity is equal to the reducing capacity of the antioxidant. Many compounds are lipophilic; therefore the antioxidant capacity of a compound depends largely on the reaction media [Huang *et al.*, 2005]. The ability of antioxidants to scavenge radicals depends on their concentration and rate of reaction, which in turn depends on their structure [Geletii *et al.*, 2002].

2.8.1.1. Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt (ABTS) radical assay

$ABTS^{\cdot+}$ is a one-electron oxidant which allows it to act with potent antioxidants. The mode of action usually involves the removal of an electron from an antioxidant. $ABTS^{\cdot+}$ is a cation (Figure 2.5) and is neutralized by accepting an electron. Phenolic antioxidants have been shown to scavenge the ABTS cation by H^+ donation and electron transfer or even a combination of both [Nenadis *et al.*, 2004]. The $ABTS^{\cdot+}$ radical reacts with antioxidants and can therefore be used over a range of pHs to determine the effect of pH on the antioxidant. It is soluble in aqueous and organic solvents so it can be used to assay lipophilic and hydrophilic antioxidants [Prior *et al.*, 2005]. This has resulted in a growing interest in natural phyto-chemicals which have the potential to act as antioxidants.

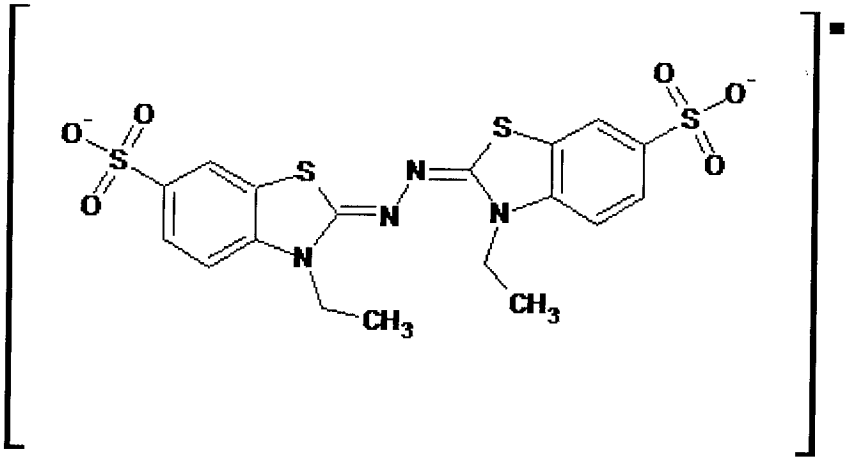

Figure 2.5: Structure of the ABTS free cation [Apak *et al.*, 2007].

The addition of antioxidants to the pre-formed $ABTS^{\cdot+}$ cation reduces it depending on how potent the antioxidant is, the concentration of the antioxidant and how long the reaction is allowed to occur [Miller and Rice-Evens, 1997]. The subsequent colour change is due to the inhibition of the ABTS radical, and is calculated in relation to a TROLOX standard under the same conditions [Re *et al.*, 1999].

The ABTS radical cation is inhibited by an antioxidant compound in a time dependant manner [Pellegrini *et al.*, 2003]. The TEAC is the equivalence between the antioxidant solution and molar (milli or micro-molar) concentrations of TROLOX. The TEAC assay indicates the ability of antioxidants to scavenge the ABTS radical in comparison to TROLOX. TEAC values for pure antioxidant compounds do not show a correlation between the TEAC values and the number of electrons a compound can theoretically donate. This may be due to the reaction rates between certain antioxidant and oxidants being different and this may not be observed due to the TEAC assay being an endpoint assay [Huang *et al.*, 2005]. The ABTS assay has been shown to take a long time to reach endpoint [Prior *et al.*, 2005] and the other disadvantage is that the $ABTS^{\cdot+}$ is not found in biological systems and is thus an unsuitable radical source.

2.8.1.2. α,α-diphenylpicrylhydrazyl (DPPH) radical assay

DPPH is a stable free radical that has a single electron delocalized over the entire molecule (Figure 2.6). When a solution of DPPH is combined with a compound(s) that can donate H^+ ions, the DPPH radical is reduced which results in a colour change [Molyneux, 2004]. The DPPH assay is based on the reducing ability of antioxidants to the DPPH, which is mainly an electron transfer reaction.

The DPPH radical can accept an electron or hydrogen ion to become a stable, diamagnetic molecule. Due to the unpaired electron, the DPPH radical has a maximum absorbance at 517 nm but once this electron is paired, the absorption disappears. The percentage DPPH remaining is proportional to the concentration of antioxidant compounds present [Blois, 1958].

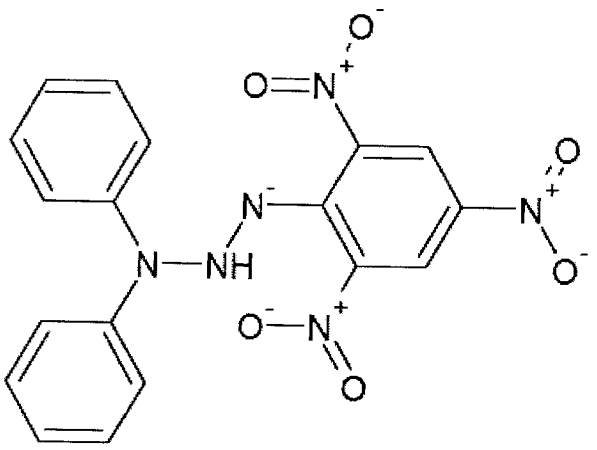

Figure 2.6: Structure of the DPPH free radical [Molyneux, 2004].

A major disadvantage is that the DPPH radical is not similar to the ROS found in biological systems. Antioxidants that react rapidly with ROS in biological systems may react slowly or not at all with the DPPH radical [Huang *et al.*, 2005] and steric interference may reduce or inhibit the interaction of antioxidants with the DPPH radical [Prior *et al.*, 2005].

2.8.1.3. Ferrozine assay

Organic molecules that possess N=C-C=N structure, have been shown to act as bidentate ligands that chelate with certain metal ions such as ferrous, cuprous and cobalt, to give a coloured species. Ferrozine (Figure 2.7) complexes with the Fe^{2+} ions and thus enables their quantification [Que *et al.*, 2006]. The major advantages of ferrozine are its water solubility and stability over the pH range of 4–9. The ferrous complex reacting with ferrozine has a maximum absorbance at 562 nm [Stookey, 1970].

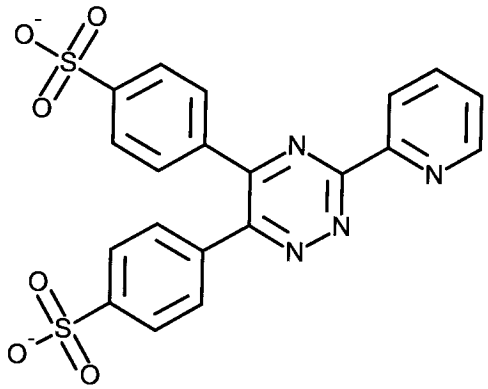

Figure 2.7: Structure of the ferrozine molecule [Adapted from Stookey, 1970].

2.8.1.4. Folin-Ciocalteu (FC) assay

The FC assay determines the reducing capacity of a sample. The FC reagent is non-specific to phenolic compounds and numerous non-phenolic compounds have been shown to reduce it (e.g. vitamin C). The pH plays a vital role in the reducing capacity of antioxidants.

In acidic conditions, the reducing capacity may be suppressed due to the protonation of the antioxidant [Huang *et al.*, 2005]. The antioxidant activity of phenolic compounds is determined by their structure and by the position and degree of hydroxylation of the ring structure. This allows delocalized unpaired electrons to stabilize the formed phenoxyl radical after the reaction with the radical species [Prior *et al.*, 2005]. Basic conditions cause the dissociation of the phenolic proton which results in a phenolate anion, which then reduces the FC reagent [Huang *et al.*, 2005]. The FC reagent has a greater molybdate portion which allows it to be more sensitive to reduction. The lithium sulphate present in the FC reagent produces a blue pigment when a reaction with phenol structures occurs [Singleton and Rossi, 1965].

2.8.2. Biological Antioxidant Assays

2.8.2.1. Lipid Peroxidation (LP)

LP is an established mechanism of cellular damage and has been extensively used as an indicator of oxidative stress. Lipid peroxides are unstable and degrade to form a complex series of compounds. PUFA peroxides produce malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) when they degrade [Oxford Biomedical Research, 2003]. LP is initiated when ROS removes a hydrogen from a CH_2 group to produce a ·CH radical. The double bond in fatty acids weakens the C-H bond on the adjacent carbon to the double bond. Thus, the PUFA side chains of membrane lipids are vulnerable to oxidation [Halliwell, 1978]. The C-radical undergoes re-arrangement to form a conjugated diene which reacts with oxygen to form a peroxyl radical which can then remove a hydrogen ion from another fatty acid and thus initiate a chain reaction. LP continues until the substrate is used or a chain-breaking antioxidant intervenes [Gutteridge and Halliwell, 1990].

$O_2^{\cdot-}$ reacts with transitional metals producing $OH^{\cdot}$ that can initiate LP by hydrogen abstraction [Gutteridge, 1995]. LP in biological membranes may result in loss of fluidity, reduce membrane potential, increased permeability to hydrogen and other ions and in serious cases cell rupture [Halliwell, 1978].

Oxidation of lipids can be detected by: i) loss of unsaturated fatty acids; ii) the primary peroxidation products and iii) the secondary carbonyls and hydrocarbon gases. The Thiobarbituric acid (TBA) assay (section 5.1.1) is probably the most commonly used assay to determine LP [Oxford Biomedical Research, 2003].

Iron is the most commonly occurring transitional metal in the human body. In normal cells iron ions are chelated by biological molecules [McCord, 2004]; however ferrous iron (Fe^{2+}) is also able to initiate LP if there is an oxidant present to oxidize Fe^{2+} to Fe^{3+}. In these cases O_2 or H_2O_2 generally acts as the oxidant [Braughler *et al.*, 1986]. H_2O_2 is able to form highly reactive free radicals in the presence of transitional metal ions. The most well-known free radical formed by H_2O_2 with a transitional metal is the hydroxyl radical ($OH^{\cdot}$). The $OH^{\cdot}$ radical is a strong oxidant that can react with most biological molecules. The $OH^{\cdot}$ radical is the most potent biological radical species that readily attacks PUFA to initiate LP [Callaway *et al.*, 1998].

Quinolinate (QUIN) is an endogenous glutamate agonist that can cause neuronal loss in vulnerable areas of the brain. QUIN can chelate ferrous ions to form $(QUIN)_2Fe^{2+}(H_2O)_3$ complex and thus is able to stimulate LP in the presence of iron ions only [Stipek *et al.*, 1997].

2.8.2.2. Superoxide anion determination (NBT assay)

All aerobes experience some sort of damage when exposed to elevated levels of oxygen. Toxicity is brought about due to the formation of the superoxide, $O_2^{\cdot-}$, which is just an oxygen molecule at a higher energy level [Halliwell, 2006[b]]. Energy is produced in the mitochondria by the electron transport chain in a process that converts oxygen to water. The reduction of O_2 to H_2O occurs due to a four electron transfer in a process that results in the formation of a number of active oxygen species [Kohen and Nyska, 2002].

The blocking of the electron transport chain, which can be achieved using CN^-, results in electrons 'leaking' from the mitochondria into the extracellular fluid of the cell. Due to the high concentration of oxygen present at the mitochondria, the 'leaked' electrons are taken up by oxygen molecules resulting in the formation of the superoxide free radical [Dawson and Dawson, 1996]. The Nitroblue tetrazolium (NBT) assay (section 5.1.2), is a commonly used superoxide anion assay to determine the ability of antioxidants to scavenge or reduce the formation of the superoxide anion [Ottino and Duncan, 1997; Halliwell, 2006[a]].

2.9. Potential Applications of Antioxidant determination tools

The use of natural products in drug design represents the progression of traditional medicine to modern healthcare. Numerous compounds with biological activity have been isolated from marine sources but few have reached clinical evaluation [Prior *et al.*, 2005]. Antioxidants are often used with drug formulations to prevent or reduce the oxidation of the active components.

Numerous drugs are manufactured in the reduced form which allows for their oxidation by atmospheric oxygen and decreases their stability. An approach based on the oxidation of the antioxidants in drug formulations depends on the antioxidant having a lower oxidation potential than the drug [Huang *et al.*, 2004]. Antioxidants that are included in drug formulations should possess a lower oxidation potential than the drug, the ability to scavenge ROS, and the ability to bind transitional metals and reduce ROS formation [Huang *et al.*, 2004].

Multi-functional antioxidants have the advantage over a combination of antioxidants as there is a reduced risk in drug-drug interactions and the mode of action is more predictable [Zhang *et al.*, 2006]. Synthetic molecules involve linking an antioxidant molecule to one or more enzyme-inhibitor pharmacophore. Numerous natural products have been shown to be multi-functional antioxidants such as curcumin [Zhang *et al.*, 2006]. The evolution of bioactive defence compounds in plants has produced compounds that may have numerous beneficial effects in biotechnology, pharmacy and medicine [de Abreu *et al.*, 2002].

2.10. Problems associated with antioxidant assays

The complexity of foods is a major factor in determining the antioxidant capacity as this would entail separating each antioxidant and studying them individually. This would also negate possible synergistic relations between antioxidants in a complex mixture. As there is a lack of an effective standard assay, results from different research groups have been difficult to compare which makes quality control of food products that contain antioxidants problematic [Frankel and Meyer, 2000]. The development of a standardised method for antioxidant analysis should guide the use of the appropriate assay; enable valid comparison of results and allow for the regulation of quality standards [Prior *et al.*, 2005].

A standardised method for antioxidant evaluation should measure the chemical reaction occurring; utilize the relevant biological species; be simple; use a pre-defined chemical mechanism and endpoint; be reproducible; be adaptable for hydrophilic and lipophilic antioxidants; and be able to be used for 'high throughput' analysis for quality control analysis [Prior *et al.*, 2005].

2.11. Electrochemical analyses of antioxidants

The use of electrochemistry to elucidate biomedical chemistry with regard to aspects of electron transfer can be useful, as there are similarities between electrochemical and biological reactions in terms of electron transfer pathways.

Electrochemically active antioxidants yield anodic peaks which can be used for identification of antioxidants [Ceballos, 2006]. Cyclic voltammetry (CV) has been used to determine the ability of selected compounds to donate an electron around the oxidation potential of the anodic peak [Huang *et al.*, 2004]. This can be correlated to the levels of Low Molecular Weight Antioxidants (LMWA). Most LMWA are reducing agents and are able to donate an electron(s) to a ROS to neutralize it. The oxidation potential (OP) indicates the reducing power of these compound(s).

The closer the oxidation potential is to zero, the greater the ability of the compound to donate an electron. The OP is specific for each individual component and not dependant on concentration.

As the oxidation scan progresses, the electrode working surface becomes sufficiently positively charged to remove an electron from the antioxidant compound and give rise to the ip_a (anodic current). The ip_a increases until the compound (s) is consumed at the electrode surface and then decreases resulting in a peak [Chevion *et al.*, 2000].

The ip_a can be used as an indication of the concentration of the compound(s) in the mixture [Chevion *et al.*, 1997]. However, if there is more than one reducing agent in the mixture, then the area under the curve (AUC) better reflects the total antioxidant capacity [Chevion *et al.*, 1997].

The use of electrochemical methods for the determination of antioxidant capacity may also entail the use and determination of the superoxide anion. The generation of the superoxide anion occurs in organic solvents as this reduces the decomposition rate of the anion. The addition of the antioxidant reduces the current response produced by the superoxide anion, which allows for the determination of the antioxidant capacity of the compound [Korotkova *et al.*, 2002]. The use of the superoxide anion in solution increases the preparation involved as careful considerations have to be taken to ensure the electrochemical cell is degassed and remains under anoxic conditions. Therefore, this method of antioxidant determination presents more disadvantages to the more commonly used electrochemical methods.

Electro-analytical methods that determine the electron-donating ability of antioxidants require little sample pre-treatment, providing information concerning mechanisms and electron transfer in biological processes [de Abreu *et al.*, 2002]. These electrochemical methods rely on the chemi-physical properties of the compounds present and not on the quenching abilities in relation to a radical species [Cosio *et al.*, 2006] as utilised in radical based antioxidant assays. These electrochemical techniques have thus been proposed to provide a means of screening of antioxidants [Diaz *et al.*, 2004].

Antioxidants present in foods, namely fruits and vegetables, are generally phenolic in structure [Apak *et al.*, 2007]. Their antioxidant properties are based on the structure of biological molecules, most importantly, the electron delocalization around the aromatic structure, nature and abundance and location of functional groups such as OH- or CH_3- groups [Huang *et al.*, 2005].

This allows for electrochemical methods to be used for both the determination and identification of antioxidant compounds [Ceballos, 2006].

However, given this claim, few studies have conducted a detailed examination or comparative critique of the use of electrochemical methods for the determination and rapid screening of antioxidants [Atayman and Osman, 1997; Chevion *et al.*, 1997; Chevion *et al.*, 2000; Kohen *et al.*, 2000; Liu *et al.*, 2000; Buratti *et al.*, 2001; Kilmartin, 2001[a]; Kilmartin *et al.* 2001[b]; Psotová *et al.*, 2001;de Abreu *et al.*, 2002; Antolovich *et al.*, 2002; Becker *et al.*, 2004; Blasco *et al.*, 2004; Campanella *et al.*, 2004; Diaz *et al.*, 2004; Ceballos *et al.*, 2006; Cosio *et al.*, 2006; Erdurak-Kiliç *et al.*, 2006; Martinez *et al.*, 2006; Raymundo *et al.*, 2007]. There also remains scope for the utilisation of electrochemical methods to provide information on the mode of action of antioxidant compounds as well as in directing the isolation of compounds with possible antioxidant properties.

Objectives

1. Evaluate the use of electrochemical methods for the determination of total antioxidant capacity of known antioxidants and crude plant samples using *Sutherlandia frutescens* as a case study.
2. Apply conventional biological and non-biological methods to validate the antioxidant properties of *Sutherlandia frutescens*.
3. Utilize electrochemical methods screen batch samples of algal extracts for antioxidant properties.
4. Apply electrochemical methods to guide the isolation of antioxidant compounds and then elucidate the structure of these compounds with NMR analysis.

CHAPTER 3: ELECTROANALYSIS OF *SUTHERLANDIA FRUTESCENS* EXTRACTS

3.1. Introduction

The plant *Sutherlandia frutescens* is a member of the pea family and has been used as a traditional medicine in Southern Africa to treat a number of diseases such as stomach cancer, tuberculosis, diabetes, influenza, anxiety, clinical depression and HIV infection [Mills *et al.*, 2005]. The leaves are the most used parts of the plant, often prepared as a tea for medicinal use; however, all the sub-terrain parts have been used. The leaves have been shown to possess a number of compounds that may possess medicinal properties such as pinitol, GABA and canavanine [Tai *et al.*, 2004]. D-pinitol is a sugar commonly found in leguminous plants [Sia, 2004] and GABA is an amino acid that acts as an inhibitory neurotransmitter [Mills *et al.*, 2005]. Canavanine is a non-protein-α amino acid with anti-tumour properties [Fernandes *et al.*, 2004]. The compounds present may act synergistically to exert the medicinal properties claimed to be derived from extracts of *Sutherlandia frutescens*.

The HW extracts of the leaves of *Sutherlandia frutescens* were shown to possess antioxidant properties using the DPPH assay [Katerere, and Eloff, 2005] and cell culture assays [Fernandes *et al.*, 2004]. The antioxidant capacity was proposed to be due to phenolic and flavonoid compounds that are present in most plants [Fernandes *et al.*, 2004]. There exists much scope in further unravelling the antioxidant mechanisms of this plant (through potential direct and indirect mechanisms) as the compounds that could potentially impart the antioxidant properties have not been clearly elucidated. [Fernandes *et al.*, 2004; Mills *et al.*, 2005].

3.1.1. Pinitol

Pinitol (Figure 3.1) is a carbohydrate with a methoxy group located at the 3-position [Bates *et al.*, 2000]. It is found in numerous plants in various tissues such as the leaves, petioles, nodules, stems and seeds [Kuo *et al.*, 1997]. This low molecular weight carbohydrate is found in high concentrations in leguminous plants, and has been shown to possess hypoglycaemic (lower blood sugar levels) properties [Bates *et al.*, 2000; Streeter, 2001].

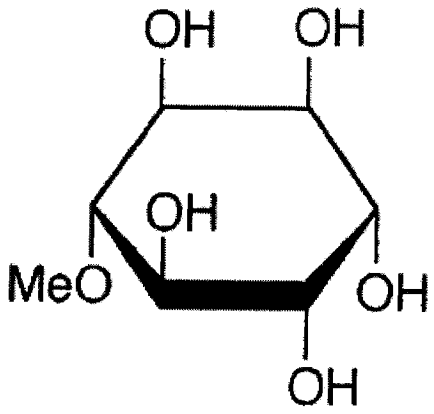

Figure 3.1: Structure of pinitol [Bates *et al.*, 2000].

3.1.2. Canavanine (α-amino-δ-guaninoxybutric acid)

Canavanine is a natural compound that contains a guanidinoxy functional group [Fearon and Bell, 1955] (Figure 3.2) and has been shown to be present in the seeds of leguminous plants. L-canavanine is structurally related to L-arginine and is found in the genera *Papilionoideae (Fabaceae),* a sub-family of *Leguminosae.* Canavanine has been shown to be toxic to numerous organisms and it is believed that its presence in seeds may be a mechanism of defence against some predators [Bleiler *et al.*, 1988].

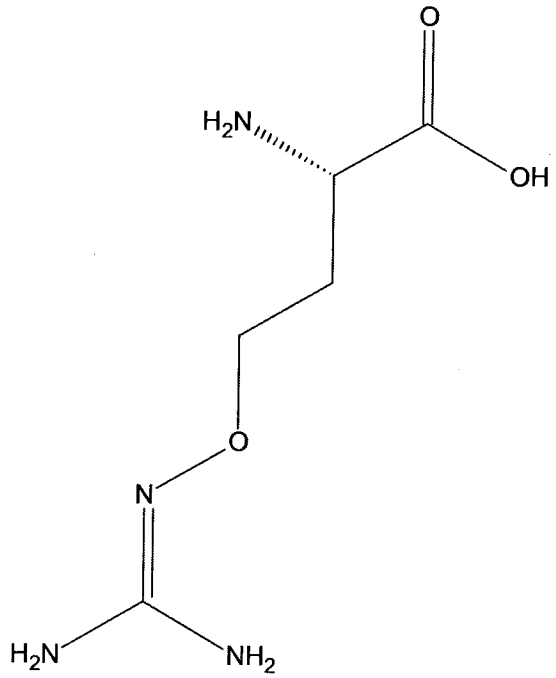

Figure 3.2: Structure of the non-protein amino acid, canavanine [Miersch *et al.*, 2000].

3.2. Electrochemical determination of antioxidants

3.2.1 The use of cyclic voltammetry (CV) for antioxidant determination.

CV has been extensively used to determine antioxidant capacity of various mediums that include plasma [Chevion *et al.*, 1997; Kohen *et al.*, 2000; Psotova *et al.*, 2001], plant material [Chevion *et al.*, 2000], pharmaceutical formulations [Campanella *et al.*, 2004; Huang *et al.*, 2004,] and herb extracts [Cosio *et al.*, 2006]. CV allows for the determination of total antioxidant capacity in biological samples (section 2.11). CV scans may be used to determine the ability of selected compounds to donate an electron around the oxidation potential of the anodic peak [Huang *et al.*, 2004]. Most LMWA are reducing agents and are able to donate an electron(s) to a ROS to neutralize it. The $E_{1/2}$ value (biological oxidation potential) indicates the reducing power of these compound(s). The closer the oxidation potential (OP) is to zero, the greater the ability of the compound to donate an electron. The ip_a (anodic current) can be used as an indication of the concentration of the compound(s) in the mixture [Chevion *et al.*, 1997]. However, if there is more than one reducing agent in the mixture, then the area under the curve (AUC) better reflects the total antioxidant capacity [Chevion *et al.*, 1997].

3.2.2. Square wave voltammetry (SWV) for the detection of antioxidants

Square wave voltammetry has been used for the determination of synthetic antioxidants [Ceballos, 2006], and ascorbic acid [Erdurak-Kilic *et al.*, 2006]. This method provides more defined peaks than CV, good discrimination against background currents and allows for more sensitive detection of analytes than CV. SWV in conjunction with CV can be used to provide valuable information regarding the antioxidant abilities of various compounds. Electrochemical methods of antioxidant assay are based on the fact that one of the modes of action of antioxidants is the ability to donate electrons [Chevion *et al.*, 1997; Chevion *et al.*, 2000; Huang *et al.*, 2004; Ceballos, 2006].

3.2.3. Adsorptive Cathodic stripping voltammetry (AdCSV) for the metal binding ability of antioxidants

AdCSV has been shown to be a sensitive method for metal chelation studies [Daniel *et al.*, 2004]. This research showed that electrochemistry, in particular stripping voltammetry, is a useful tool to determine the ability of compounds that are prospective antioxidants to bind transitional metals (such as Fe^{3+}).

3.3. Overall Objective

The overall objective of this chapter was to evaluate the use of electrochemical methods for the determination of total antioxidant capacity of known antioxidants and crude plant samples using *Sutherlandia frutescens* as a case study.

3.4. Aims

1. Characterise known antioxidants using electrochemical methods,
2. Electrochemically characterise canavanine and pinitol,
3. Characterise the HW and MeOH extracts of the leaves of *Sutherlandia frutescens* using the electrochemical methods used for Aim 1,
4. Compare the sensitivity of CV to that of SWV for the determination of antioxidants in crude samples.
5. Use the method of AdSV to determine the metal-chelating ability of canavanine, pinitol and the crude extracts of *Sutherlandia frutescens*.

3.5. Materials and Methods

D-pinitol (95 %), $FeCl_3$ (5wt % on silica gel), L-canavanine sulfate salt (> 99 %), melatonin (99 %), resorcinol (98 %), p-coumaric acid (≥ 98 %), quercetin dehydrate (≥ 98 %) and alumina oxide were obtained from Sigma-Aldrich (South Africa). L (+) ascorbic acid (99 %) was sourced from Merck, South Africa. Vanillic acid (≥ 97 %) and 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (TROLOX) were obtained from Fluka, South Africa. A plant specimen, obtained from Ryneath farm, Salem, South Africa, was prepared for positive identification and was lodged with the Selmar Schonland Herbarium, Albany Museum, Grahamstown, South Africa.

The specimen was registered as *Sutherlandia frutescens* (L.) R.Br. (Ragubeer 001 (GRA)). Water used for all experiments was obtained from a Millipore MilliQ system. pH measurements were made using a WTW pH 330i pH meter (Germany), coupled to a SenTix 41 pH electrode. The electro-analytical procedures were performed using a Potentiostat/Galvanostat 30 (PGSTAT 30) from Autolab. Analysis of electrochemical data was performed on the General Purpose Electrochemical System (GPES) software. The $E_{1/2}$ and AUC values were calculated using the peak search function present in the GPES software. The peak search function provides the AUC for each peak in Coulombs. This function also provides the values required to calculate the $E^1/_2$ using the formula: $E^1/_2 = ½(E_{pc} + E_{pa})$ for the CV scans. Using the peak search function the $E^1/_2$ was determined as it is indicated by the software. A three electrode system (Figure 2.1) was used for all electrochemical analysis. The PGSTAT 30 was used with a glassy carbon electrode (working electrode), 3 mm diameter (BioAnalytical Systems (BAS)). The reference electrode was an Ag/AgCl electrode (in 3 M KCl). A platinum wire (BAS) was used as the auxiliary electrode.

3.5.1. Sample Preparation

MeOH was used as it has been shown to extract lipophilic antioxidant compounds [Nakamura *et al.,* 1998; Exarchou *et al.*, 2005[a]]. Hot water (HW) extracts of the leaves of *Sutherlandia frutescens* have previously been shown to possess antioxidant properties, which is linked with its medicinal use in tea [Fernandes *et al.*, 2004; Katerere and Eloff, 2005], therefore HW extracts of the leaves were used for electrochemical analysis.

The leaves of *Sutherlandia frutescens* were dried at 60 °C for 3 days. Thereafter, the dried leaves were subjected to extraction with methanol and hot water. The methanolic extraction was performed by incubating 20 g of the dried leaves with methanol (200 ml, 12 h). The mixture was filtered and the supernatant was concentrated to produce the MeOH extract.

The hot water extraction involved the addition of dried leaves (20 g) to hot water (60 °C, 200 ml) and was allowed to stand for 12 h and then filtered. The supernatant was then lyophilised (-40 °C, overnight) to concentrate the sample.

The samples to be analysed were prepared at a concentration of 1 mg/mL, as the concentrations of compounds present in the plant extracts were unknown. This would allow for a level a comparison of results between MeOH and HW extracts. Stock solutions (1 mM) of melatonin, resorcinol, p-coumaric acid, quercetin dehydrate; ascorbic acid and vanillic acid were prepared for electro-analysis.

3.5.2 Electrochemistry

A working volume of 2 ml was used for all analytical procedures unless stated otherwise. Consideration was taken to ensure that all the electrodes were equidistant from each other.

3.5.2.1 Preparation of the glassy carbon electrode (GCE) working electrode

The GCE working surface was rinsed with milliQ water, polished on a Buehler felt pad (BAS) with a slurry of alumina oxide powder and rinsed with milliQ water to remove any traces of alumina oxide. In addition the GCE was rinsed in 5 % nitric acid solution, to remove residual organics and metal ions, and finally rinsed with milliQ water. Unless stated otherwise, the GCE working surface was cleaned and polished after each and every scan. The sample was introduced into the electrochemical cell that included the buffer solution and a scan was performed. Thereafter, the working electrode (GCE) was removed, polished and rinsed, then returned to the cell. Sample was added to the cell (stirred solution) and another scan was performed.

CV and SWV analysis of the pure compounds was performed in Tris-HCl (0.1 M, pH 7.4) while the CV and SWV analysis of complex samples (plant extracts) was performed in PBS (0.1 M, pH 7.4) as these buffers provided the best resolution in terms of peak shape. Other tested buffers included sodium phosphate buffer (0.1 M, pH 7.4) and Tris-HCl (0.1 M, pH 4 and 5).

AdCSV was performed in Tris-HCl (0.1 M, pH 7.4) for iron III (Fe^{3+}). The deposition potential of 0.1 V *vs.* Ag/AgCl was applied for 30 sec (stirred solution) to allow for optimum adsorption of the metal species. The potential was then scanned from 0 to -1.0 V [Lambat *et al.*, 2002]. For AdCSV, the buffer solution was deaerated with nitrogen and a nitrogen blanket was maintained over the solution during the analysis. The solution must be deaerated to ensure that there is no interference due to dissolved oxygen [Daniel *et. al.*, 2004].

Once a sufficiently deaerated solution was obtained, the Fe^{3+} solution (100 µL, 1 mM) was added and scanned. The AdCSV of Fe^{3+} was performed in the presence of increasing concentrations of the test samples. All scans were performed at a scan rate of 0.1 V/s. The current response is determined during the stripping step due to the reduction of the metal species [Daniel *et al.*, 2004].

All glassware was washed with warm soapy water and rinsed with milliQ water. The glassware was then soaked overnight in a 5 % nitric acid bath and rinsed with milliQ water prior to use. The above methods, reagents and instruments were used throughout the project for electrochemical procedures unless stated otherwise.

3.6 Results and Discussion

3.6.1 Electrochemical characterisation of known antioxidant compounds

The antioxidant properties of the following compounds have been documented; melatonin [Tan *et al.*, 2002], ascorbic acid [Machlin and Bendich, 1987], p-coumaric acid resorcinol, vanillic acid [Kilmartin *et al*, 2001] and quercetin [Kilmartin, 2001] and these compounds were electrochemically characterised for comparative purposes (Figure 3.3 and Figure 3.4). 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (TROLOX), a well known and commonly used synthetic vitamin E analogue, was also electrochemically characterised.

All the antioxidant compounds examined using CV (Figure 3.3) and SWV (Figure 3.4) produced anodic peaks and resulted in good linearity between area under the curve (AUC) and increasing concentration of antioxidant compound. (Standard curves of known antioxidants are shown in Appendix A).

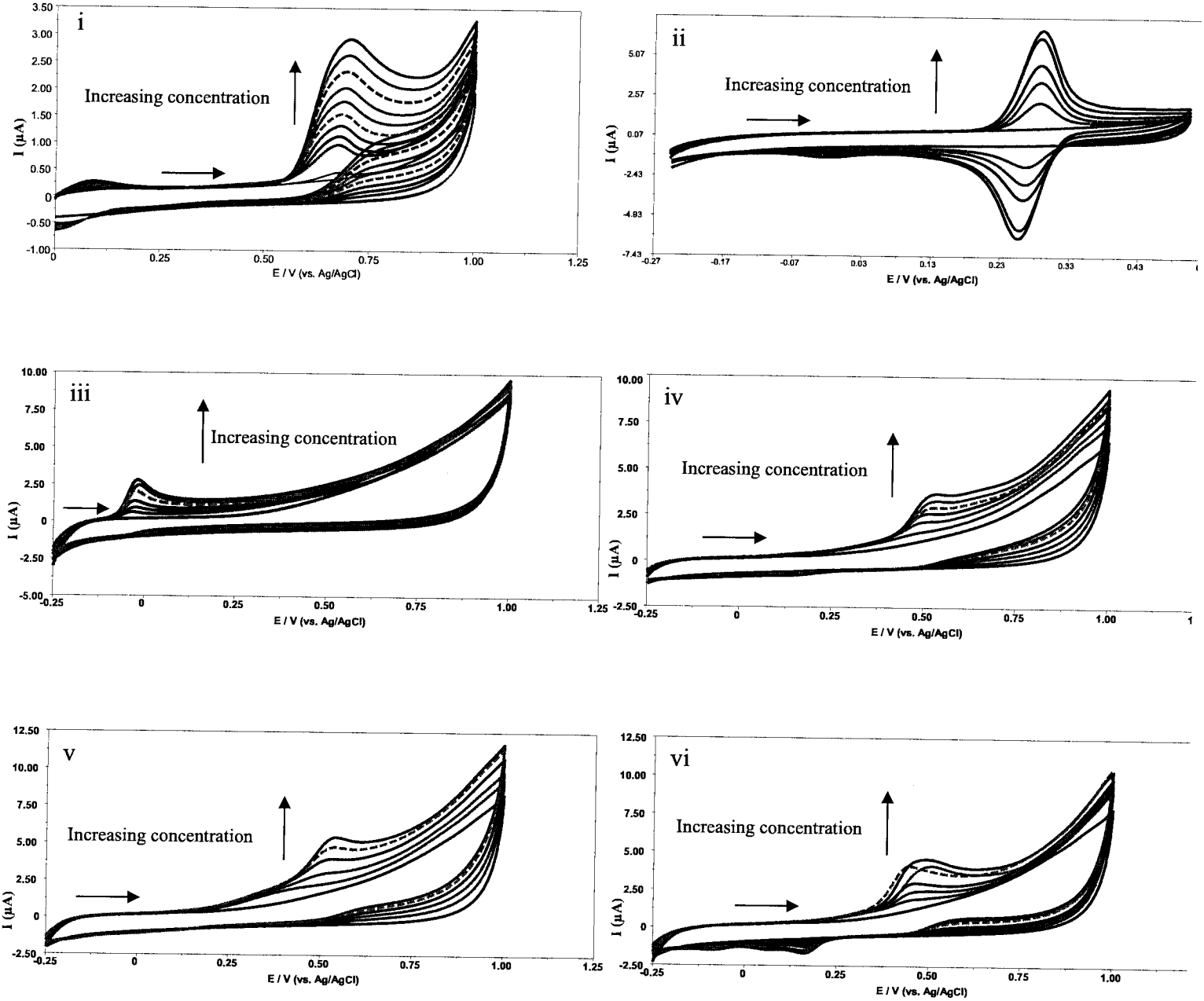

Figure 3.3: **Cyclic voltammograms of (i) melatonin, (ii) TROLOX, (iii) ascorbic acid, (iv) p-coumaric acid, (v) resorcinol and (vi) vanillic acid (0 – 130 µM) *vs.* Ag/AgCl. (scan rate = 0.05 V/s), 0.1 M Tris-HCl, pH 7.4.**

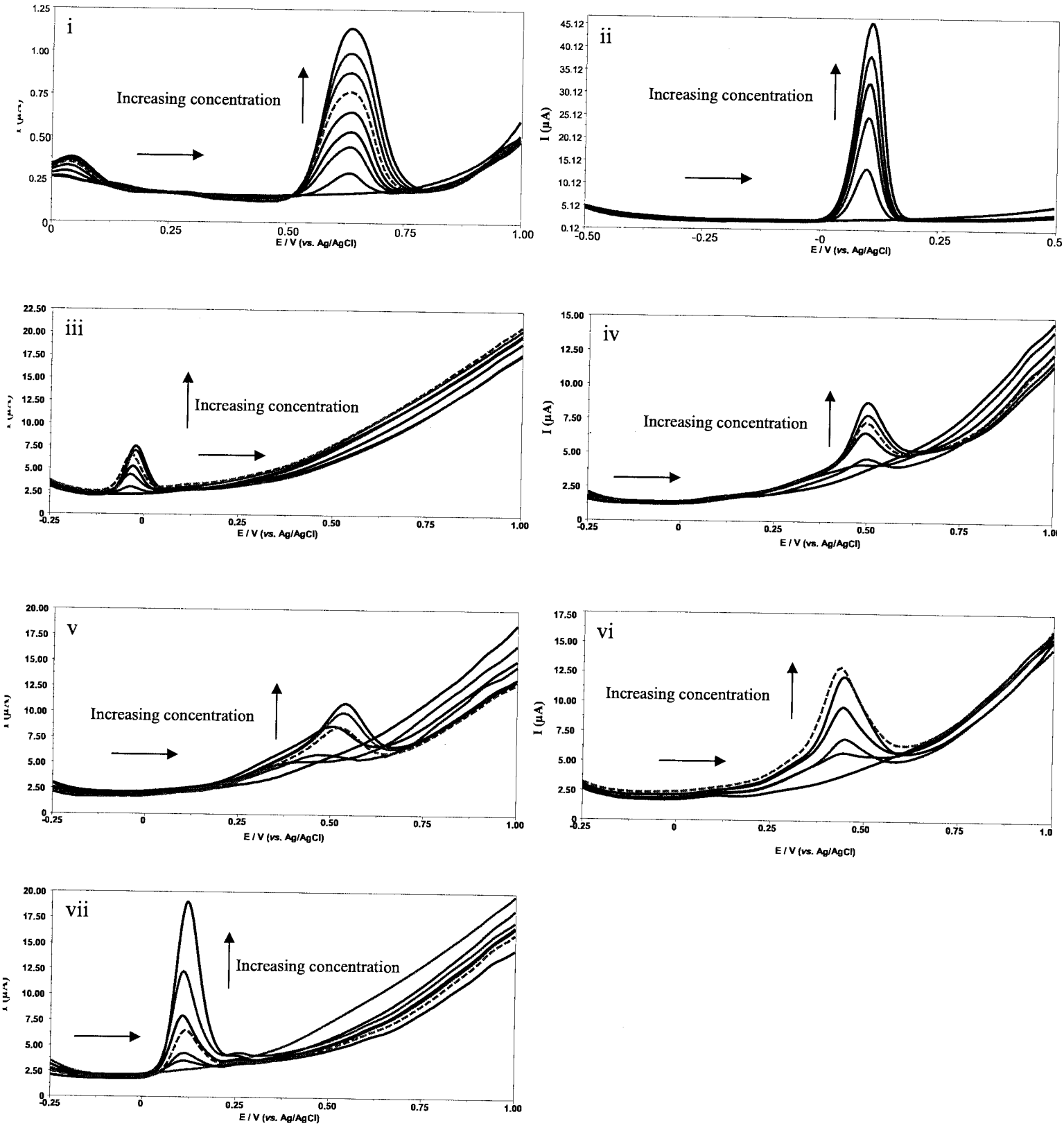

Figure 3.4: **Square wave voltammograms of (i) melatonin, (ii) TROLOX, (iii) ascorbic acid, (iv) p-coumaric acid, (v) resorcinol, (vi) vanillic acid and (vii) quercetin (0 – 130 µM) *vs.* Ag/AgCl. (scan rate = 0.1275 V/s), 0.1 M Tris-HCl, pH 7.4.**

Table 3.1: Known antioxidant compounds characterised electrochemically (130 μM).

	Ascorbic acid	Coumaric acid	Resorcinol	Vanillic acid	Quercetin	Melatonin	TROLOX
CV (E)	-0.019 V	0.510 V	0.536 V	0.466 V	----	0.730 V	0.287 V
$E_{1/2}$	-0.05 V	0.470 V	0.470 V	0.427 V	----	0.647 V	0.08 V
R^2 value	0.991	0.995	0.994	0.992	----	0.995	0.997
SWV (E)	-0.023 V	0.500 V	0.528 V	0.445 V	0.118 V	0.630 V	0.104 V
$E_{1/2}$	-0.043 V	0.455 V	0.452 V	0.393 V	0.083 V	0.570 V	0.068 V
R^2 value	0.994	0.993	0.996	0.991	0.995	0.998	0.994

There was a potential shift closer to zero when using SWV for some samples. SWV has been used as a sensitive method for the determination of antioxidants [Ceballos, 2006] and produced distinct peaks for quercetin while CV did not. The closer the oxidation peak is to zero, the more potent the antioxidant [Huang *et al.*, 2004]. The results (Table 3.1) indicate that the antioxidant compounds can be characterised based on the oxidation potentials, thus allowing these compounds to be ranked in the order: **ascorbic acid> quercetin > TROLOX > vanillic acid > coumaric acid > resorcinol > melatonin**. Literature values of ascorbic acid and coumaric acid indicate that at lower pHs the OP for ascorbic acid and coumaric acid were 0.42 V and 0.73 V, respectively [Sousa *et al.*, 2004]. At lower pHs vanillic acid and quercetin exhibited OP of 0.923 V and 0.526 V, respectively [Kilmartin, 2001]. However, for the purposes of this study all antioxidants were examined at physiological pHs to determine their effectiveness at this pH and thus cannot be compared to studies at lower pHs.

3.6.2. Electrochemical characterisation of canavanine and pinitol.

Canavanine produced a large irreversible anodic peak at 0.69 V ($E_{1/2}$ = 0.65 V) (Figure 3.5) at the surface of the GCE utilising CV. The scans of SWV (Figure 3.6) confirmed the results of the CV scans as canavanine produced a single irreversible peak at 0.66 V ($E_{1/2}$ = 0.62 V). Canavanine cannot be compared to the LMWA in terms of structure as it is an amino acid, however in terms of the OP canavanine is not as potent an antioxidant when compared to the LMWA examined in Table 3.1. The LMWA present in plants (Table 3.1) possess lower oxidation potentials than canavanine and would be more effective as antioxidants.

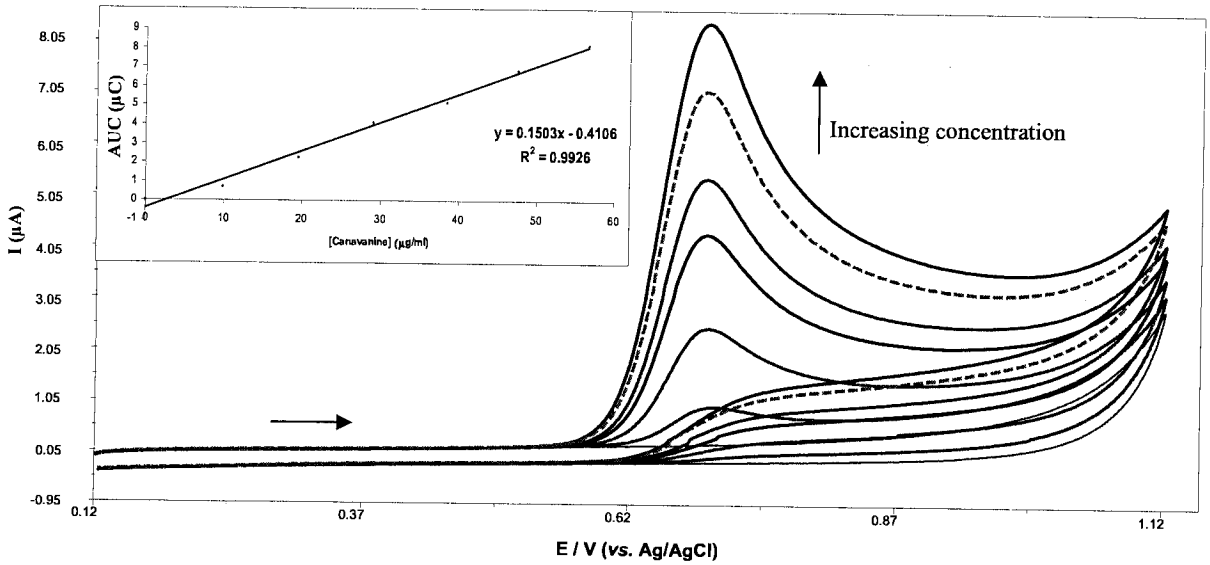

Figure 3.5: **CV characterisation of canavanine (0-57 µg/mL) *vs.* Ag/AgCl. (scan rate = 0.05 V/s), 0.1 M Tris-HCl, pH 7.4.**

However, due to its electro-activity, it may impart some antioxidant properties. Canavanine may exert an antioxidant effect in other ways. Canavanine has four amino substituents that provide sufficient electron-donating ability to the compound. The AUC of the CV increased with concentration, with $R^2 = 0.993$ (Figure 3.5) and SWV indicated a concentration dependant relationship with AUC with $R^2 = 0.994$ (Figure 3.6).

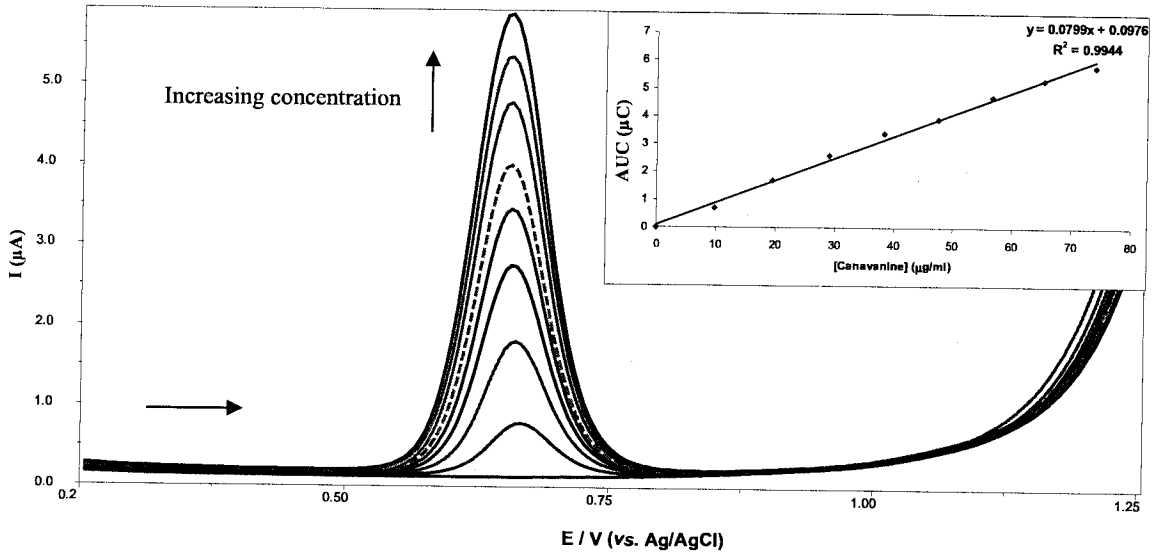

Figure 3.6: **SWV characterisation of canavanine (0-74 µg/mL) *vs.* Ag/AgCl. (scan rate = 0.1275 V/s), 0.1 M Tris-HCl, pH 7.4.**

CV of pinitol (Figure 3.7 A) indicated that it was not electro-active at the surface of the GCE as no anodic peaks were detected. The SWV (Figure 3.7 B) scans of pinitol confirmed that the compound possesses no electrochemical activity at the GCE. The electrochemical characteristics of analytes depend largely on their structural features [Cosio *et al.*, 2006]. Pinitol may be more difficult to oxidize using electrochemistry with a GCE.

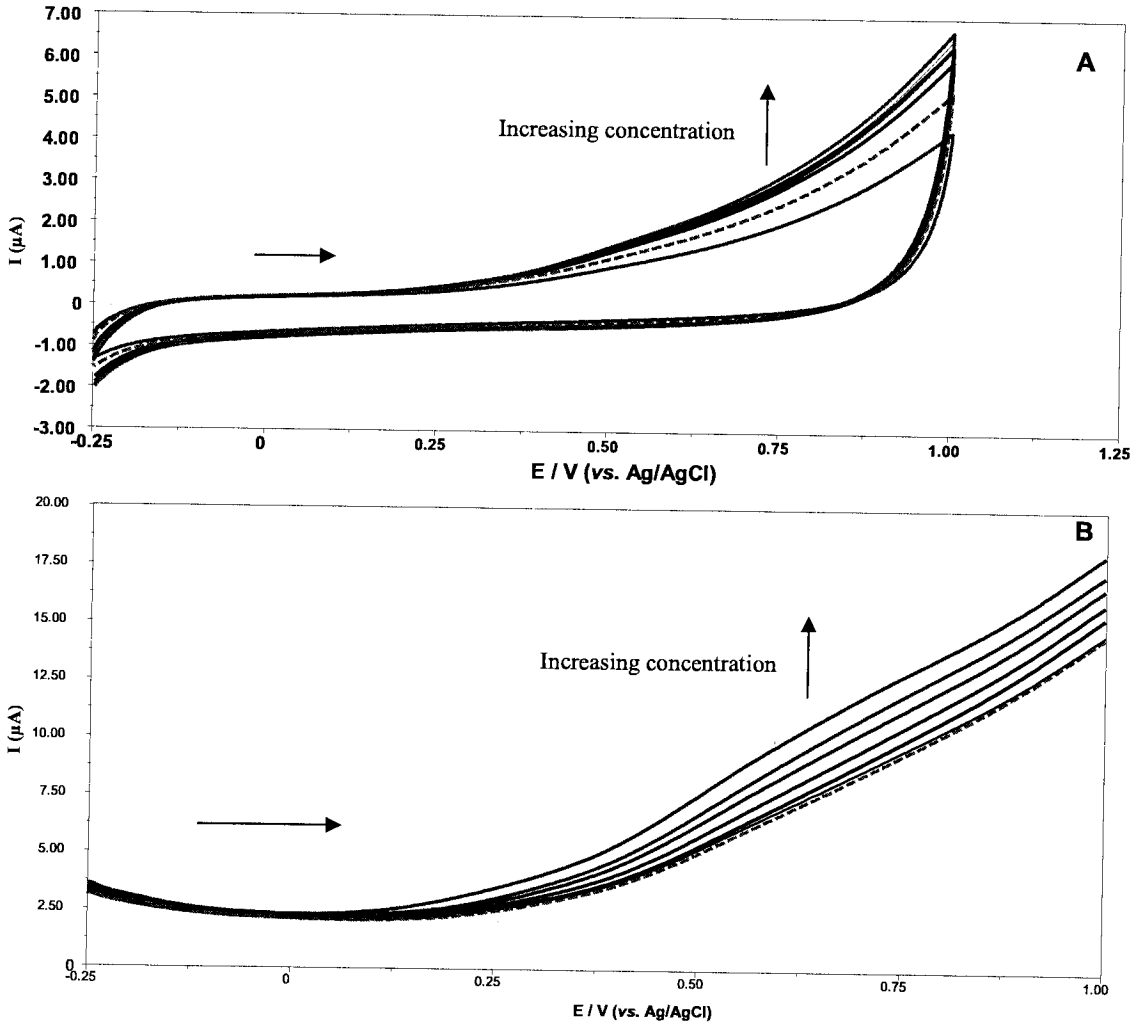

Figure 3.7: **CV (A) (scan rate = 0.05 V/s) and SWV (B) (scan rate = 0.1275 V/s) characterisation of pinitol (0-47 µg/mL) *vs.* Ag/AgCl, 0.1 M Tris-HCl, pH 7.4.**

Antioxidants may possess a mode of action that does not include electron donation. Therefore, a major drawback for antioxidant determination utilising electrochemical methods is that antioxidants that are not electro-active cannot be detected.

Furthermore, some antioxidants with electron donating capacities possess sluggish electron transfer kinetics and are not readily detectable at a bare glassy carbon electrode.

3.6.3. Electrochemical Characterisation of HW and MeOH extracts of Sutherlandia frutescens.

Plant extracts contain various compounds that may possess antioxidant properties [Balasundram *et al.*, 2006] and the use of CV would allow for the total antioxidant capacity to be determined [Buratti *et al.*, 2001]. The CV of the HW extracts of *Sutherlandia frutescens* (Figure 3.8) scans showed the presence of at least two electro-active compounds as confirmed by the SWV scan (Figure 3.9). CV of the HW extract showed two peaks at 0.26 V and 0.69 V. As shown by Kilmartin [2001], a mixture of compounds results in peaks that are broad. This may be the reason why the second peak produced by the HW extract of *Sutherlandia frutescens* is a broad peak.

The peaks obtained from the HW extract are attributed to the water-soluble antioxidants extracted form the leaves of *Sutherlandia frutescens*. The HW extraction procedure removes the water-soluble antioxidants as water is ineffective in extracting lipophilic antioxidants. The CV analysis showed the presence of a reversibly oxidizable compound at 0.26 V.

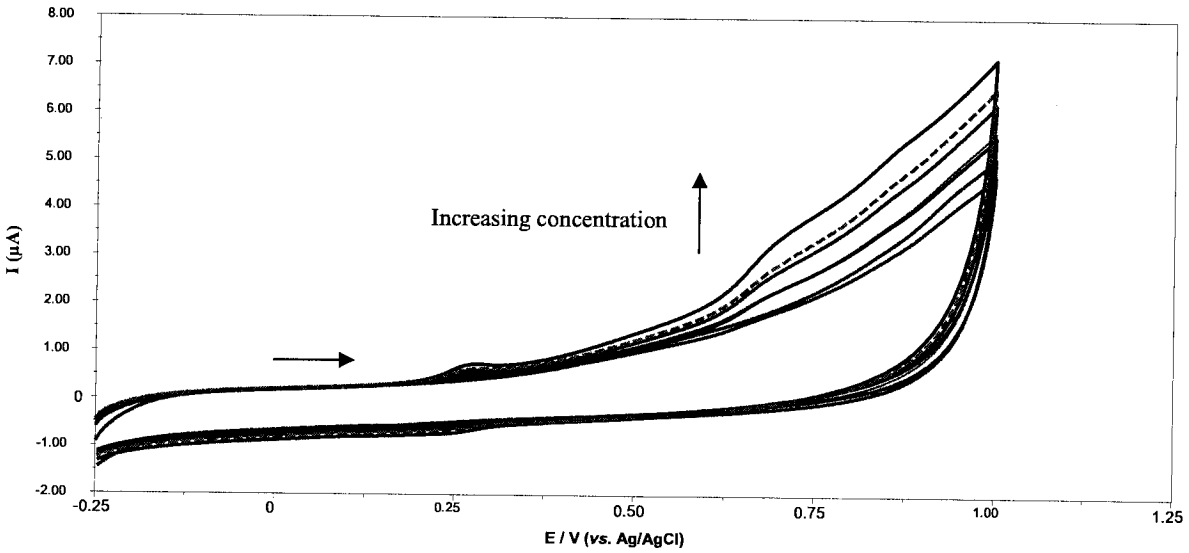

Figure 3.8: CV characterisation (scan rate = 0.05 V/s) of HW extracts (0–130 µg/mL) of *Sutherlandia frutescens* vs. Ag/AgCl., 0.1 M PBS (pH 7.4).

The AUC of these anodic peaks, as determined by SWV (Figure 3.9), indicated that these compounds were present at low concentrations [Chevion *et al.*, 2000]. The HW extract (130 µg/mL), produced two peaks with an AUC of 0.24 µC (at 0.26 V) whilst the second peak had an AUC of 0.047 µC (at 0.69 V).

The AUC values determined cannot be compared with that of pure compounds determined before given the presence of unknown compounds and differing concentrations. The use of the AUC value for the determination of the total antioxidant capacity may present problems as there are no well defined standards for comparative studies. The use of ascorbic acid as a standard compound for the comparison of plant material examined has however been shown [Kim *et al.*, 2002]. Thus, the ascorbic acid equivalence (AAE) was determined for the extracts of *Sutherlandia frutescens* using the AUC obtained for ascorbic acids (Figure A6) and the SWV analysis of the crude plant material. The HW extract of *Sutherlandia frutescens* was shown to possess an AAE value of 45.3 µg, while the MeOH extract possessed an AAE value of 39.5 µg. These values indicate that 130 µg/mL of the HW extract produces an equivalent AUC of 45.3 µg of ascorbic acid and 130 µg/mL of the MeOH extract produces an equivalent AUC of 39.5 µg of ascorbic acid. OP however allows for a readily defined means of comparing antioxidants [Chevion *et al.*, 1997].

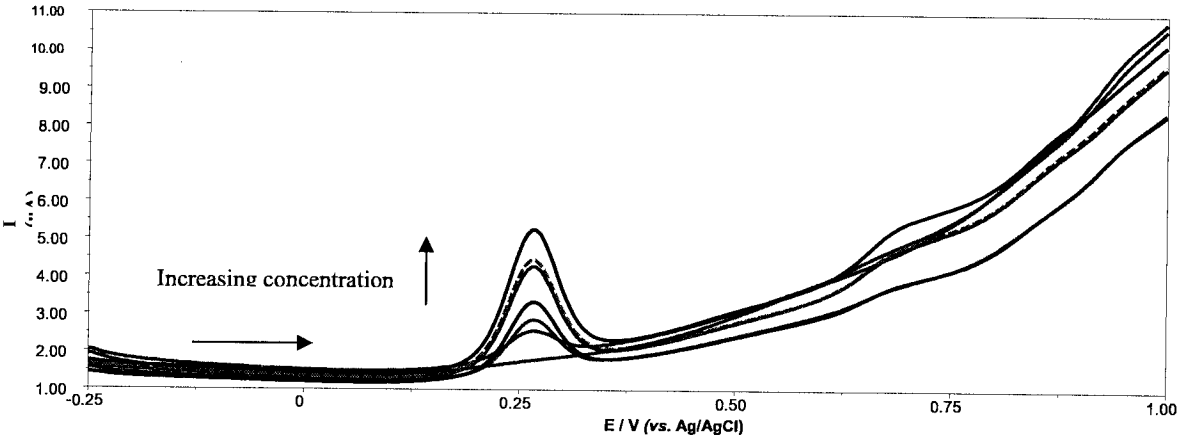

Figure 3.9: **SWV scans (scan rate = 0.1275 V/s) of HW extracts (0–130 µg/mL) of *Sutherlandia frutescens* vs. Ag/AgCl., 0.1 M PBS (pH 7.4).**

SWV clearly showed the two anodic peaks from the HW extract. The second peak at 0.69 V may be due to the water soluble canavanine as the CV of canavanine (Figure 3.5) indicates that the anodic peak produced by canavanine occurs at this potential. However, this would still need to be confirmed.

The compound(s) in the HW extract that produced the anodic peak at 0.26 V ($E_{1/2}$ = 0.20 V) possesses potent antioxidant properties when compared to the oxidation potentials of ascorbic acid and quercetin (Table 3.1).

The CV of the MeOH of *Sutherlandia frutescens* (Figure 3.10) and SWV, (Figure 3.11) show a single irreversible anodic peak present at 0.04 V ($E_{1/2}$= -0.01 V). The compound (s) that cause the anodic peak are electro-active and possess potent antioxidant capabilities as the oxidation potential is close to zero. The SWV analysis of the MeOH extracts of *Sutherlandia frutescens* verified the CV scans of these extracts. The anodic peak of the MeOH extract at 0.04 V and the peaks of the HW extract (0.26 and 0.69 V) were more distinctive using SWV analysis than that of the CV scans. The LMWA (Table 3.1) indicate that only ascorbic acid has a lower oxidation potential than the compound (s) in the MeOH extract. This indicates the presence of a potent lipophilic antioxidant (s). The AUC of the anodic peak was used as an indication of the total antioxidant capacity of all the antioxidant compounds present in this sample.

The AUC for the anodic peak for the MeOH extracts (130 µg), using SWV was 0.25 µC. The anodic peaks observed for the MeOH and HW extracts of *Sutherlandia frutescens* are observed at different potentials which is an indication of the presence of different lipophilic and hydrophilic antioxidants in the leaf extracts, as the MeOH extracted the lipophilic electro-active compounds and the hot water extracted the water-soluble electro-active compounds.

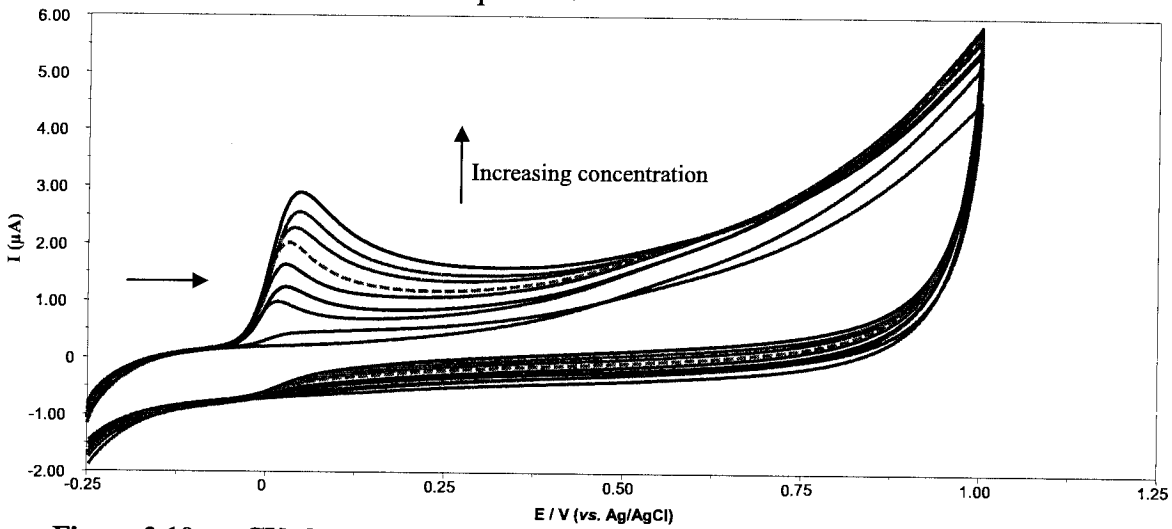

Figure 3.10: CV characterisation (scan rate = 0.05 V/s) scans of MeOH extracts (0–130 µg/mL) of *Sutherlandia frutescens* vs. Ag/AgCl., 0.1 M PBS (pH 7.4).

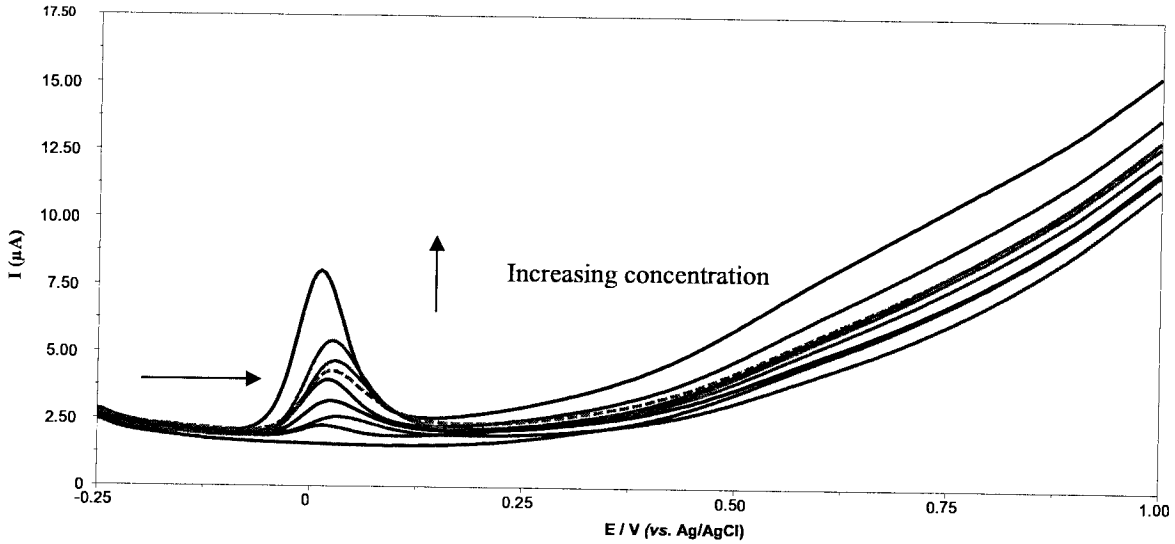

Figure 3.11: **SWV characterisation (scan rate = 0.1275 V/s) scans of MeOH extracts of *Sutherlandia frutescens* (0–130 µg/mL) *vs.* Ag/AgCl., 0.1 M PBS (pH 7.4).**

The AUC of the HW and MeOH extracts of *Sutherlandia frutescens* indicate that both extracts possess similar total antioxidant capacity although the MeOH extract has more potent antioxidant compounds due to its lower oxidation potential. This is shown in Table 3.2.

Table 3.2: **Summary of the OP, $E_{1/2}$ and AUC of extracts of *Sutherlandia frutescens* and canavanine (130 µg/mL)**

	E (V)	$E_{1/2}$ (V)	AUC (µC)
Canavanine	0.69	0.65	0.5
HW extract	0.26 and 0.69	0.2	0.24
MeOH extract	0.04	-0.01	0.25

3.6.4. Adsorptive Cathodic Stripping Voltammetry

AdCSV of Fe^{3+} resulted in a peak at -0.375 V due to the stripping of iron from the electrode. The addition of canavanine (Figure 3.12) resulted in a decrease in the peak height which indicated that this compound was able to bind to Fe^{3+}. The strength of the metal-ligand bond is important as this determines the extent of the decrease of the Fe^{3+} peak [Lambat *et al*, 2002; Daniel *et al*., 2004].

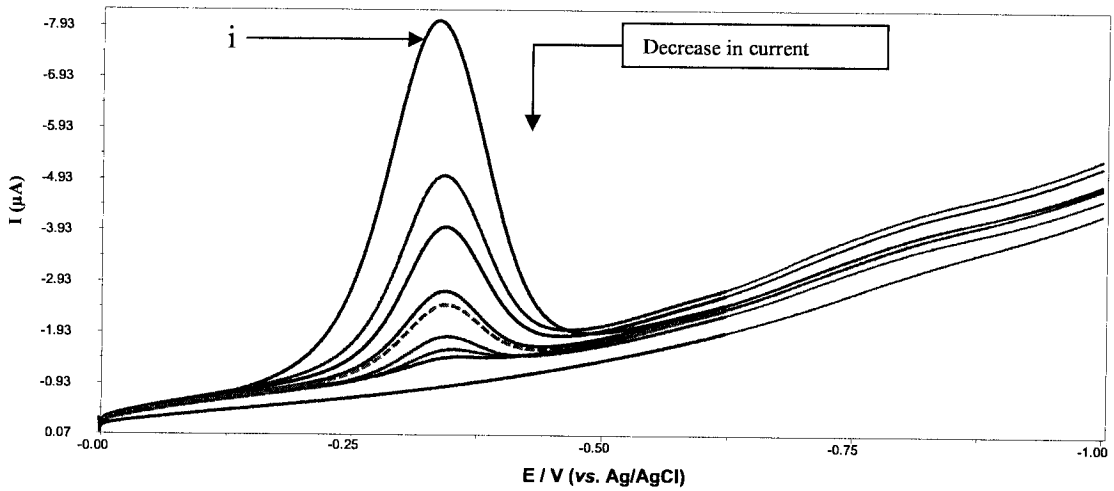

Figure 3.12: AdCSV of Fe^{3+} alone (i) and the addition of increasing concentrations of canavanine (0–23 µg/mL), 0.1 M Tris-HCl, pH 7.4.

The presence of substituents on antioxidant compounds have been shown to play a role in metal chelation [Huang *et al.*, 2006]. Canavanine (Figure 3.12) may be able to chelate Fe^{3+} which may be due to the presence of –NH substituents. Pinitol also indicated an ability to bind to Fe^{3+} as indicated by the decrease in the Fe^{3+} peak (Figure 3.13). Pinitol was not electro-active at the surface of the GCE indicating that it may not possess an electron donating ability. The ability to chelate transitional metals is another property of antioxidants and this may a mode of action for pinitol. Pinitol (Figure 3.13) has numerous –OH groups which may enable metal-binding [Huang *et al.*, 2006].

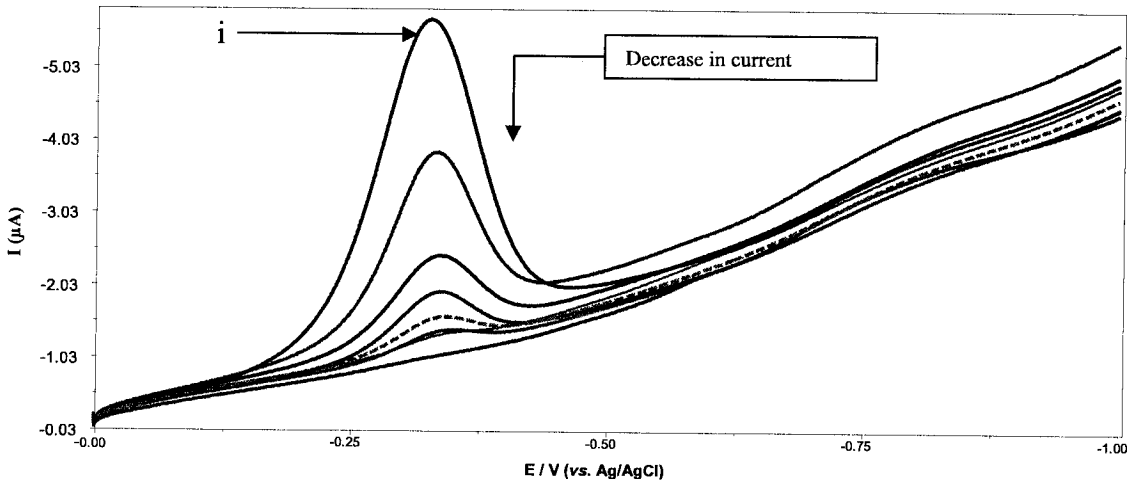

Figure 3.13: AdCSV of Fe^{3+} alone (i) and the addition of increasing concentrations of pinitol (0–23 µg/mL), 0.1 M Tris-HCl, pH 7.4.

The HW and MeOH extracts of *Sutherlandia frutescens* (Figures 3.14 and 3.15, respectively) showed the ability to bind to Fe^{3+} using AdCSV. The HW extracts of *Sutherlandia frutescens* indicated the presence of possible antioxidant compounds that are able to chelate Fe^{3+}.

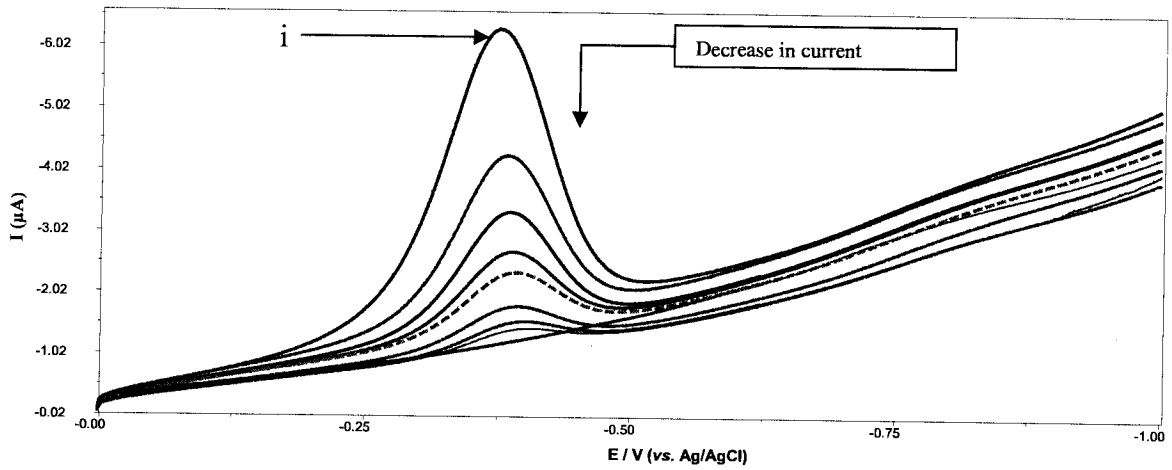

Figure 3.14: **AdCSV of Fe^{3+} alone (i) and the addition of increasing concentrations (0–23 µg/mL), of the HW extracts of *Sutherlandia frutescens*, 0.1 M Tris-HCl, pH 7.4.**

The ability of the plant extracts to bind to the metals may be attributed to the presence of polyphenolic compounds present. These compounds have been shown to possess the ability to chelate metals [Hagerman *et al.*, 1998; Khokhar *et al.*, 2003].

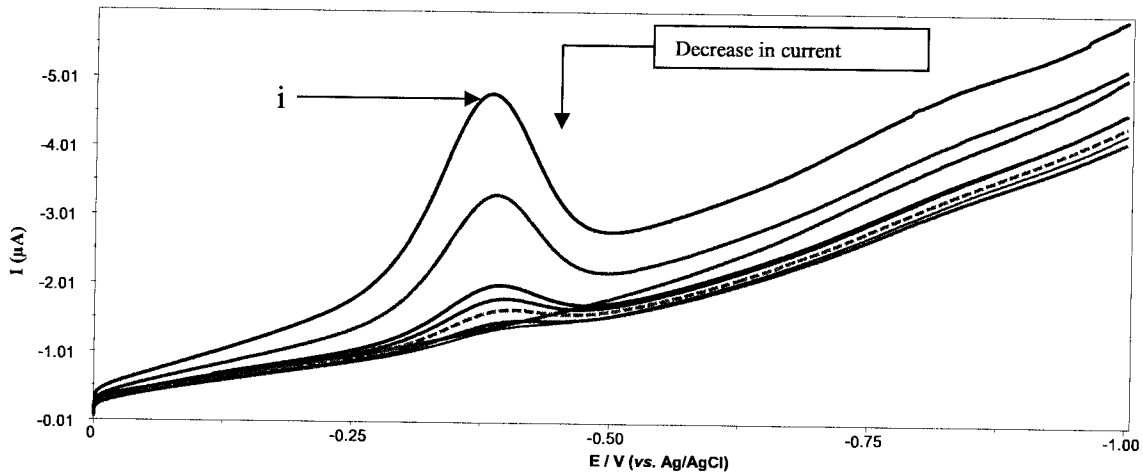

Figure 3.15: **AdCSV of Fe^{3+} alone (i) and the addition of increasing concentrations (0–23 µg/mL), of MeOH extracts of *Sutherlandia frutescens*, 0.1 M Tris-HCl, pH 7.4.**

The MeOH extract reduced the Fe^{3+} peak at 23 µg/mL (Figure 3.15) to the base-line which is an indication of the presence of compounds that chelate Fe^{3+} strongly. The MeOH extract of *Sutherlandia frutescens* was shown to possess compounds which were more effective at metal-binding than those in the HW extract, at higher concentrations (23 µg/mL). The MeOH extracts of *Sutherlandia frutescens* may contain lipophilic antioxidant compounds that may be more effective at metal chelation than the antioxidant compounds present in the HW extract.

The peak produced by Fe^{3+} alone was assigned a value of 100%. The current response in the presence of the 'ligands' was then calculated as a percentage of that value. Figure 3.16 shows the decrease in current response for each of the 'ligands'.

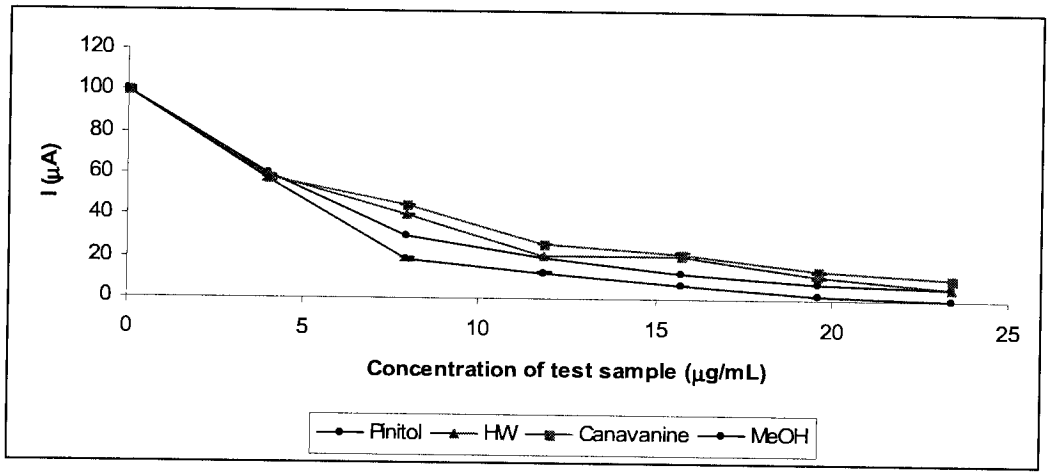

Figure 3.16: An indication of the decrease in current produced by Fe^{3+} before and after the addition of the individual analytes.

Pinitol reduced the current response at lower concentrations compared to canavanine, while the MeOH extract showed greater Fe^{3+} binding than the HW extract, however all showed metal binding ability. Pinitol and canavanine present in the hot extracts of the *Sutherlandia frutescens* may contribute to the metal-binding ability of these plant extracts.

3.7. Conclusions

Electrochemical techniques can be used to characterise known antioxidants that are known LMWA, electro-active neuro-hormones or synthetic antioxidants. Utilising these methods, these studies showed that the compound canavanine is electro-active but pinitol is not electrochemically active at the bare GCE.

However, canavanine is not a potent antioxidant in terms of the ability to donate electrons, as it has a greater oxidation potential than known antioxidants characterised. The HW extract of *Sutherlandia frutescens* indicated the presence of two possible antioxidant compounds (0.26 V and 0.69 V), whilst the MeOH extract showed the presence of a single electro-active species. The HW and MeOH extracts of *Sutherlandia frutescens*, both indicated the presence of potent, electro-active compounds (as yet unidentified) that may contribute to the antioxidant properties of *Sutherlandia frutescens.*

SWV was observed to be a more sensitive method for the determination of the anodic potential of antioxidants in crude samples and was thus used in further studies conducted. The AUC of the extracts of *Sutherlandia frutescens* indicated that the HW extracts possessed a similar total antioxidant capacity (0.24 μC at 0.26 V and 0.047 μC at 0.69 V) compared to the MeOH extract (0.25 μC at 0.04 V), however the MeOH extract possessed more potent electron-donating compound (s) as indicated by the low OP. AdCSV, using Fe^{3+}, was utilised for the determination of the ability of the compounds and extracts to bind this metal as a potential antioxidant mechanism. The MeOH extracts of *Sutherlandia frutescens* were shown to be more effective as a ligand for Fe^{3+} than HW extracts of *Sutherlandia frutescens*, while pinitol was more effective than canavanine.

The voltammetric methods applied correspond with previous studies indicating the antioxidant properties of *Sutherlandia frutescens* [Katerere, and Eloff, 2005, Fernandes *et al.*, 2004]. This study also shows for the first time the electrochemical characterisation of canavanine, also suggesting a role of this compound in conferring some of the antioxidant benefits of this plant. Pinitol was not electrochemically active indicating that this compound may not confer direct antioxidant properties, through electron donation.

However, as there are several factors which need to be considered when assessing the electro-activity of a compound, for example optimisation of pH, stability of the compound, solubility in aqueous media, and considering the possibility of sluggish electron transfer kinetics which may inhibit its electrochemical detection, this cannot be excluded.

The study does suggest that a *possible* mode of antioxidant activity may be *via* Fe^{3+} binding. Additional studies would need to be conducted to assess the reliability thereof.

CHAPTER 4: NON-BIOLOGICAL RADICAL ASSAYS FOR ANTIOXIDANT DETERMINATION

4.1. Introduction

Free radical generated assays are commonly used for the determination of antioxidants in complex samples such as beverages, foodstuff and in clinical studies [Antolovich *et al.*, 2002; Becker *et al.*, 2004; Ozgen *et al.*, 2006; Thaipong *et al.*, 2006]. These include the α,α-diphenylpicrylhydrazyl (DPPH) assay, the azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay, the Folin-Ciocalteu (FC) assay and the ferrozine assay [Prior *et al.*, 2005; Apak *et al.*, 2007] as described in section 2.8.1. These assays are easy to perform, cost-effective and do not require specialised equipment [Sanchez-Moreno, 2002].

4.2. Overall objectives

Utilise conventional non-biological methods to examine the antioxidant properties of *Sutherlandia frutescens.*

4.3. Aims

1. Determine the antioxidant properties of canavanine, pinitol and the HW and MeOH extracts of *Sutherlandia frutescens* with the conventionally used antioxidant assays: the DPPH, TEAC, ferrozine and FC assays.
2. Assess the degree of correlation of the conventional assays to the results of the electrochemical characterisation.

4.4. Materials and Methods

D-pinitol (95 %), 2, 2-diphenyl-1-picryl hydrazyl (DPPH), 3-(2-pyridyl)-5, 6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), Fe_2SO_4, Folin-Ciocalteu reagent and L-canavanine sulphate salt (> 99%) were obtained from Sigma-Aldrich, South Africa. Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (TROLOX) were obtained from Fluka. All other reagents used were of analytical grade. Spectrophotometric analyses were performed on a PowerWave spectrophotometer supplied with 96 well plates and analysed with the KC Junior software programme.

Plant extracts were prepared as described in section 3.3.1. Pinitol and canavanine were dissolved in milliQ H_2O to yield stock solutions of 1 mg/mL.

4.4.1. α,α-diphenylpicrylhydrazyl (DPPH) assay

The DPPH assay was adapted from Blois (1958). DPPH (0.3 mM) dissolved in methanol was used to determine the free radical scavenging ability of canavanine, pinitol, HW and MeOH extracts (1 mg/ml stock solution). The reaction mixture consisted of DPPH solution (2.9 mL) and was incubated with the test solution (0.1 mL) (increasing concentrations) for 20 minutes, then determined at 517 nm. The percentage (%) inhibition of the DPPH radical was calculated:

$$\textbf{\% inhibition} = \frac{A_0 - A_1}{A_0} \times 100 \quad \text{.....equation 1.}$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the samples at different concentrations. The control consisted of DPPH solution (2.9 mL) and milliQ H_2O (0.1 mL).

4.4.2. TROLOX Equivalence Antioxidant Capacity (TEAC) assay

The TROLOX Equivalence Antioxidant Capacity (TEAC) assay was performed using the $ABTS^{\cdot+}$ radical. The $ABTS^{\cdot+}$ radical was formed by following the protocol of Re *et al.*, (1999). The ABTS (7 mM) solution was mixed with $K_2S_2O_8$ (2.45 mM) (final concentration). This mixture was allowed to stand in the dark for 12-16 hours to produce the ABTS radical. The absorbance was adjusted to 1 ± 0.05 before use. The reaction mixture consisted of the $ABTS^{\cdot+}$ radical solution (2.9 mL) incubated with the test solution (0.1 mL) for 30 minutes. A_0 was the control of $ABTS^{\cdot+}$ solution (2.9 mL) and milliQ H_2O (0.1 mL). A standard curve was produced using TROLOX (1 mM stock solution) and the % inhibition was calculated using equation 1. The scavenging ability of the test samples was determined from the TROLOX standard curve (Appendix B) and represented as TEAC values.

4.4.3. Ferrozine assay

The ferrozine assay was used to determine the ability of canavanine, pinitol, HW extract and MeOH extract to chelate the transitional metal ion, Fe^{2+} [Que *et al.*, 2006].

Fresh solutions of ferrozine (5 mM) and Fe_2SO_4 (0.2 mM) in deaerated milliQ H_2O were prepared just prior to use. An aliquot of ferrozine (0.2 mL), Fe_2SO_4 (0.1 mL) and deaerated milliQ H_2O (3.7 mL) was incubated with the test samples (0.5 mL) for 20 minutes, and thereafter the absorbance was read at 562 nm [Que *et al.*, 2006]. The chelating ability was determined using equation 1. A_o consisted of ferrozine (0.2 mL), Fe_2SO_4 (0.1 mL) and deaerated milliQ H_2O (4.2 mL).

4.4.4. Folin-Ciocalteu (FC) assay

The total phenol concentration of the plant extracts (10 mg/ml stock solution) was determined using the Folin-Ciocalteu (FC) assay [Singleton and Rossi, 1965]. The test sample (1 mL) (2.5, 5, and 10 mg/mL) was diluted with milliQ H_2O (46 mL). Thereafter, the FC reagent (1 mL) was added to the sample and agitated. After three minutes, Na_2CO_3 solution (3 ml, 25 g/100 mL) was added and agitated. The reaction mixture was incubated for 2 hours and the absorbance was determined at 760 nm. The phenol concentration was determined and represented as Gallic acid equivalence (GAE).

4.5 Results and Discussion

4.5.1. α,α-diphenylpicrylhydrazyl (DPPH) assay

At higher concentrations, the HW extract was the most effective at scavenging the DPPH radical based on the % inhibition of the DPPH radical (Figure 4.1).

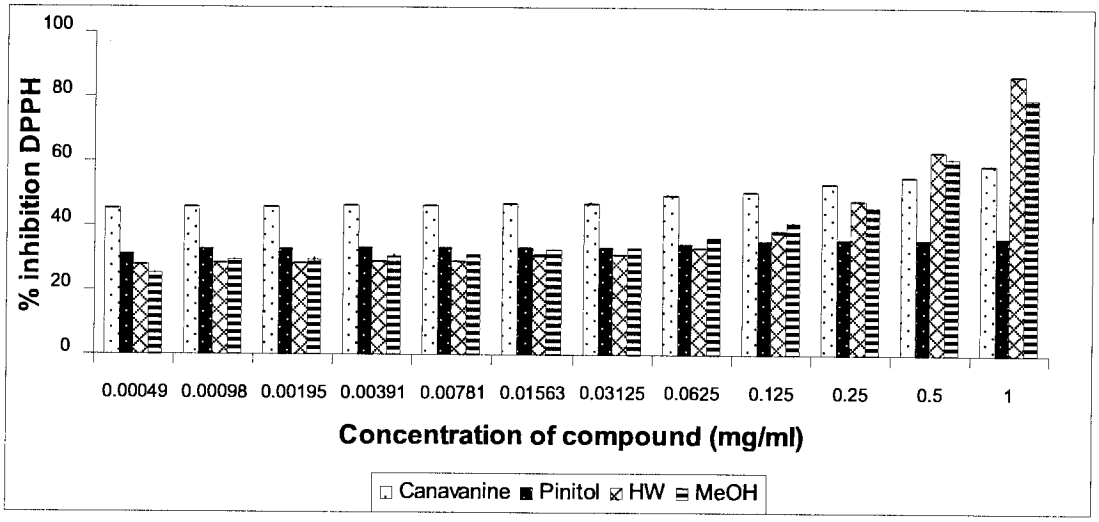

Figure 4.1: The scavenging abilities of different concentrations of canavanine, pinitol, and HW and MeOH extracts of *Sutherlandia frutescens* using the DPPH radical.

The DPPH radical functions by either accepting a H-ion or electron from the antioxidant compound [Kumaran and Karunakaran, 2007]. Thus, the HW extract has a greater H-ion or electron donating capacity than the MeOH extract. However, for both extracts, as the concentration increases so did the scavenging capacity. The HW and MeOH extracts showed an inhibition of the DPPH radical of 87 % and 80 %, respectively at 1 mg/mL. Canavanine inhibited the DPPH radical by 60 % while pinitol showed a 36 % inhibition at 1 mg/mL. A recent study has shown ascorbic acid (0.05 mg/mL) to inhibit the DPPH radical by 92.8 % [Yanga *et al.*, 2007].

Only pinitol was not able to inhibit the DPPH radical by 50 % or more indicating that it does not possess any significant radical scavenging ability. The structure of canavanine leads to the conclusion that its antioxidant ability is due to the ability to donate electrons or accept a H-ion. Antioxidants that have the ability to donate H-ions are able to inhibit the DPPH radical to a greater extent; however, compounds that are able to donate electrons may inhibit the DPPH radical but not as significantly [Prior *et al.*, 2005].

Pinitol has the lowest scavenging ability of all the test compounds. Pinitol is a carbohydrate with a similar structure to glucose. DPPH has been shown not to be able to react with glucose as it does not have a high enough redox potential [Blois, 1958]. This may be the reason that pinitol has a low scavenging ability.

4.5.2. TROLOX Equivalence Antioxidant Capacity (TEAC) assay

The HW extract, canavanine, MeOH extract and pinitol had TEAC values of 0.22, 0.21, 0.15 and 0.05, respectively (Figure 4.2). Pinitol has the lowest TEAC value indicating that it does not possess the antioxidant ability of donating electrons nor H ions, confirming results obtained using the DPPH assay and the lack of electro-activity. The test samples have relatively low TEAC values as the TEAC value of 1 is assigned to TROLOX. Numerous antioxidants have been show to possess TEAC values closer to 1 such as ascorbic acid and α-tocopherol possessing values of 0.99 and 0.97 (2.5 μM), respectively [van den Berg *et al.*, 1999].

The highest TEAC is 0.22 for the HW extract (at 1mg/ml) which is significantly lower than that of ascorbic acid. This is in contrast to the antioxidant abilities shown for the DPPH assay. The ABTS radical in the TEAC assay has been shown to take a long time to reach endpoint [Prior *et al.*, 2005] and the other disadvantage is that the $ABTS^{\cdot+}$ is not found in biological systems and is thus an unsuitable radical source. The ABTS radical is a stable radical and cannot be used as a representative radical source for ROS found in biological systems. However, for comparative purposes canavanine exhibited a greater TEAC value at all concentrations than pinitol, while the MeOH extraxt yielded a greater TEAC than the HW extract.

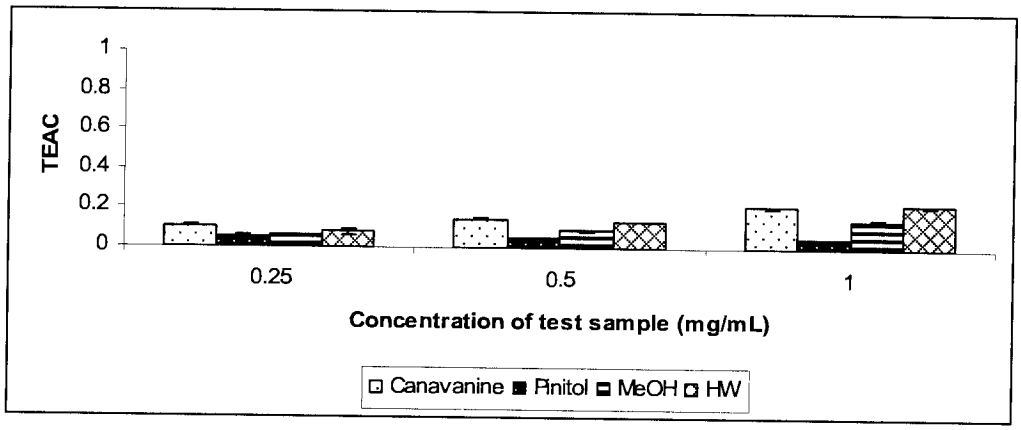

Figure 4.2: **The TEAC of canavanine, pinitol and the HW and MeOH extracts of *Sutherlandia frutescens* using the ABTS radical.**

4.5.3. Ferrozine assay

Canavanine, pinitol, the MeOH and HW extracts of *Sutherlandia frutescens* had the binding ability of 27, 27, 28 and 44 %, respectively at 1 mg/mL (Figure 4.3). The metal-binding ability of test compounds indicates their ability to reduce the formation of free radicals that are produced through reactions with transitional metals, however, considering that the highest concentration of 1 mg/mL examined did not show a binding ability greater than 44 % these results do not provide strong evidence for Fe^{2+} binding as an antioxidant mechanism.

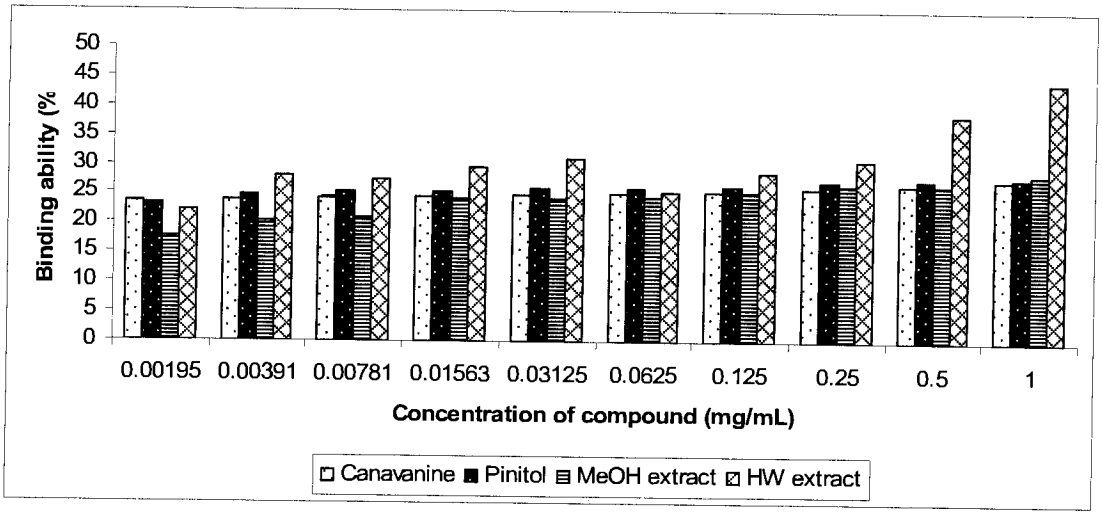

Figure 4.3: **The binding ability of the test compounds obtained using the ferrozine assay (n=3).**

Plant extracts have been shown to chelate Fe^{2+} ions due to numerous compounds in the extracts [Khokhar and Apenten., 2003]. The HW extract may have performed better than the MeOH extract as the ferrozine preparation is aqueous and the lipophilic antioxidant compounds present in the MeOH extract may not have been able to function at optimum.

4.5.4. Folin-Ciocalteu (FC) assay

The total phenol content was expressed as mg per mL of plant sample used. At 10 mg/mL the HW and MeOH extracts had GAE of 0.55 and 0.37 (Figure 4.4). This correlates with the DPPH and ABTS radical assays as they show the HW extract to possess greater radical scavenging ability than the MeOH extract.

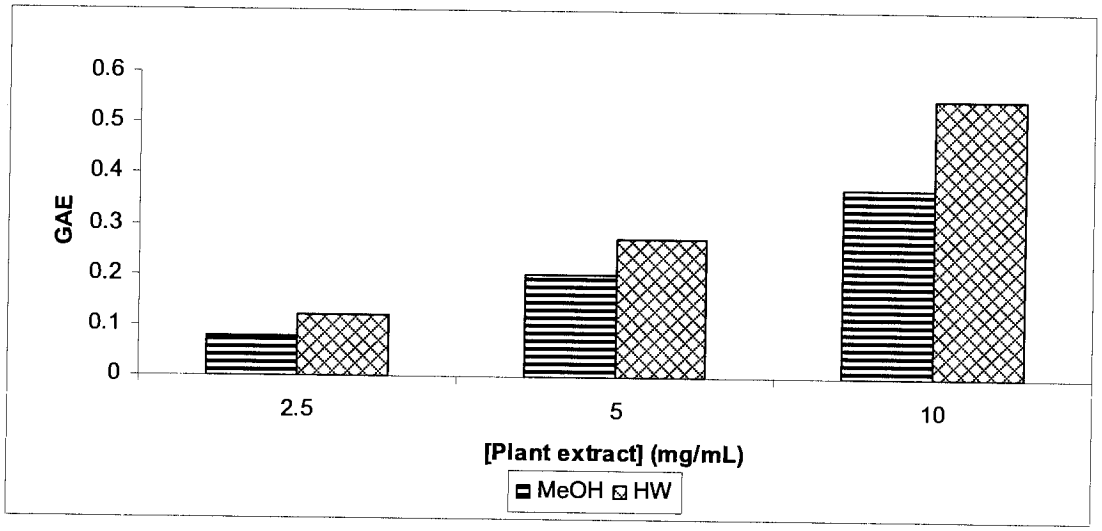

Figure 4.4: **Graph showing the Gallic acid equivalence of both extracts of *Sutherlandia frutescens*.**

Phenolic compounds have been shown to possess antioxidant properties due to their structure [Peschel *et al.*, 2006]; however, not all phenolics possess antioxidant properties, a shortcoming of this method. They have been shown to contribute to the antioxidant activity of crude extracts therefore the phenolic content is often determined to indicate the portion of the plant extracts that may be phenolic in nature [Prior *et al.*, 2005]. The HW extracts indicated a greater phenolic content than the MeOH extracts that may explain the greater inhibition of the DPPH radical at higher concentrations and the higher TEAC values compared to the MeOH extracts.

4.6. Conclusions

The HW and MeOH extracts of *Sutherlandia frutescens* inhibited the DPPH radical by 80 % or more indicating that both extracts contain compounds that are able to donate electrons (confirming voltammetric analysis) or hydrogen ions. Canavanine showed reasonable ability to quench the DPPH radical while pinitol was shown to be the least effective. The $ABTS^{\cdot+}$ radical assay indicated that the HW extract of *Sutherlandia frutescens* and canavanine were able to act as antioxidants by donating electrons to quench the $ABTS^{\cdot+}$ radical. Pinitol was again the least effective at quenching the $ABTS^{\cdot+}$ radical. This result concurs with the voltammetric characterisation of this compound which indicated that electron donation was not a potential mode of action of this compound.

The ferrozine assay indicated that the test samples did not possess considerable ability to chelate the transitional metal Fe^{2+}. None of the test compounds possessed the ability to bind Fe^{2+} greater than 44 %. However, the ferrozine assay has numerous hindrances that include extensive preparation required for the assay. The solutions and buffers must be amply deaerated to ensure that the Fe^{2+} is not oxidized by O_2 to form iron hydroxyides (this is dependant on pH). The incubation period may also allow for inference to occur due to oxygen diffusion into the reaction mixture. Another shortfall of the ferrozine assay is that the ferrozine molecule binds to Fe^{2+} and Fe^{3+} [Viollier *et al.*, 2000]. Therefore, the presence of both states of the transition metal may result in an error in the results.

The HW extract of *Sutherlandia frutescens* was shown to possess a greater total phenolic content than the MeOH extract, which may contribute to its antioxidant properties and may be the reason it was able to scavenge the DPPH radical and ABTS radical to a greater extent than the MeOH extract.

Both plant extracts were shown to possess electro-active compounds (section 3.5.3). As the DPPH and TEAC assay rely on the ability of the antioxidant compound to donate electrons or H^+ ions, it can be determined that both plant extracts possess compounds that are electron donors. The HW extract however, possesses compounds that are more effective at donating electrons than the MeOH extract based on the non-biological assays.

CHAPTER 5: LIPID PEROXIDATION AND SUPEROXIDE ANION ASSAYS WITH *SUTHERLANDIA FRUTESCENS* EXTRACTS

5.1. Introduction

The use of rapid radical assays have been criticised as these systems are not based on physiological parameters [Arnao, 2000]. Methods that use substrates that may be affected by ROS are often used to elucidate the effectiveness of antioxidant compounds [Jackson, 1999; Abuja and Albertini, 2001]. Some of the substrates include lipids, proteins or nucleic acids that imitate the biological systems that are often affected by ROS [Laguerre *et al.,* 2007]. This would allow for the analysis of antioxidant compounds which can be used, for example, in prevention diseases such as atherosclerosis [Katsube *et al.*, 2004]. The most common biological assays include the thiobarbituric acid (TBA) assay [Garcia *et al.*, 2005] and the superoxide anion (NBT) assays [Callaway *et al.,* 1998].

5.1.1. Lipid peroxidation (LP)

The thiobarbituric acid assay has been widely used as a measure of LP in animal tissue [Abuja and Albertini, 2001]. The assay involves the reaction of one of the end products of LP, malondialdehyde (MDA), with thiobarbituric acid (TBA). One molecule of MDA reacts with two molecules of TBA resulting in a pink chromogen with an absorbance at 532 nm [Oxford Biomedical Research, 2003]. Antioxidants are often used to reduce lipid peroxidation [De Ruvo *et al.*, 2000; Fang *et al.*, 2002]. The reduction of lipid peroxidation in the presence of test compounds, as indicated by a decrease in absorbance, provides an indication of their ability to blunt lipid peroxidation as an antioxidant mechanism. However, a variety of plant species contain compounds that may interfere with the absorbance determination of the MDA-TBA complex at 532 nm. Despite this, the MDA/TBA assay is still used for the determination of LP, because the spectrophotometric analysis of MDA-TBA complex provides a rapid and simplistic assay for LP without extreme manipulation [Hodges *et al.*, 1999].

5.1.2. Nitroblue tetrazolium (NBT) assay

It has been shown that cytochrome-mediated conversion of O_2 to H_2O can be inhibited by CN^- [Freeman and Crapo, 1981]. CN^- has been used to block cytochrome *b* and prevent the transfer of electrons through the mitochondria that can react with an oxygen molecule resulting in the formation of a superoxide free radical [Dawson and Dawson, 1996]. The NBT assay is a simple, commonly used assay to determine the superoxide radical [Ottino and Duncan, 1997]. During the assay the NBT dye is reduced by the generated superoxide radical to form NBD that is extracted with glacial acetic acid with the concentration determined spectrophotometrically at 560 nm. The change in absorbance in the presence of test compounds is attributed to the superoxide scavenging ability of antioxidants present (Halliwell, 2006[a]).

5.2 Overall Objective

Utilise conventional biological methods of lipid peroxidation and the superoxide anion assay to validate the antioxidant properties of *Sutherlandia frutescens.*

5.3. Aims

1. Examine the antioxidant capacity of canavanine, pinitol, the HW and MeOH extracts of *Sutherlandia frutescens* using the pharmacological methods of lipid peroxidation and superoxide anion generation.
2. Utilise the biological methods to validate the results of the electrochemical analysis.

5.4. Materials and Methods

2,3-Pyridinedicarboxylic acid (QA), butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), nitroblue diformazan (NBD), nitroblue tetrazolium (NBT), KCN, L-canavanine sulphate salt (> 99 %), D-pinitol (95 %) and Fe_2SO_4 were obtained from Sigma–Aldrich (South Africa). L (+) ascorbic acid (99 %) was sourced from Merck (South Africa). Trichloroacetic acid (TCA), glacial acetic acid, hydrogen peroxide, EDTA and butanol were obtained from Merck (Saarchem, South Africa). All rat livers used were obtained from the Faculty of Pharmacy, Rhodes University.

5.4.1. Lipid peroxidation (LP)

The HW extract, MeOH extract, pinitol and canavanine were used in the assays to determine whether they possessed the ability to reduce the production of MDA. The concentration of the stock solutions of the test compounds ranged from 0.625-10 mg/mL. All biological assays are represented as the mean ± SE. The results were analyzed using the one-way analysis of variance (ANOVA). The means were compared to the control using Student-Nueman Keuls Multiple Range Test (Statistica 7). Two models of generating lipid peroxidation were examined, that involving the quinolinic acid and Fe^{2+} as well as H_2O_2 in the presence of Fe^{2+}.

5.4.1.1. QA- Fe^{2+} induced LP

This lipid peroxidation (LP) assay was modified from that of Duncan and Ottino (1997). Fe^{2+} (0.5 mM) and QA (1 mM) were used to induce LP. QA stimulates LP *in vitro* and combined with Fe^{2+} LP is initiated [Stipek *et al.*, 1997]. The reaction mixture contained rat liver homogenate (0.7 mL), QA (0.1 mL, 1 mM), Fe^{2+}(0.1 mL, 0.5 mM) and the specific test sample at increasing concentrations (0.0625; 0.125; 0.25; 0.5; and 1 mg/mL, final concentrations). This reaction mixture was incubated at 37 °C for 1 h.

Thereafter, BHT (0.5 mL, 0.5mg/mL in absolute EtOH) and TCA (1 mL, 25 %) was added and the vessel sealed and heated for 10 minutes in a boiling water bath. The samples were removed from the water-bath and cooled to 4 °C. The samples were centrifuged (20 minutes, 2000 × g) and the protein free supernatant (2 mL) was removed from each vessel. TBA (0.5 mL, 33 %) was added to each tube and heated for 1 h at 95 °C. Once cooled, the TBA-MDA complex was extracted with butanol (2 mL) and the absorbance determined at 532 nm. MDA concentrations were determined from a MDA standard curve (Appendix C). Final results were expressed as nano-moles MDA/mg tissue.

5.4.1.2. H_2O_2- Fe^{2+} induced LP

LP was also performed using H_2O_2 to determine metal-binding properties of the test compounds as a potential mechanism of reducing LP, serving also as a complementary method to the ferrozine assay.

H_2O_2 can be found in the cell and is often an intracellular signalling pathway messenger [Sziraki *et al.*, 1998]; however in the presence of Fe^{2+}, H_2O_2 is reduced to form the highly reactive hydroxyl ($OH^{\cdot}$) radical [Gutteridge *et al.*, 1982] in the Fenton reaction.

The reaction mixture contained rat liver homogenate (0.8 mL), H_2O_2 (0.1 mL, 2.6 mM), Fe^{2+} (0.1 mL, 0.25 mM), ascorbic acid (0.1 mL, 1.3 mM), EDTA (0.1 mL, 1.3 mM) and the specific test sample at increasing concentrations (0.05; 0.125; 0.25; 0.5; and 1 mg/mL, final concentrations). This reaction mixture was incubated at 37 °C for 1 h. Thereafter, BHT (0.5 mL, 0.5 mg/mL in absolute EtOH) and TCA (1 mL of 25 %) were added and the vessel sealed and heated for 10 minutes in a boiling water bath. The samples were removed from the water-bath, cooled to 4 °C and centrifuged (20 minutes, 2000 × g). The protein free supernatant (2 mL) was removed form each vessel, TBA (0.5 mL, 33 %) was added to each and heated 1 h, 95 °C). Once cooled the TBA-MDA complex was extracted with butanol (2 mL) and the absorbance read at 532 nm. MDA concentrations were determined from a MDA standard curve generated (Appendix C). Final results are expressed as nano-moles MDA/mg tissue.

5.5.2. Superoxide anion assay (NBT)

The superoxide anion assay (NBT) was performed according to Duncan and Ottino (1997), with slight modifications. This assay is commonly used to indicate the presence of superoxide, and used here to indicate the ability of test compounds to scavenge these radicals.

The NBT is reduced by the free radical to insoluble NBD, which is then extracted using glacial acetic acid and the concentration determined at 560 nm. NBD concentrations were determined from a NBD standard curve generated (Appendix C). Final results are expressed as nano-moles NBD/mg tissue. The change in absorbencies is due to the superoxide scavenging ability of the antioxidant [Halliwell, 1995].

The reaction solution consisted of rat liver homogenate (0.9 mL), KCN (0.1 mL, 15 mM), NBT (0.4 mL, 0.1 %) and the specific test sample at increasing concentrations (0.04; 0.08; 0.16; 0.33; and 0.67 mg/mL, final concentrations).

The reaction mixture was incubated at 37 °C for 1h and then centrifuged for 20 minutes (2000 × g). The pellet was re-suspended in glacial acetic acid (2 ml) and centrifuged (20 minutes, 2000 × g); the absorbance of the supernatant was read at 560 nm.

5.6. Results and Discussion

5.6.1 Lipid peroxidation (LP)

5.6.1.1. QA- Fe^{2+} induced LP

The addition of QA and Fe^{2+} induced LP that resulted in an overproduction of free radicals which oxidized polyunsaturated fatty acids. The complexation of QA and Fe^{2+} has been shown to initiate lipid peroxidation to a greater extent than QA alone [Stipek *et al.*, 1997]. This accounts for the increase in the MDA production. The addition of the test samples resulted in a decrease in the production of MDA.

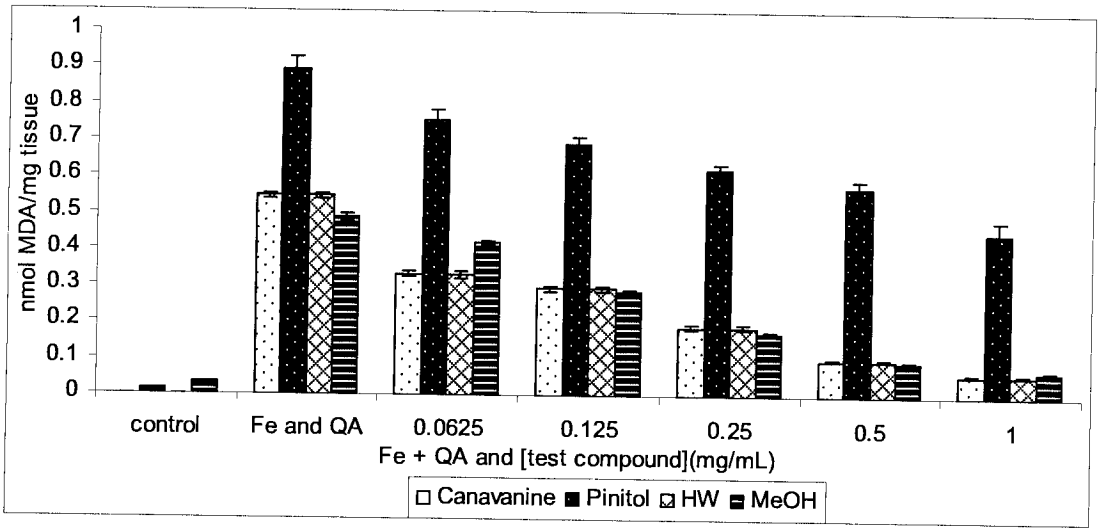

Figure 5.1: Effect of HW, MeOH extracts of *Sutherlandia frutescens* and pinitol and canavanine on QA and Fe^{2+} lipid peroxidation. Each bar represents the mean ± SE; n=3. The control contained only the rat liver homogenate.

The results (Figure 5.1) indicate that canavanine, HW extract and MeOH extract possess antioxidant properties as they reduce lipid peroxidation in a concentration dependant manner.

As the concentration of canavanine, HW and MeOH extracts of *Sutherlandia frutescens* increased LP was significantly reduced compared to the Fe^{2+} and QA only.

These compounds may be acting in two possible ways to reduce lipid peroxidation. Either, they directly scavenge the free radicals, or they compete with the QA for the Fe^{2+} ions and reduce the formation of the QA-Fe^{2+} complex which initiates lipid peroxidation.

Table 5.1: The statistical analysis of the effect of each compound on Fe^{2+} and QA induced lipid peroxidation of rat liver homogenate.

	Fe^{2+} and QA	0.0625mg/mL	0.125mg/mL	0.25mg/mL	0.5mg/mL	1mg/mL
Canavanine	#	***	***	***	***	***
Pinitol	#	***	ns	ns	ns	***
HW	#	***	***	***	***	***
MeOH	#	***	***	***	***	***

($p<0.001$) in comparison to the control; ns ($p>0.05$) and *** ($p<0.005$) compared to Fe^{2+} and QA.

The statistical data (Table 5.1) indicates that LP in the liver homogenate was significantly reduced by canavanine, the HW and MeOH extracts of *Sutherlandia frutescens*. The analysis of LP indicated that pinitol was not as effective at reducing the production of MDA compared to canavanine, the HW and MeOH extracts even though pinitol did decrease the production of MDA.

5.6.1.2. H_2O_2- Fe^{2+} induced LP

The $OH^{\cdot}$ free radical is the most reactive known biological free radical and has been shown to oxidize biological molecules in the immediate vicinity of its production site [Halliwell, 2006[b]]. Figure 5.2 shows the ability of all four test compounds to reduce MDA production. As the concentration of test sample was increased (Figure 5.2), the LP due to the Fenton reaction was reduced.

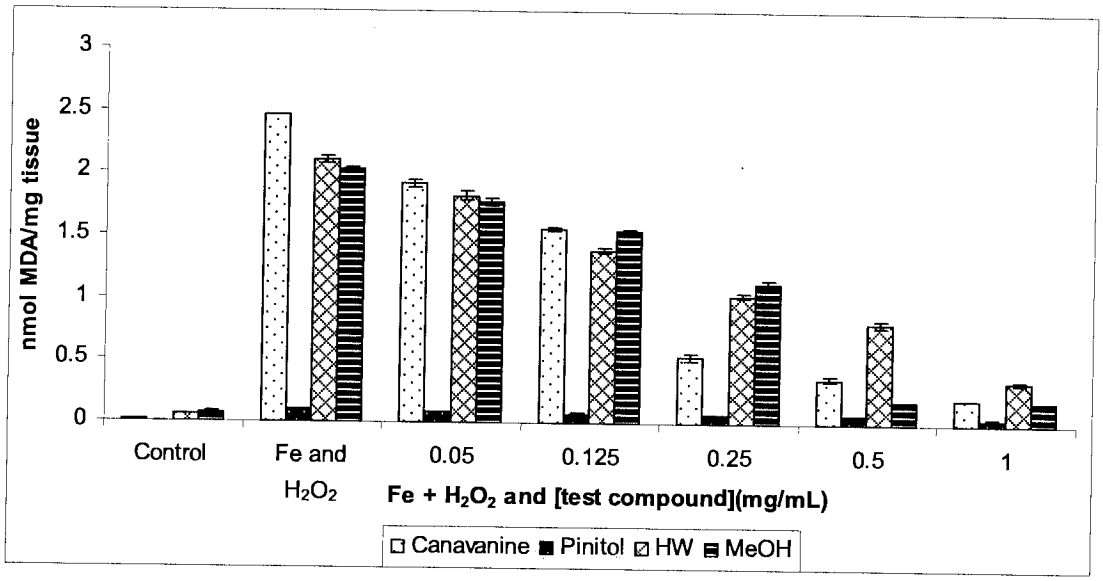

Figure 5.2: **Effect of hot water and methanolic extracts of SF, and of pinitol and canavanine on H_2O_2 and Fe^{2+} induced lipid peroxidation. Each bar represents the mean ± SE; n=3. The control contained only the rat liver homogenate.**

The test samples reduced LP either by binding the Fe^{2+} metal or quenching the $OH^{\cdot}$ radical. Canavanine and HW extract significantly reduced H_2O_2- Fe^{2+} induced LP at all concentrations shown (Table 5.2), indicating comparatively, that these two samples are either able to inhibit the $OH^{\cdot}$ radical or bind Fe^{2+} to a greater extent than the other test samples examined.

Table 5.2: **The statistical analysis of the effect of each compound on Fe^{2+} and H_2O_2 induced lipid peroxidation of rat liver homogenate.**

	Fe^{2+} and H_2O_2	0.05mg/mL	0.125mg/mL	0.25mg/mL	0.5mg/mL	1mg/mL
Canavanine	#	***	***	***	***	***
Pinitol	#	***	***	ns	***	***
HW	#	***	***	***	***	***
MeOH	#	***	***	***	***	ns

($p<0.001$) in comparison to the control; ns ($p>0.05$) and *** ($p<0.005$) compared to Fe^{2+} and QA.

5.6.2. Superoxide anion (NBT) assay

All the samples assessed for the NBT assay reduced the production of NBD in a concentration-dependent manner (Figure 5.3). The addition of KCN induced the generation of the superoxide anion and the addition of the test compound showed the scavenging ability of each of the samples as observed by the decrease in generation of NBD.

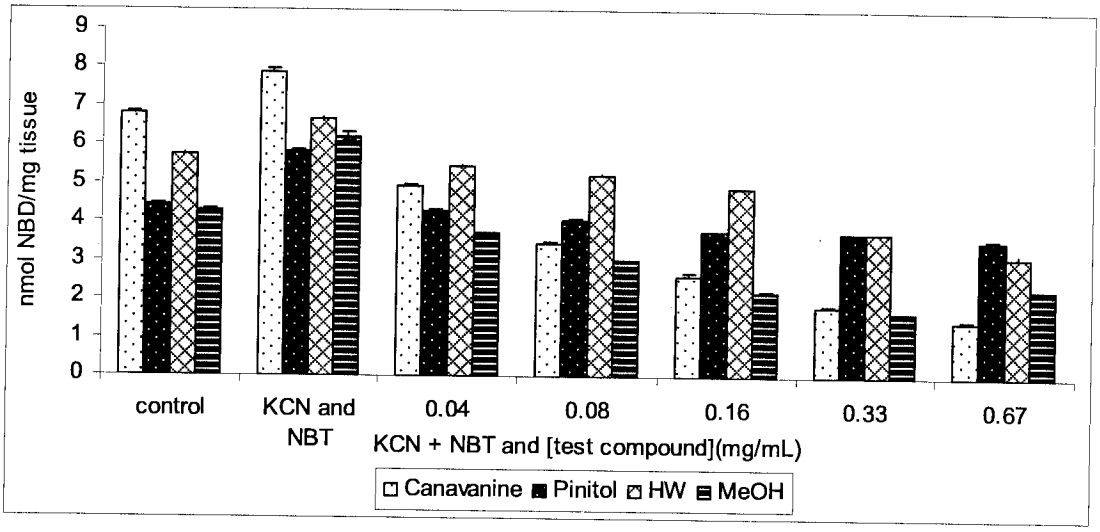

Figure 5.3: Comparison of superoxide scavenging ability of: pinitol, HW, MeOH extracts of *Sutherlandia frutescens* and canavanine.

Both extracts of *Sutherlandia frutescens* showed the ability to reduce the production of NBD with only the HW extract significantly decreasing the production of NBD at all concentrations, in contrast to the MeOH extract of *Sutherlandia frutescens* (Table 5.3). This may occur as the MeOH extract possesses lipophilic antioxidants that are not able to affect their optimum antioxidant potential in the aqueous medium [Suhaj, 2004].

Table 5.3: The statistical analysis of the ability of the test compounds to scavenge the O_2^- radical anion.

	KCN and NBT	0.04 mg/mL	0.08mg/mL	0.16mg/mL	0.33mg/mL	0.67mg/mL
Canavanine	#	***	***	***	***	***
Pinitol	#	***	***	***	ns	***
HW	#	***	***	***	***	***
MeOH	#	***	***	ns	***	***

($p<0.001$) in comparison to the control; ns ($p>0.05$) and *** ($p<0.005$) compared to KCN and NBT.

The MeOH extract of *Sutherlandia frutescens* reduced NBD production at all concentrations except at 0.16 mg/mL, however, at 0.67 mg/mL there was an increase in the absorbance reading of the assay. This may be due to the presence of compounds, such as anthocyanins, in the plant extract that have been shown to interact with NBD to produce an increased reading at higher concentrations [Hodges *et al.*, 1999].

Canavanine was shown to possess better superoxide scavenging abilities than pinitol. Canavanine possesses a number of amine groups that would enable it to scavenge the superoxide by donating an electron. The double bonds present would allow for the stabilisation of the canavanine radical which then forms. Canavanine may become a stable free radical due to the donation of a hydrogen ion or an electron.

Pinitol does, however, scavenge the superoxide. Carbohydrates, such as glucose and mannitol, scavenge the $OH^{\cdot}$ free radical indicating that compounds with carbohydrate structures are capable of scavenging free radicals [Blake *et al.*, 1987]. In this assay, too, canavanine and the HW extract were shown to perform better than the MeOH extract and pinitol (Table 5.3) as they significantly reduced NBD production at all concentrations.

5.7. Conclusions

Canavanine, the HW and MeOH extracts of *Sutherlandia frutescens* indicated the ability to significantly reduce ($p<0.005$) LP generated by Fe^{2+} and QA. These samples may act through a number of ways such as binding to Fe^{2+}, preventing Fe^{2+} from binding with QA to form a radical species or by scavenging the radical species formed or reacting with chain propagating radical to reduce continued H^{+} extraction from fatty acid side chains. Canavanine and the HW extract of *Sutherlandia frutescens* were the only samples that significantly reduced LP generated by the $OH^{\cdot}$ radical at all concentrations. The ability of samples to scavenge the superoxide anion is an important function of antioxidants as this would reduce the formation of other ROS. Canavanine and the HW extract of *Sutherlandia frutescens* were the only samples that significantly reduced the formation of NBD at all concentrations.

The electrochemical analysis indicated that the HW and MeOH extracts of *Sutherlandia frutescens* possessed potential antioxidant compounds that have the ability to donate electrons as an antioxidant mode of action. The Fe^{2+}-QA induced LP was reduced for canavanine and the extracts of *Sutherlandia frutescens* upon the addition of these samples to the reaction mixture indicating that these samples do possess the ability to scavenge radical species.

The DPPH and TEAC assays function either by electron donation, hydrogen donation or both. The electrochemistry characterisation of the extracts of *Sutherlandia frutescens* indicated the presence of compounds that are able to donate electrons. These radical assays are in agreement with the electrochemical analysis of the HW, MeOH and canavanine as they also show that these compounds possess the antioxidant property of electron donation.

The biological and non-biological assays indicate that the use of electrochemical methods to evaluate antioxidant compounds provides valuable information with regards to the total antioxidant capacity of crude samples and possible modes of action of antioxidant compounds.

CHAPTER 6: THE USE OF ELECTROCHEMISTRY AS A RAPID SCREENING TECHNIQUE FOR THE DETERMINATION OF ANTIOXIDANT COMPOUNDS IN MARINE ALGAE.

6.1. Introduction

The search for safe and effective naturally occurring antioxidants is now focused on edible plants, marine organisms, herbs and spices [Arabshahi *et al.*, 2007; Zhang *et al.*, 2007]. Almost all photosynthesising plants, including seaweeds, are exposed to both light and high oxygen concentrations that lead to the formation of free radical and other strong oxidising agents, but they seldom suffer from any serious photodynamic damage during metabolism [Masuda *et al.*, 1999]. This indicates the presence of some protective antioxidative mechanisms and compounds [Heo *et al.*, 2005]. Plants produce large quantities of antioxidants to combat free radical damage and are thus a potential source of novel antioxidant compounds [Mosquera *et al.*, 2007].

Algal biomass and algae-derived compounds have a wide range of potential applications, from animal feed and aqua-culture, to human nutrition and health products. Some algae are considered as rich sources of natural antioxidants [Li *et al.*, 2007]. Marine algae have been extensively researched and have been a valuable source of new secondary metabolites that have novel activity in biological systems [Takamatsu *et al.*, 2003].

6.2. Screening for natural antioxidant products

Bio-assays in a screening program should be rapid, simple, relevant, capable of automation, cost-effective and able to allow for high through-put. Appropriate technology should be used to enable low limits of detection. Natural product screens need to be buffered against extremes of pH and ionic strength and should not be affected by the presence of colour [Gad, 2005]. Mass screening methods have been replaced by molecular biology and modelling, followed by computer-aided molecular design. First generation biotechnology use methods for drug design, such as proteins, receptors and antibodies, to determine structures and activities of new drugs. Second generation biotechnology combines molecular biology with sensor technology for drug discovery [Taylor and Schultz, 1996].

Electrochemical methods and parameters can be used for biomedical chemistry as they provide vast amounts of information about the mechanism of biological electron-transfer processes [de Abreu *et al.*, 2002]. As there is a large number of plant species potentially available for study [Katalinic *et al.*, 2006], it is vital to have effective systems for rapid chemical and biological screening of the plant extracts selected [Hostettman, 1998]. The use of electro-analysis in biomedical chemistry is advantageous as it is a relatively clean chemical system, easy to control, is not affected by turbid solutions of analytes and can be used to analyse radical species in organic or aqueous solvents [Buratti *et al.*, 2001, de Abreu *et al.*, 2002].

Discovery of compounds that possess medicinal properties is essential for the development and commercialisation of new pharmaceuticals, providing the basis for new drugs and new therapies [CIBA Foundation Symposium, 1990]. The basic advantages of electrochemical sensors used in analysis are high specificity and sensitivity, portability, real-time output, cost-effectiveness and user friendliness. [Taylor and Schultz, 1996].

6.3 Overall objective

Utilise electrochemical methods to screen batch samples of algal extracts for antioxidant properties

6.4. Aims

1. Use electrochemical techniques (Chapter 3) to screen a large number of algal extracts to evaluate the use of electrochemistry for the determination of antioxidant compounds.
2. Compare results of electrochemistry, DPPH and FC assays as methods for antioxidant determination.

6.5. Materials and Methods

One hundred and seven concentrated solvent extracts of algae were obtained from, Dr D.R. Beukes, of the Rhodes University, Faculty of Pharmacy, Pharmaceutical Chemistry. A blind study was performed on these samples where initially no information was provided about the marine algae. The samples were labelled RU 1 - RU 107.

As different masses of the various extracts were provided, the screening protocol was preformed on all the samples at 1 mg/mL. Stock solutions of each sample were made by dissolving the sample in 1 mL of absolute EtOH. Thereafter, a working solution of 1 mg/mL was prepared from the stock solution. The working solution consisted of H_2O:EtOH at a ratio of 1:1.

The samples were analysed using voltammetry, the DPPH and FC assays. The electrochemistry was performed (section 3.5.2) using the electrochemical methods of SWV and CV *vs.* Ag/AgCl reference electrode and an auxiliary Pt wire in PBS (0.1 M; pH 7.4). The samples that produced current responses from SWV analysis were further analysed by CV to determine if the compounds produced electrochemically reversible peaks. The samples that produced OP below 0.5 V were selected. The DPPH assay has been used to screen for potential antioxidants in marine algae [Marxen *et al.*, 2007]. The DPPH assay was performed (section 4.3.1) and only samples that inhibited the DPPH radical above 50 % at 1 mg/mL were deemed to possess sufficient antioxidant potential. Likewise, using the FC assay (section 4.3.4) only compounds that possessed a GAE greater than 0.05 mg/ 1 mg were selected.

6.6. Results and Discussion

Thirteen samples yielded positive results of the three assays: voltammetry, DPPH and FC assay (shaded blue in Table 6.1).

There were 31 samples that produced peaks in the positive anodic potential range (Table 6.1), as indicated by the samples with fraction codes in bold (shaded yellow green and blue). Twelve of these samples possessed compounds that produced peaks in the negative potential range. Eighteen of these 31 samples possess more than one compound that was electro-active and a potential antioxidant. As for chapter 3, the cut-off potential was assigned to a value of 0.5 V. No samples were excluded as all generated at least one anodic peak below 0.5 V.

Twenty one samples were able to reduce the DPPH radical greater than 50 % (shaded blue and green). Ten of the samples which generated electro-active compounds did not yield positive results for DPPH nor FC (shaded yellow).

This may be due to the reaction kinetics, or that the antioxidant compound was more hydrophilic than lipophilic. It has been shown that hydrophilic antioxidants are not as effective in organic solvents and the DPPH radical was prepared in methanol [Prior *et al.*, 2005]. Eight samples yielded positive results for both the DPPH assay and the electrochemical analysis but not for the FC assay (shaded green). This may indicate the presence of non-phenolic antioxidants.

There were 16 samples that possessed a phenolic content of at least 0.05 mg/mL of sample. Thirteen of these 16 samples were also detected by electrochemical analysis and DPPH (shaded blue). This is a good indication of the link between electrochemistry and the antioxidant properties of phenolic compounds. Four of the samples only generated positive results for the FC assay (shaded purple). This may indicate the presence of non-antioxidant phenolics.

The results indicated that 29 % of the algal extracts possessed compounds that were electro-active, while 20 % of the algal extracts were able to scavenge the DPPH radical (> 50 %) at 1 mg/mL. The FC assay indicated that 15 % of the algal extracts possessed GAE greater than 0.05 mg/1 mg.

SWV was able to detect compounds that were readily oxidizable and at low concentrations (Appendix D1). CV analysis of these compounds (Appendix D2) indicated that 19 of the 31 samples that were detected with SWV analysis possessed compounds that produced electrochemically reversible peaks (Table 6.2). This is an indication of the ability of compounds present in these samples to accept and donate electrons. As there are numerous phytochemicals present that may be electro-active, there was no definitive way of proving that the samples which produced current responses possessed known antioxidants.

The FC assay was used to determine the total phenolic content of each sample. There was a clear relation between the samples that were shown to possess higher phenolic content and the electro-analysis. This can be attributed to the structure of the phenolic compounds that allows them to readily donate electrons and be resonance stabilised. There have been studies relating phenolic compounds to antioxidant function [Hagerman *et al.*, 1998].

Table 6.1: Screening of various solvent extracts of different algae using electrochemistry, DPPH and the FC assay.

Vial	Collection code	Fraction code	Potential (V)	DPPH analysis : % Inhibition					FC assay: GAE	
				1mg/mL	0.5mg/mL	0.25mg/mL	0.125mg/mL	0.0625mg/mL	1mg/mL	2mg/mL
RU 1	KB 06 – 3	AA1-7a		4.35	3.91	2.91	3.32	2.61	0.02	0.04
RU 2		AA1-7b		6.66	5.11	4.42	3.17	3.44	0.02	0.05
RU 3		AA1-7c		3.04	3.01	2.86	2.77	2.23	0.01	0.02
RU 4	KB 06 – 8	**AA1-37a**	**0.29**	3.21	1.90	1.62	1.34	1.56	0.02	0.04
RU 5		AA1-37b		1.92	2.46	1.40	1.86	0.65	0.02	0.04
RU 6		AA1-37c		0.45	1.75	1.95	1.78	0.60	0.02	0.02
RU 7		**AA1-37d**	**0.19**	2.85	2.42	1.84	0.80	0.65	0.02	0.02
RU 8		AA1-37e		3.32	2.87	2.19	2.74	1.43	0.02	0.03
RU 9	KB 06 – 9	**AA1-39a**	**0.22**	3.98	3.20	1.60	0.20	0.11	0.02	0.03
RU 10		**AA1-39b**	**0.23**	**89.18**	**54.84**	**27.38**	**6.44**	**2.53**	**0.05**	**0.11**
RU 11		**AA1-39c**	**0.07; 0.23**	**90.24**	**77.10**	**53.23**	**22.87**	**8.37**	**0.07**	**0.16**
RU 12		**AA1-39d**	**0.07; 0.24; 0.71**	**92.15**	**92.28**	**75.16**	**46.25**	**22.18**	**0.12**	**0.22**
RU 14	KB 06 – 5	**AA1-40a**	**0.18**	6.65	2.79	2.01	0.86	0.20	0.02	0.05
RU 15		**AA1-40b**	**0.19**	3.10	2.46	1.55	1.31	1.09	0.02	0.04
RU 16		AA1-40c		2.25	2.89	2.09	0.75	0.32	0.02	0.02
RU 17		**AA1-40d**	**-0.04; 0.06; 0.39**	**85.37**	**69.92**	**55.07**	**41.23**	**24.57**	**0.16**	**0.29**
RU 18		**AA1-40e**	**-0.05; 0.24; 0.55**	**66.13**	**43.42**	**30.59**	**14.61**	**6.60**	**0.08**	**0.14**
RU 19	KB 06 – 4	AA1-41a		8.28	8.46	1.37	0.56	0.45	0.03	0.04
RU 20		AA1-41b		9.44	9.20	5.56	1.85	1.74	0.02	0.02
RU 21		AA1-41c		9.88	7.74	6.32	1.78	0.26	0.02	0.03
RU 22		AA1-41d		6.49	4.15	2.50	2.46	0.77	0.02	0.02
RU 23		AA1-41e		7.24	2.42	2.91	1.14	0.63	0.03	0.04

Electrochemically only
Electrochemically and DPPH
FC assay only
Electrochemically, DPPH and FC assay

Vial	Collection code	Fraction code	Potential (V)	DPPH analysis : % Inhibition					FC assay: GAE	
				1mg/mL	0.5mg/mL	0.25mg/mL	0.125mg/mL	0.0625mg/mL	1mg/mL	2mg/mL
RU 24	NDK 06 – 6	AA1-42a		3.51	2.24	1.24	1.33	1.31	0.02	0.07
RU 25		AA1-42b		2.62	1.81	1.38	0.38	0.28	0.03	0.04
RU 26		AA1-42c		1.97	1.59	0.54	0.06	0.06	0.02	0.02
RU 27		**AA1-42d**	**0.24**	32.74	17.58	7.10	2.84	2.67	0.04	0.07
RU 28		AA1-42e		13.54	5.12	4.96	3.13	0.98	0.03	0.04
RU 29	NDK 06 – 9b	AA1-43a		3.53	3.33	3.29	1.97	1.05	0.03	0.05
RU 30		AA1-43b		4.76	3.73	3.26	2.14	1.83	0.03	0.05
RU 31		AA1-43c		3.63	1.53	0.84	0.61	0.38	0.02	0.03
RU 32		AA1-43d		4.43	2.93	1.92	1.16	0.75	0.02	0.03
RU 33		AA1-43e		7.78	7.37	4.69	1.49	1.27	0.04	0.06
RU 34	NDK 06 – 5	**AA1-44a**	**-0.078; 0.31**	**69.62**	**38.35**	**19.39**	**7.94**	**1.50**	0.04	0.06
RU 35		**AA1-44b**	**-0.074; 0.29**	**87.72**	**86.49**	**81.68**	**68.37**	**57.79**	**0.10**	**0.23**
RU 36		**AA1-44c**	**-0.079; 0.29**	**91.25**	**74.93**	**48.51**	**26.51**	**14.79**	0.03	0.03
RU 37		**AA1-44d**	**-0.079;0.39;0.49**	**92.46**	**93.36**	**93.00**	**92.73**	**86.77**	**0.18**	**0.34**
RU 38		**AA1-44e**	**-0.074; 0.30**	**95.08**	**94.72**	**87.64**	**50.33**	**28.53**	-	-
RU 39	NDK 06 – 10	**AA1-45c**	**-0.043**	10.46	6.44	2.86	1.93	0.35	0.02	0.02
RU 40		AA1-45d		40.33	33.73	32.19	32.09	1.11	0.03	0.04
RU 41		AA1-45e		28.39	23.94	22.39	21.49	20.53	0.04	0.06
RU 42	NDK 06 – 1b	AA1-46b		4.89	3.83	2.99	1.06	-0.88	0.03	0.04
RU 43		AA1-46c		6.09	4.75	2.46	2.00	1.30	0.04	0.06
RU 44		AA1-46d		3.72	2.72	2.45	2.35	2.14	0.02	0.02
RU 45		AA1-46e		7.28	5.58	3.81	3.70	3.12	0.05	0.07

Electrochemically only
Electrochemically and DPPH
FC assay only
Electrochemically, DPPH and FC assay

Vial	Collection code	Fraction code	Potential (V)	DPPH analysis : % Inhibition					FC assay: GAE	
				1mg/mL	0.5mg/mL	0.25mg/mL	0.125mg/mL	0.0625mg/mL	1mg/mL	2mg/mL
RU 48	NDK 06 – 19	**AA1-47a**	**0.21; 0.39**	**61.91**	**43.95**	**42.58**	**31.60**	**19.85**	**0.05**	**0.08**
RU 49		**AA1-47b**	**0.21; 0.36**	**91.15**	**89.26**	**69.42**	**42.71**	**21.62**	0.01	0.01
RU 50		**AA1-47c**	**0.21; 0.36**	**72.68**	**93.25**	**92.93**	**92.62**	**91.51**	0.02	0.02
RU 51		**AA1-47d**	**0.08; 0.24; 0.39**	**93.23**	**93.07**	**92.16**	**91.59**	**62.27**	0.04	0.04
RU 52		**AA1-47e**	**0.22; 0.35**	**93.33**	**92.78**	**92.17**	**90.63**	**76.16**	0.03	0.04
RU 53	TS 06 – 14	AA1-66a		3.08	3.53	2.052	1.24		0.02	0.03
RU 54		AA1-66b		6.81	4.62	2.76	1.39	0.48	0.03	0.05
RU 55		AA1-66c		15.77	4.35	3.45	1.99	0.53		
RU 56		AA1-66d		3.87	3.39	1.92	1.20	0.45	0.04	0.06
RU 57		**AA1-66e**	**-0.08**	3.69	3.46	2.57	0.99	0.71	0.03	0.04
RU 58	TS 06 – 11	AA1-72a		2.89	2.63	1.74	1.38	1.18		
RU 59		AA1-72b		4.07	2.92	2.37	2.25	1.70	0.02	0.02
RU 60		AA1-72c		4.58	2.23	1.46	1.36	0.93	0.02	0.03
RU 61		AA1-72d		7.77	4.35	2.71	2.18	0.94	0.04	0.06
RU 62		AA1-72e		1.84	1.13	1.01	0.71	0.71	0.03	0.05
RU 63	TS 06 - 10	**AA1-75a**		18.91	6.93	2.41	1.53	0.59	**0.06**	**0.11**
RU 64		AA1-75b		17.32	15.56	13.02	10.83	4.05	0.04	0.08
RU 65		AA1-75c		3.43	3.38	2.52	1.77	0.23	0.02	0.03
RU 66		**AA1-75d**	**-0.02; 0.48**	**92.89**	**91.22**	**90.22**	**73.50**	**54.28**	**0.29**	**0.56**
RU 67		**AA1-75e**		48.52	34.85	20.29	13.26	8.18	**0.06**	**0.12**
RU 70	TS 06-09	A		3.26	1.68	0.84	0.29	0.29	0.03	0.04
RU 71		B		4.46	3.79	3.41	1.63	0.77	0.03	0.04
RU 72		C		3.74	2.79	2.65	2.04	0.19	0.02	0.02
RU 73		D		3.33	1.98	0.68	0.43	0.19	0.03	0.07

Electrochemically only	Electrochemically and DPPH
FC assay only	Electrochemically, DPPH and FC assay

Vial	Collection code	Fraction code	Potential (V)	DPPH analysis : % Inhibition					FC assay: GAE	
				1mg/mL	0.5mg/mL	0.25mg/mL	0.125mg/mL	0.0625mg/mL	1mg/mL	2mg/mL
RU 74	KOS06-1	CRUDE		19.74	12.45	7.52	5.31	3.34	**0.05**	**0.09**
RU 75		D		9.39	8.89	7.69	3.82	3.13	0.04	0.06
RU 76	KOS06-13b	B		3.08	2.89	2.71	2.25	0.87	0.04	0.07
RU 77		C		1.40	1.57	1.18	0.56	0.28		
RU 78		D		4.22	3.29	3.02	2.47	2.14	0.02	0.03
RU 79	NDK06-6&11	CRUDE		2.38	2.28	1.79	1.36	1.14	0.03	0.04
RU 80	KOS06-15	**A**	**0.082; 0.21**	24.69	14.24	8.90	5.81	3.09	0.03	0.06
RU 81		**CRUDE**	**0.067; 0.20; 0.34**	**90.22**	**75.82**	**41.07**	**20.19**	**10.28**	**0.05**	**0.13**
RU 82	KOS06-17	CRUDE								
RU 83	KOS06-2	A		10.84	5.78	3.62	3.54	2.09	0.02	0.05
RU 84		B		5.55	3.52	1.34	0.94	0.29	0.03	0.05
RU 85		C		3.25	2.75	2.14	2.07	1.57	0.03	0.03
RU 86	KOS06-20	A		10.62	4.30	3.94	1.85	1.06	0.04	0.05
RU 87		B		4.59	2.73	2.29	1.55	1.42	0.03	0.04
RU 88		D								
RU 89		C		4.35	3.98	1.91	1.68	1.12	0.03	0.05
RU 90	TS06-07	A		4.04	2.00	1.74	0.85	0.09	0.04	0.04
RU 91		B		2.49	2.45	1.99	1.38	1.23	0.04	0.07
RU 92		C		10.08	9.02	6.38	4.30	1.13	**0.07**	**0.12**
RU 93		D		3.56	2.09	1.65	1.24	1.01	0.03	0.05

Electrochemically only	Electrochemically and DPPH
FC assay only	Electrochemically, DPPH and FC assay

Vial	Collection code	Fraction code	Potential (V)	DPPH analysis : % Inhibition					FC assay: GAE	
				1mg/mL	0.5mg/mL	0.25mg/mL	0.125mg/mL	0.0625mg/mL	1mg/mL	2mg/mL
RU 94	KOS06-3	A		7.70	6.43	4.94	3.93	3.10	0.03	0.07
RU 95		B		8.12	4.56	2.57	0.43	0.22	0.04	0.06
RU 96		C		0.82	0.72	0.61	0.61	0.12	0.01	0.02
RU 97		D		8.82	4.26	2.96	2.46	1.96	0.03	0.03
RU 98	KOS06-16	**A**	**-0.069**	**62.21**	**29.32**	**20.49**	**10.29**	**7.93**	0.03	0.08
RU 99		**B**	**-0.044**	**74.49**	**85.19**	**62.42**	**30.98**	**13.93**	**0.11**	**0.19**
RU 100		**C**	**0.04; 0.49; 0.66**	**91.73**	**92.22**	**92.22**	**51.69**	**2.86**	**0.20**	**0.35**
RU 101	KOS06-6	C		3.84	2.62	2.38	1.12	0.99	0.02	0.02
RU 102		**D**	**0.26**	17.09	14.27	13.79	11.11	10.79	0.03	0.04
RU 103	KOS06-15	**B**	**0.088; 0.22; 0.36**	**91.51**	**90.51**	**57.26**	**28.34**	**15.48**	**0.09**	**0.16**
RU 104	KOS06-23	CRUDE		2.85	2.71	2.28	2.04	0.34	0.02	0.02
RU 105	KOS06-11	CRUDE		10.62	7.39	4.05	3.79	2.69	0.03	0.05
RU 106	KOS06-6	CRUDE		9.01	5.68	3.64	2.15	0.37	0.03	0.26
RU 107	KOS06-3	CRUDE		3.75	2.86	2.76	2.09	0.51	0.02	0.03

Electrochemically only
Electrochemically and DPPH
FC assay only
Electrochemically, DPPH and FC assay

The 31 samples (green, blue, red and black dots) that produced peaks in the positive anodic potential range are shown in Figure 6.1. Some produced peaks in the negative range, indicated by black dots, which was an indication of potentially very potent antioxidant compounds. A clear correlation between the samples that are electro-active and those that inhibit the DPPH radical above 50 % was observed (Figure 6.1). All samples that scavenged the DPPH radical were electro-active.

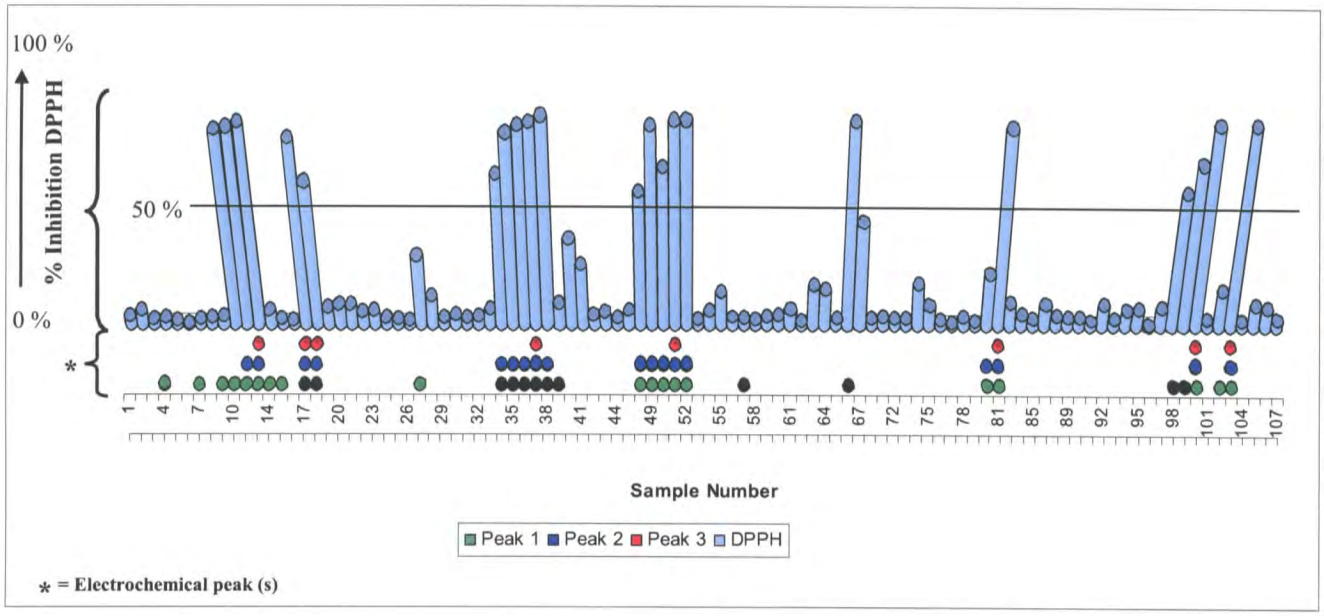

Figure 6.1: A graphical comparison of the DPPH results and electrochemical analysis.

Some of the solvent extracts in this study that exhibited electrochemical properties were not able to scavenge the DPPH radical. The DPPH method has limitations that are associated with the fact that it is a stable radical species. This suggests that it may not be as effective as abstracting electrons or hydrogen ions from antioxidant compounds as ROS found in biological systems. The DPPH radical may react slowly or not at all with antioxidants that would react rapidly with ROS in biological systems [Huang *et al.*, 2005]. Steric interference may also reduce or inhibit the interaction of antioxidants with the DPPH radical [Prior *et al.*, 2005].

There have been numerous health benefits attributed to phenolic compounds which are often consumed through the diet [Huang *et al.*, 2005]. These benefits have been associated with the antioxidant properties of phenolic compounds [Balasundram *et al.*, 2006]. The FC assay allowed for the determination of the total phenolic content of the algal samples and the comparison of these results with the results obtained from the electrochemical analysis (Figure 6.2) indicate that there is a link between the electro-active properties of the algal extracts and the total phenolic content.

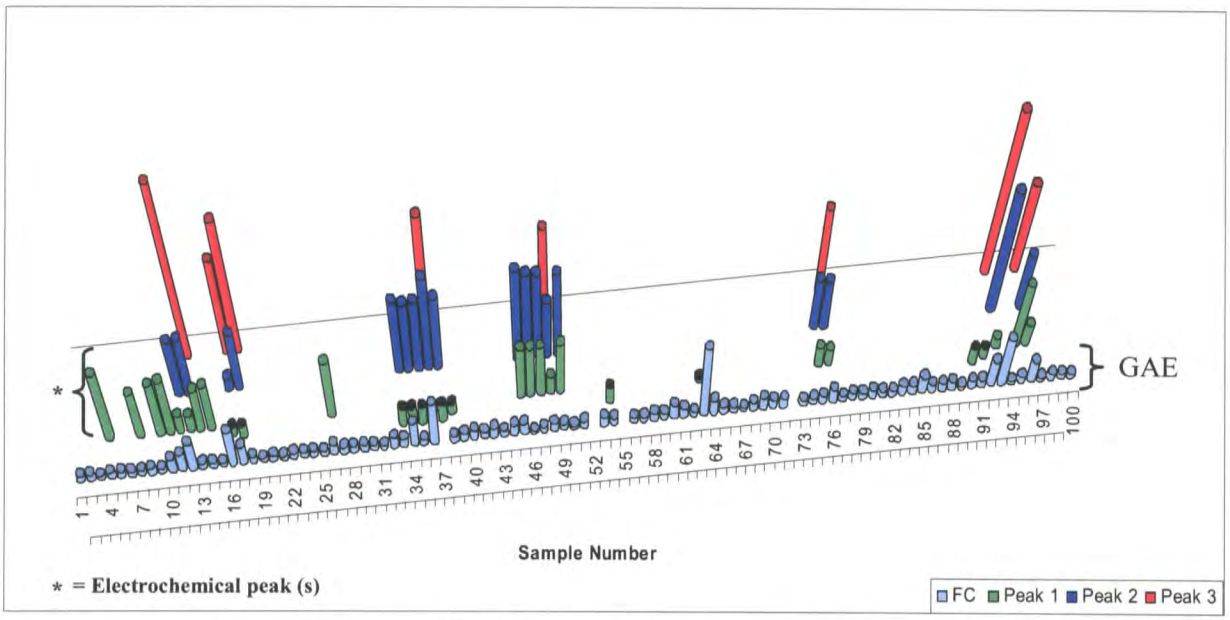

Figure 6.2: A representation of the algal solvent that possess high total phenolic content and those that produced electrochemical responses.

The FC assay, in combination with the DPPH assay, is often used to determine the reducing ability of extracts. Electron transfer (ET) reactions are generally slow, which results in the antioxidant capacity being determined by percentage decrease rather than kinetics [Prior *et al.*, 2005]. However, the FC assay is non-specific and numerous compounds that are not phenolic have been shown to reduce the FC reagent. Another disadvantage is that not all phenolic compounds possess antioxidant properties.

Table 6.2 is an indication of the AUC produced by the electrochemically active samples. The area under the peak is directly proportional to the concentration of the antioxidants [Chevion *et al.*, 2000].

As biological samples may contain more than one compound with antioxidant properties which may have the same potential, the area under the anodic peak may be more pertinent than the peak height for the determination of antioxidant capacity [Chevion *et al.*, 2000].

Table 6.2: AUC for algal samples that provided electrochemical responses with SWV analysis.

		AUC (SWV) (μC)		
Sample number	**Reversible**	**1**	**2**	**3**
RU 4	No	0.00527		
RU 7	No	0.00228		
RU 9	No	0.0165		
RU 10	Yes	0.262		
RU 11	Yes	0.00223	0.405	
RU 12	Yes	0.00224	0.579	0.00319
RU 14	No	0.000393		
RU 15	No	0.000049		
RU 17	No	0.0187	0.00152	0.0186
RU 18	No	0.00115	0.00544	0.0000615
RU 27	Yes	0.0558		
RU 34	Yes	0.0946	0.00703	
RU 35	Yes	0.425	0.00846	
RU 36	Yes	0.0185	0.0657	
RU 37	Yes	0.312	0.000083	0.000846
RU 38	Yes	0.47	0.015	
RU 39	No	0.0172		
RU 48	Yes	0.11	0.000572	
RU 49	Yes	0.409	0.0393	
RU 50	**Yes**	**6.57**	**0.0188**	
RU 51	**Yes**	**0.0082**	**5.9**	**0.012**
RU 52	**Yes**	**3.24**	**0.0511**	
RU 57	No	0.00187		
RU 66	No	0.043	0.00472	
RU 80	No	0.00517	0.0455	
RU 81	Yes	0.00742	0.643	0.0326
RU 98	Yes	0.0986		
RU 99	Yes	0.588		
RU 100	Yes	0.58	0.0172	0.00995
RU 102	No	0.00138		
RU 103	Yes	0.00403	0.0317	0.0182

The samples RU 50 – RU 52 (bold) were shown to possess the largest AUC of all the samples that contained electro-active compounds at the same concentration. Thus, this suggests that samples contained the highest concentration of potential antioxidant compounds. This is another advantage of electro-analysis.

Table 6.3 provides a list of the species names provided for all samples which were electro-active and potential antioxidants. All the extracts of *Sargassum heterophyllum* were shown to possess compounds that were electro-active. Extracts of *Polysiphonia incompta* were also shown to possess the highest concentration of electro-active compounds.

Table 6.3: Collection and fraction code with the species name of algal samples that were detected using electrochemical methods.

Sample number	Fraction code	*Species name*
RU 4	AA1-37a	*Caulacanthus ustulatus*
RU 7	AA1-37d	
RU 9	AA1-39a	*Pterosiphonia cloiophylla*
RU 10	AA1-39b	
RU 11	AA1-39c	
RU 12	AA1-39d	
RU 14	AA1-40a	*Bifurcaria brassicaformis*
RU 15	AA1-40b	
RU 17	AA1-40e	
RU 18	AA1-41a	
RU 27	AA1-42d	*Laurencia flexuosa*
RU 34	AA1-44a	*Sargassum heterophyllum*
RU 35	AA1-44b	
RU 36	AA1-44c	
RU 37	AA1-44d	
RU 38	AA1-44e	
RU 39	AA1-45c	*Gelidium capense*
RU 48	AA1-47a	*Polysiphonia incompta*
RU 49	AA1-47b	
RU 50	AA1-47c	
RU 51	AA1-47d	
RU 52	AA1-47e	
RU 57	AA1-66e	*Gelidium pristoides*
RU 66	AA1-75d	Unknown
RU 80	CRUDE	*Polysiphonia incompta*
RU 81	CRUDE	
RU 98	A	*Sargassum heterophyllum*
RU 99	B	
RU 100	C	
RU 102	D	*Laurencia flexuosa*
RU 103	B	*Polysiphonia incompta (with some Ceramium sp.)*

Species of *Chlorophyta* (green algae), *Phaeophyta* (brown algae) and *Rhodophyta* (red algae) have been analysed and have been shown to possess antioxidant compounds [Zubia *et al.*, 2007]. Marine algae have been used as food sources and for medicinal purposes in Eastern cultures. Extensive research has shown extracts of *Gelidium amansii*, *Gloiosiphonia capillaris*, *Polysiphonia urceolata*, *Sargassum kjellmanianum*, *Desmarestia viridis*, and *Rhodomela tere* have been shown to possess strong antioxidant properties [Yan *et al.*, 1998]. *Sargassum spp.* have been shown to possess significant antioxidant properties [Anggadiredja *et al.*, 1997; Heo *et al.*, 2005]. Extracts of the marine algae of the genus *Polysiphonia* have been analysed for antioxidant properties using the DPPH assay and have shown very good scavenging ability of the DPPH radical [Duan *et al.*, 2006].

6.7. Conclusions

The electrochemical methods of SWV and CV, in combination, can be used as preliminary screening tools for a large sample size of crude extracts. The use of SWV enabled the rapid, sensitive screening of a large sample size for antioxidant compounds. The low oxidation potentials for the ten samples which exhibited electro-active properties but did not yield positive results for DPPH nor FC assay (shaded yellow) may thus indicate a) that they possess electron donating abilities but may not be effective antioxidants given that they were unable to scavenge the DPPH radical; b) that they are indeed antioxidants but may not be effective electron donors in lipophilic systems (a shortcoming of the DPPH assay). These compounds may not be biological electron donors and thus may not be detectable by the DPPH assay. If the latter is considered then the data suggests that electrochemical methods may be more reliable indicators of the presence of possible electron donating antioxidants than the DPPH assay. The FC assay was able to detect compounds that were not electro-active; however, this may be due to the FC reagent being non-specific and interferences that result in false results.

There were more extracts of the *Sargassum spp.* that possessed electro-active compounds than any of the other algal species. This indicates that the *Sargassum spp.* may possess a variety of compounds that can be determined electrochemically and thus requires a more in-depth study.

CHAPTER 7: ELECTROCHEMICALLY GUIDED ISOLATION OF ANTIOXIDANT METABOLITES FROM *SARGASSUM ELEGANS*

7.1 Introduction

Natural products obtained from plants contribute to new drug discovery as they may be modified to produce new drugs and/or are used to determine new pharmacological action [CIBA Foundation Symposium, 1990]. In the process of drug development from plants, once the crude extract has been identified to possess valuable biological properties, a bioassay must be developed to determine which chemical fraction possesses the biological activity, followed by a purification step(s) which is ideally optimized until the active compound(s) is determined [Acamovic and Brooker, 2005]. The classical process leading from the plant to a pharmacologically active, pure, constituent has always been a long and tedious process requiring substantial human and financial resources [Vuorela *et al.*, 2004].

7.2. Current methods of isolating natural metabolites.

A natural product isolation protocol normally incorporates the following steps. Solvent extraction is performed to remove compounds that may possess valuable properties. The crude extract is then partitioned using organic solvents that allow for the separation of compounds based on their solubility in these solvents. A separation step involving the use of column chromatography allows for the fractionation of the solvent partitions. The fractions that are shown to possess interesting or novel activities are then purified using high performance liquid chromatography (HPLC). HPLC is the most widely used technique for the separation of complex mixtures [Pukalskas *et al.*, 2005].

The main components of an HPLC system are a high pressure pump, a column, an injection system and a detector. The system works as follows: the eluent is filtered and pumped through a chromatographic column and the eluent is monitored using a detector and recorded as 'peaks' [Hamilton and Sewell, 1977]. HPLC with a UV photo-diode array detector has been widely used for analysis of plant extracts as the UV spectrum provides information about the type of constituents such as polyphenols [Gad, 2005].

7.3. Structural characterization of Natural products

Hyphenated techniques such as LC-MS and LC-NMR allow for the determination of individual compounds before they have been totally purified. HPLC with nuclear magnetic resonance (NMR) is important for structure evaluation of natural products [Tsao and Deng, 2004]. LC-NMR is one of the most powerful of the hyphenated techniques used by phytochemists but still suffers from a lack of sensitivity [Macomber, 1998; Exarchou *et al.*, 2005[b]].

7.4. Marine algae as a source of antioxidant metabolites.

Natural resources, from aquatic to terrestrial, are gaining focus as sources of potential pharmaceutical applications as numerous plants are used as traditional remedies [Keusgen, 2002]. Screening of natural products can provide a greater structural diversity than standard synthetic chemistry on its own, and offers significant opportunities for finding novel low molecular weight lead compounds. Marine micro-fauna and micro-flora provide a large diversity of secondary metabolites [Gad, 2005]. Seaweeds or their extracts have been studied as novel sources of antioxidants and other compounds that have been shown to possess biological and medicinal value [Heo *et al.*, 2005]. Natural products from marine products provides for a continuous source of novel compounds such as antioxidants due to the biodiversity of algae [Li *et al.*, 2007].

Marine algae have been shown to produce numerous compounds that have potential as medicines due to the biological activity they possess [Anggadiredja *et al.*, 1997]. Numerous algal species including *Sargassum* species have been used as alternative sources of food and food ingredients [Plaza *et al.*, 2007]. The *Sargassum* species have been shown to be a source of antioxidants [Lim *et al.*, 2002]. This may be attributed to the high phenolic content present in the extracts [Cho *et al.*, 2007]. The genus *Sargassum* has been shown to be present in tropical and sub-tropical regions around the world. There have been intensive studies performed on a mixed *Sargassum* species population at Reunion Rocks, KwaZulu-Natal, South Africa including *Sargassum elegans* and other *Sargassum spp.*, of which *Sargassum elegans* made up the majority of the population [Gillespie and Critchley, 1999].

The traditional methods for natural product analysis in general includes fractionation of a crude extract, separation and isolation of the individual components using a range of available chromatographic methods and structure determination using various spectroscopic methods (UV, IR, NMR, and MS). Although these methods are time-consuming and costly, they are still extensively used for natural product analysis [Clarkson *et al.*, 2006]. There exists scope for the evaluation of alternative rapid screening methods for guiding the isolation of potential antioxidants from crude fractions.

7.5. Overall Objective

The overall objective of this study was to evaluate electrochemical methods to guide the isolation of antioxidant compounds and then to elucidate the structure of these compounds with NMR analysis.

The extracts of *Sargassum spp.* were shown to possess the highest activity in electrochemical screening, DPPH assay and the FC assay (Table 6.3). Thus, the electrochemical methods were examined to direct the isolation of compounds present in *Sargassum elegans*.

7.6. Aims

1. Acquire solvent partitions of *Sargassum elegans* and perform electrochemistry on these partitions to determine which partitions to focus on for further purification.
2. Fractionate the partitions that are shown to possess electro-active compounds using silica gel chromatography.
3. Analyse these fractions using electrochemical methods to determine which fractions contain electro-active compounds that may be antioxidants.
4. Isolate the compounds in these fractions that are electro-active, using semi-preparatory HPLC.
5. Voltammetrically analyse the isolated compounds to determine which one of the compounds imparts possible antioxidant properties.
6. Elucidate the structure of these compounds using NMR.

7.7. Materials and Methods

7.7.1. Plant material

Samples of *Sargassum elegans* (NDK07-1b) were collected at low tide from Noordhoek near Port Elizabeth and identified by comparison with voucher specimens in the Faculty of Pharmacy.

7.7.2. Equipment

Compounds were purified using a Spectra-Physics IsoChrom LC HPLC system, which consisted of a rheodyne injector, a Waters R401 differential refractometer, and a Rikadenki chart recorder. Normal phase chromatography was utilized with a Whatman Magnum 10 Partisil 9 column. The ^{1}H (400 MHz), ^{13}C (100 MHz), NMR spectra were all determined using a Bruker Avance 400 NMR spectrometer. Spectra were referenced to residual protonated solvent resonances ($CHCl_3$ δ_H 7.25, δ_C 77.0). The electro-analytical procedures were performed using a Potentiostat/Galvanostat 30 (PGSTAT 30) from Autolab using the electrochemical cell (section 3.3.2).

7.7.3 Extraction and solvent partitioning of Sargassum elegans (NDK07-1b)

Table 7.1 summaries the methodological approach. The frozen *Sargassum elegans* was submerged in MeOH to reduce the water content (1 h, 4 °C). The MeOH was decanted and a DCM:MeOH (2:1) solution was used to perform the extractions. The solution was heated (30 min, 40 °C) and the supernatant decanted. The temperature of the solution was monitored such that it did not rise above 40 °C, as above this temperature active compounds may degrade. This was performed three times and the supernatant was concentrated to yield the crude extract.

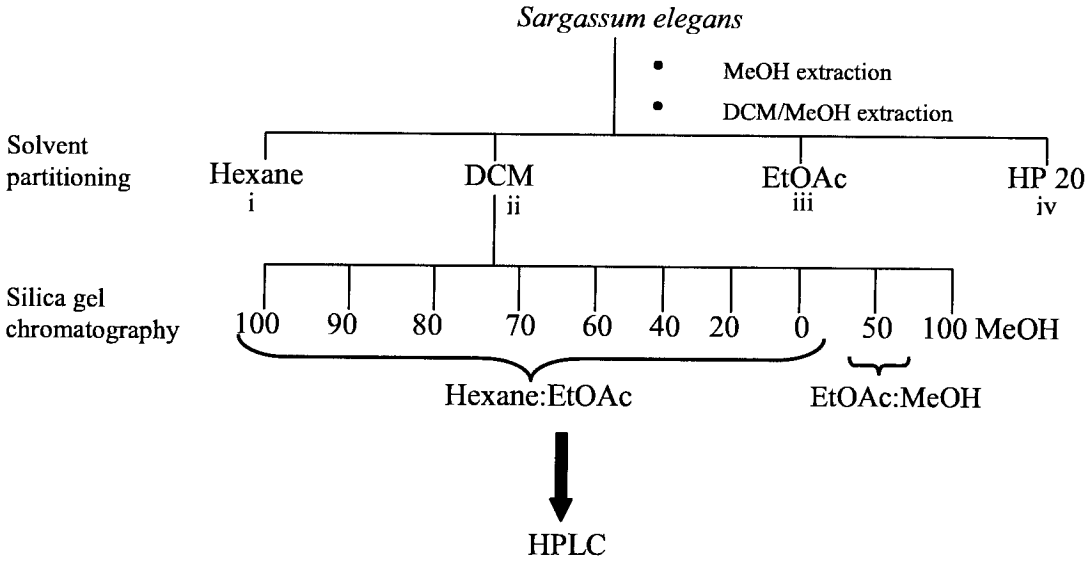

Figure 7.1: **Schematic of the purification process of the partitions and fractions of *Sargassum elegans*.**

(i) Hexane partition

The concentrated sample was re-constituted in MeOH (90 mL). The remaining sample was re-suspended with hexane (100 mL) and the hexane mixture was added to the separating funnel. Thereafter, H_2O (10 mL) was added to the MeOH/Hexane mixture, which causes the solvents to separate as hexane is not miscible in water. The aqueous layer was removed and the organic layer was collected. The extraction procedure was performed three times on the aqueous layer with hexane. The hexane portion was concentrated to yield the hexane partition (1497.4 mg).

(ii) DCM partition

DCM (100 mL) was added to the aqueous portion that remained from the hexane extraction. Thereafter, H_2O (30 mL) was added and the organic layer was collected. The extraction was performed on the aqueous portion three times. The organic layer was dried to yield the DCM partition (2737 mg).

(iii) EtOAc partition

EtOAc (100 mL) was added to the aqueous portion that remained from the DCM extraction, and the organic layer was collected. Thereafter, the extraction was performed three times using EtOAc (50 mL). The organic layer was dried to yield the EtOAc partition (120.7 mg).

The remaining aqueous portion was added to the aqueous portion from the MeOH extraction to yield an aqueous partition for further partitioning.

(iv) Aqueous (HP 20) partition

The aqueous partition was passed through a column consisting of HP 20 resin. The aqueous partition was eluted using EtOAc and then concentrated to yield the aqueous partition (145.8 mg).

Labels assigned to the partitions of *Sargassum elegans*:

NDK -07 -1b (A) → Hexane partition

NDK -07 -1b (B) → DCM partition

NDK -07 -1b (C) → EtOAc partition

NDK -07 -1b (D) → Aqueous partition

Electro-analysis of each of the partitions using SWV and CV as described in section 3.3.2 was performed.

7.7.4. Silica gel Column Chromatography

Each partition was re-constituted in DCM. This mixture was mixed with silica beads (Merck 7734 Kieselgel 60 silica, 70-230 mesh) to form a slurry. The solvent was removed to bind the partition to the beads. Silica beads (Merck 9385 Kiesgel 60 silica, 230-440 mesh) were mixed with hexane to form a slurry. This was packed into a column. Thereafter, the silica beads with the bound partition were stacked onto the fine silica beads. The column was eluted with a mixture of hexane and EtOAc (75 mL) at various ratios (Figure 7.1, the number value indicates % hexane). After the final wash the column was eluted with a EtOAc:MeOH (50:50) solution.

A final wash was performed with MeOH (100 %). The supernatant was collected and dried to produce the different fractions. This procedure was followed for each partition. Electrochemistry was performed on each of the fractions using SWV and CV as described in section 3.3.2.

7.7.5. HPLC of the 60 % DCM partition

The 60 % fraction of the DCM partition was used for HPLC. The mobile phase used was Hexane:EtOAc (40:60). The partitions and fractions were stored at 4 °C in a dessicator to reduce the degradation of the compounds present. Electrochemistry was performed on each of the HPLC fractions using SWV and CV as described in section 3.3.2.

7.8. Results and Discussion

7.8.1. Electro-analysis of solvent partitions of Sargassum elegans

SWV analysis of the DCM, hexane, EtOAc and aqueous partition indicated that all except the aqueous partition contained electro-active compounds (Figure 7.2). Table 7.1 summarises the anodic potentials and half wave potentials obtained for each partition.

Table 7.1: SWV analysis of the partitions (90 µg/mL) of *Sargassum elegans*.

Sample Code	Potential (V)	$E_{1/2}$ (V)	AUC (µC)
A (Hexane)	-0.09	-0.13	0.63
	0.3	0.25	0.07
B (DCM)	0.01	-0.05	1.24
C (EtOAc)	-0.02	-0.035	0.04
	0.1	0.05	0.54
	0.49	0.4	0.93
D (Aqua)	No peak		

The hexane, DCM and EtOAc partitions show the presence of compounds with OP that are close to zero and below 0 V *vs* Ag/AgCl. Thus, these partitions suggest the presence of compounds that have the properties of potent antioxidants.

The CV scans (Figure 7.3) indicated that the compounds in the DCM partition were reversible while those present in the hexane partition were not, even though they both possessed similar anodic potentials. CV of the EtOAc partition (Figure 7.3C) also suggested the presence of several electro-active compounds. These were largely irreversible except for the redox wave at potentials below 0 V. The aqueous partition indicated the presence of a low concentration of electrochemically reversible compounds as evidenced by a low AUC value compared to the other partitions.

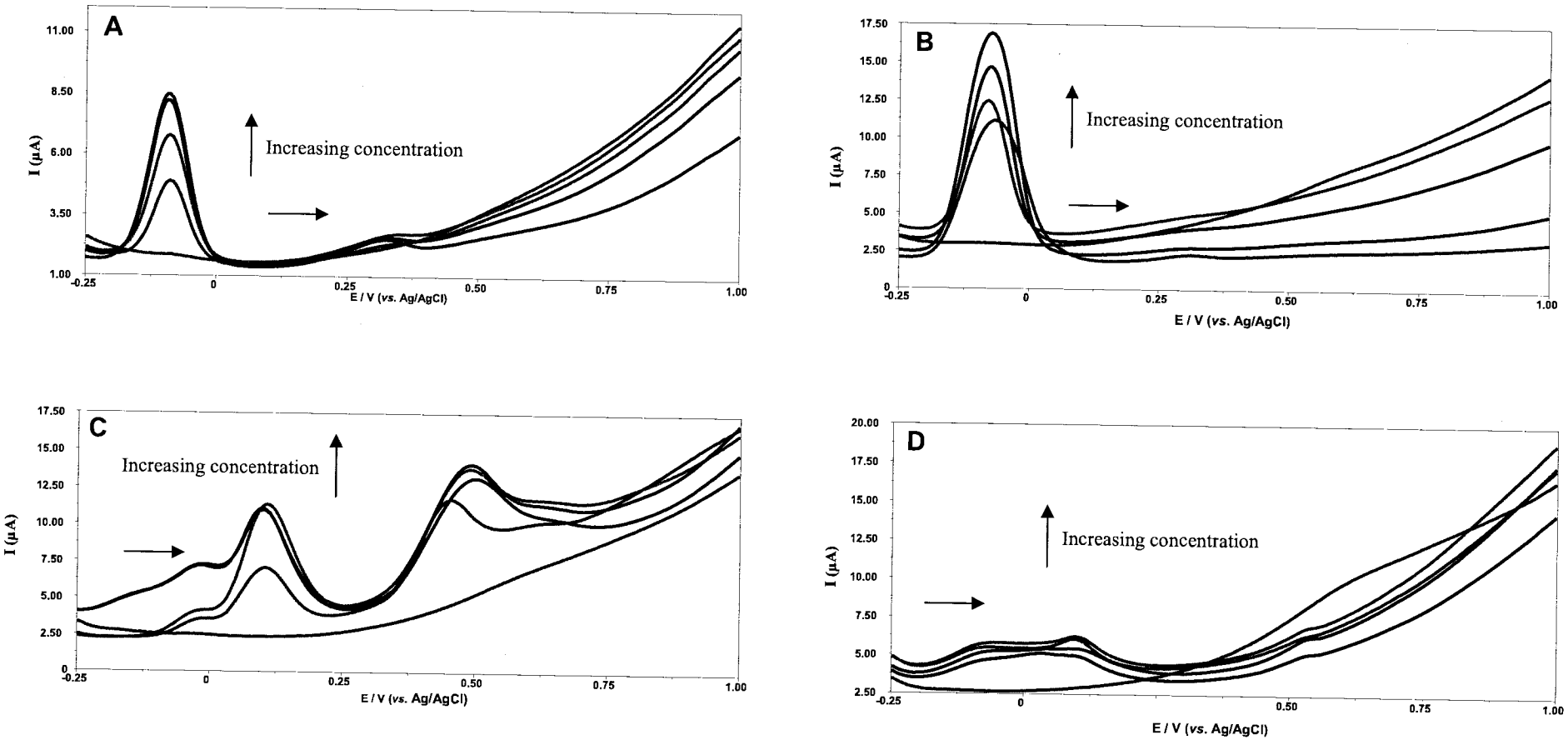

Figure 7.2: **SWV scans of the (A) Hexane, (B) DCM, (C) EtOAc and (D) aqueous partitions of *Sargassum elegans* (0-90 µg/mL).**

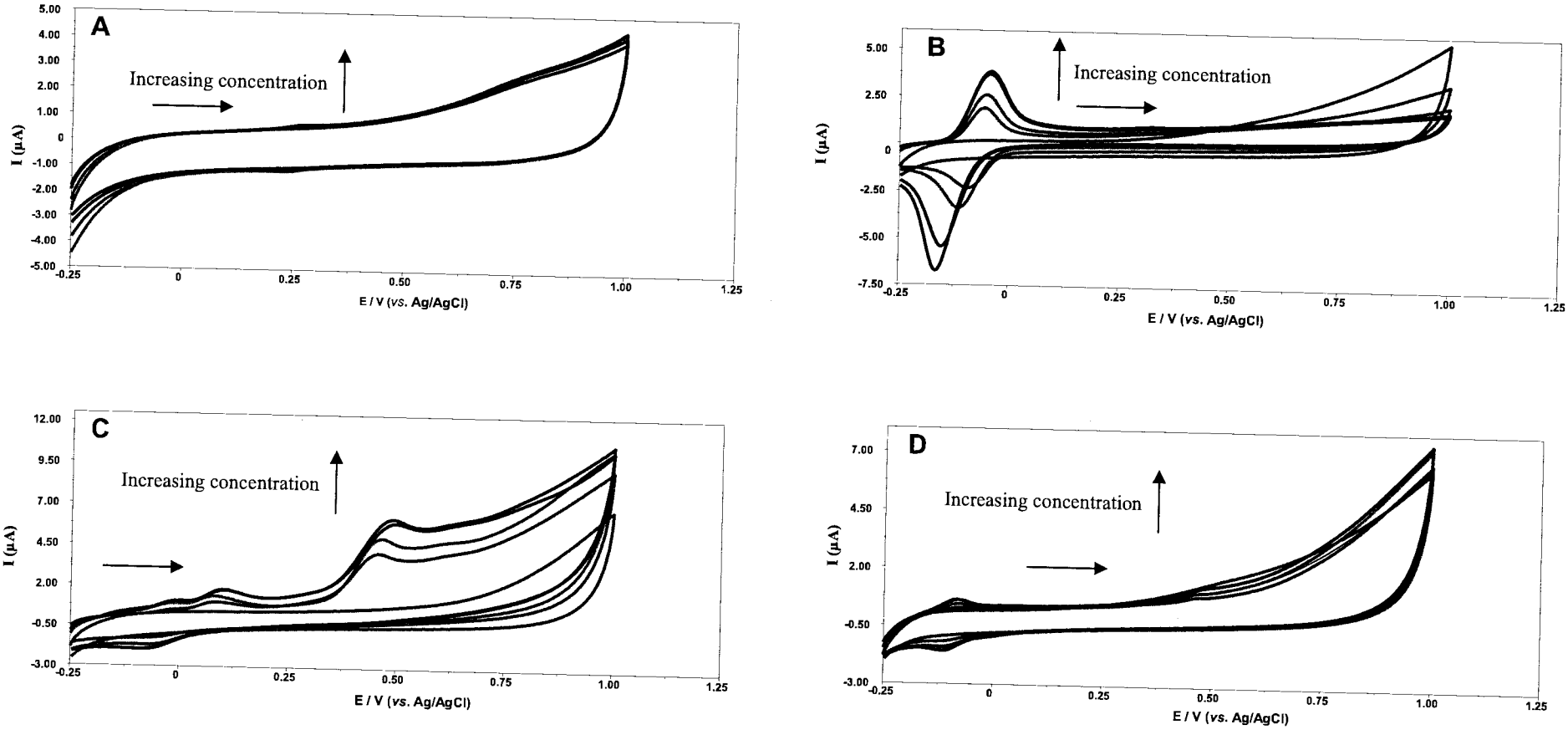

Figure 7.3: **CV scans of (A) Hexane, (B) DCM, (C) EtOAc and (D) aqueous partitions of *Sargassum elegans* (0-90 µg/mL).**

The DCM, hexane and EtOAc partitions were then fractionated to further isolate the electro-active compounds (Schematic 7.1). The fractions that possessed the greatest AUC indicated the highest concentration of electro-active compounds. The fractions that possessed electro-active compounds were purified further.

7.8.2. Electrochemistry of fractions of DCM, hexane and EtOAc partitions of Sargassum elegans.

Three (green, red and blue) of the nine hexane fractions contained compounds that were electro-active (Figure 7.4) as shown by the anodic waves in SWV analysis of these fractions. The current responses produced by the hexane *fractions* were lower than that of the hexane *partition*.

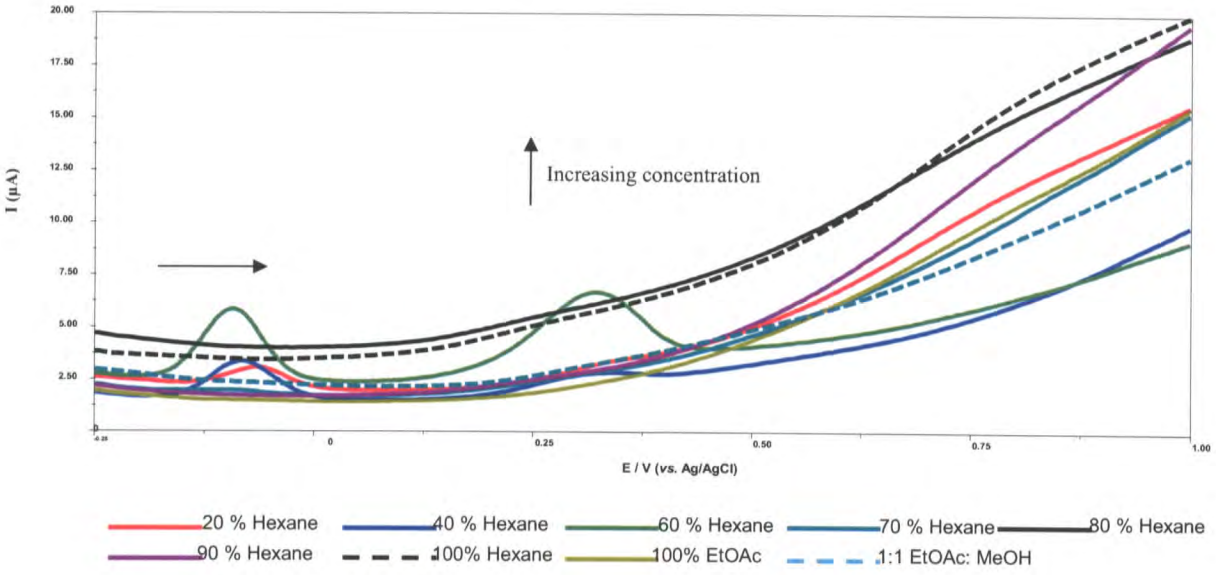

Figure 7.1: SWV scans of the different fractions of the hexane partition (90 µg/mL).

As illustrated in Figure 7.5, there was a decrease in the AUC of peak 1 (5.63 µC) of the hexane partition (**i**) when compared to that of the AUC of peak 1 (2.76 µC) of the 60 % hexane fraction. There was however, an increase in the AUC of peak 2 (4.57 µC) of the 60 % fraction (**ii**) when compared to peak 2 (0.09 µC) of the hexane partition.

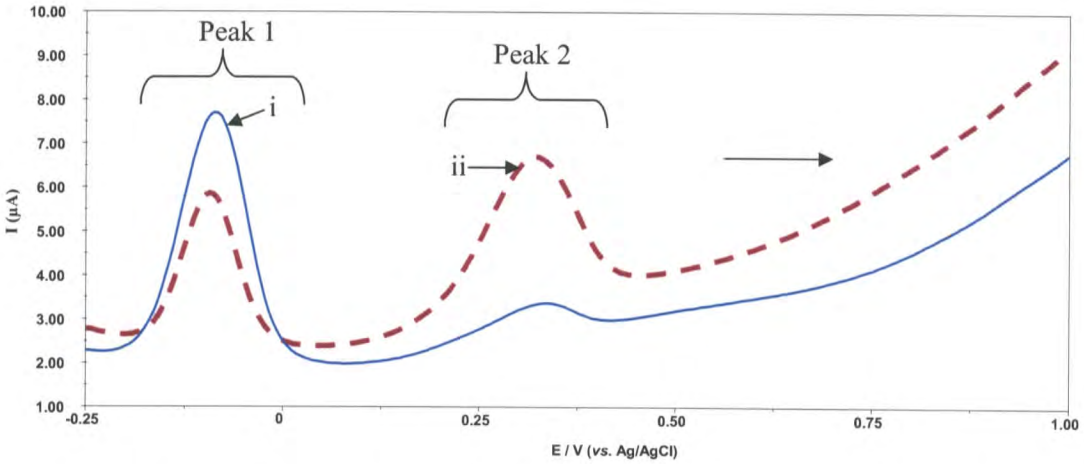

Figure 7.2: Comparison of the SWV of hexane partition (i) and 60 % hexane fraction (ii) (90 µg/mL).

This indicates that there may be degradation of the compound (s) that forms peak 1 in the hexane partition to form the compound (s) that produces peak 2 in the 60 % fraction.

Five (green, blue, cyan, red and brown) of the nine DCM fractions contained compounds that were electro-active (Figure 7.6).

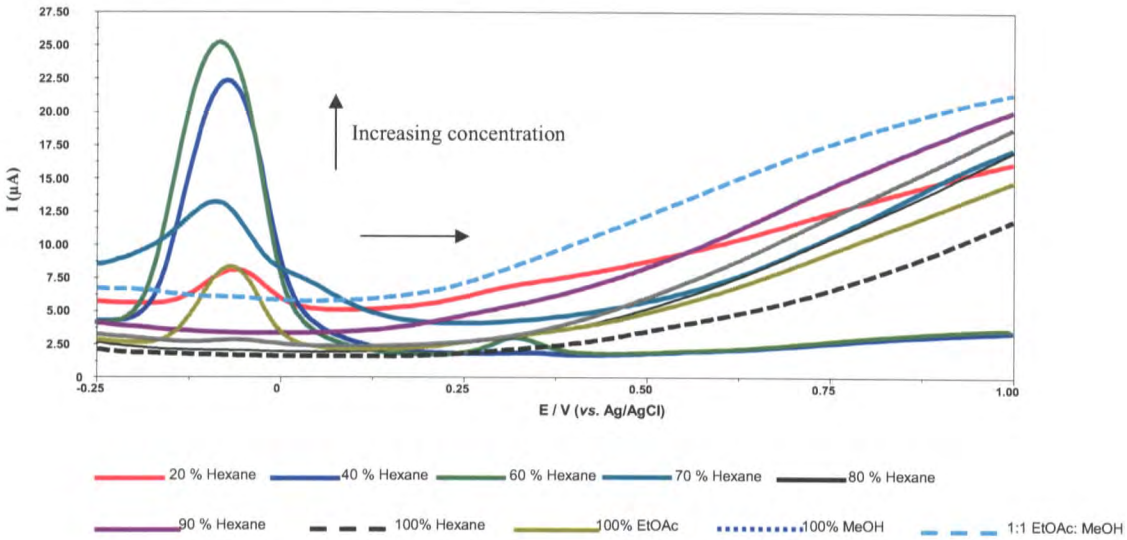

Figure 7.3: SWV scans of the different fractions of the DCM partition (90 µg/mL).

When compared to the DCM partition, (Figure 7.7), the electro-active compounds in the 40 % and 60 % fractions produced a greater AUC, indicating a high concentration of electro-active compounds from the DCM partition. This can be attributed to a concentration effect as a result of the purification process. Thus, the 60 % fraction was selected for further purification. The 60 % DCM fraction also showed the presence of a second anodic peak that may have occurred due to the degradation of the compound (s) that produced peak **i**.

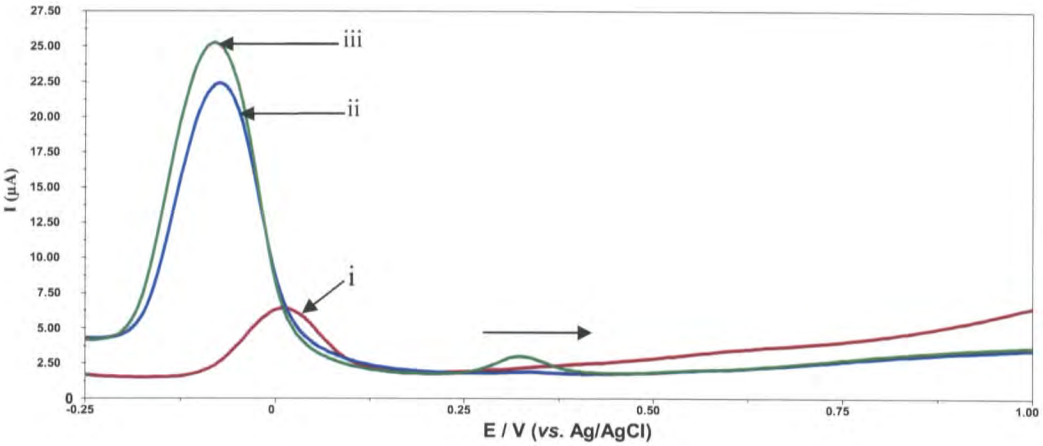

Figure 7.4: Comparison of the (i) DCM partition, the (ii) 40 % DCM fraction and the (iii) 60 % DCM fraction (90 µg/mL).

The anodic potential indicated that a shift in potential towards lower potentials occurred as the electro-active compounds of the DCM partition were purified; when compared to the 40 % and 60 % fractions (Figure 7.7). The broad peak of the DCM partition may be due to all electro-active compounds in the partition. These compounds may interfere with each other through co-adsorption effects resulting in the lowered AUC value. As the fractions are purified the electro-activity of the substituents present may become enhanced.

Only two (red and brown) of the EtOAc fractions contained compounds that were electro-active but these produced low AUC, indicating low concentrations of these compounds (Figure 7.8) when compared to the EtOAc partition.

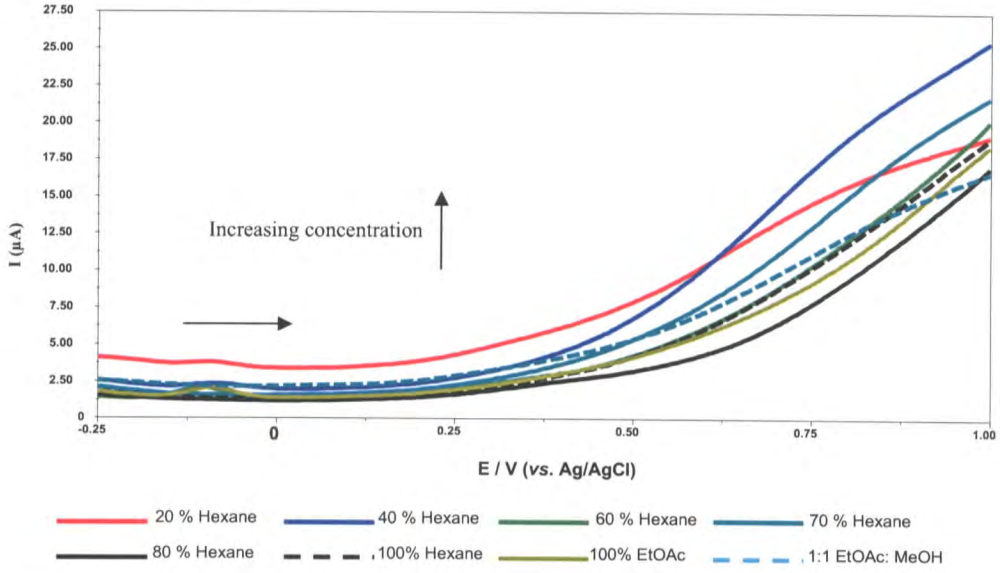

Figure 7.5: SWV scans of the different fractions of the EtOAc partition (90 µg/mL).

The compounds present in the EtOAc partition (Figure 7.2C) may have degraded over time due to their high reactivity. Thus, by utilising electro-analysis, the partitions with electro-activity can be fractionated sooner and potentially analysed prior to degradation to elucidate the compounds that possess antioxidant activity.

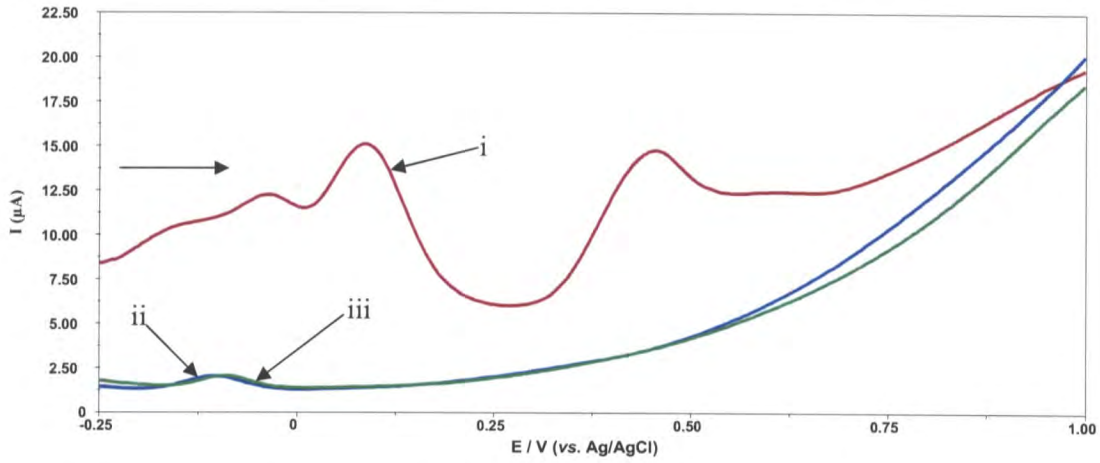

Figure 7.6: Comparison of the EtOAc partition (i), the 60 % EtOAc fraction (ii) and the 100 % EtOAc fraction (iii) (90 µg/mL).

As the EtOAc fractions produced low AUC, indicating a low concentration of electro-active compounds (Figure 7.9) when compared to the AUC of the DCM and hexane fractions, these were therefore not considered for further analysis.

7.8.3. HPLC isolation of electro-active compounds of the 60 % DCM fraction

The 60 % DCM fraction produced the highest AUC of all the fractions and was used for semi-preparatory HPLC analysis. The HPLC chromatograph of the 60 % DCM fraction (Figure 7.10) produced four distinct peaks. These peaks indicated the presence of four individual compounds and these compounds were isolated and analysed electrochemically.

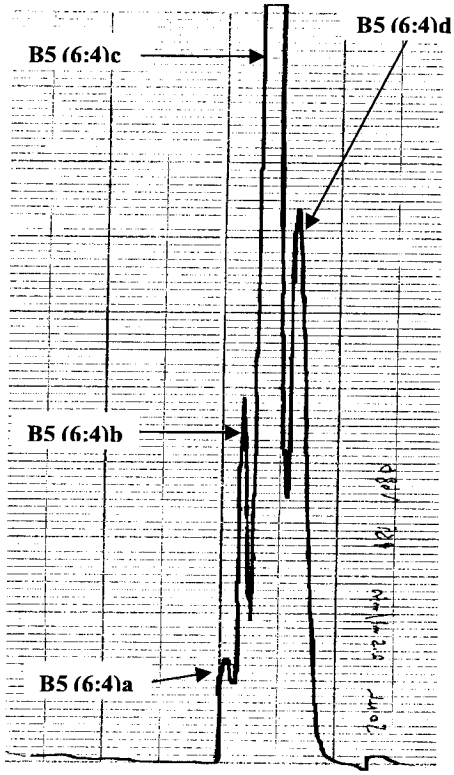

Figure 7.10: HPLC chromatograph of the 60% DCM fraction.

The individual compounds of the HPLC analysis (Figure 7.10) were collected and concentrated. These were made up to a concentration of 1 mg/mL and assayed using SWV. Three of the pure compounds from the HPLC analysis were shown to produce anodic waves that were within the range of the anodic waves observed for the SWV of the 60 % DCM fraction (between -0.25 V and 0.175 V) (Figure 7.11). The oxidation potentials and AUC are summarised in Scheme 7.1.

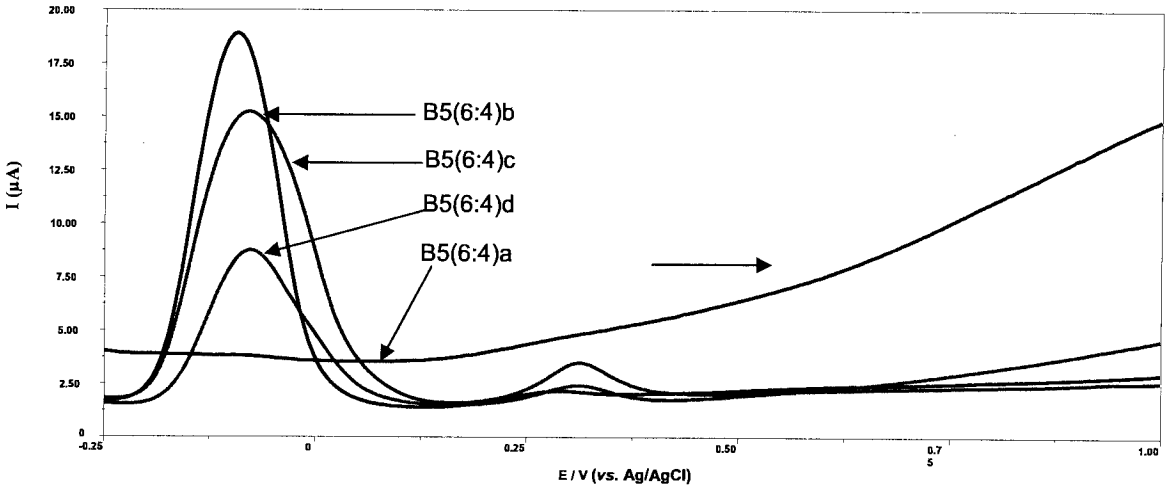

Figure 7.11: SWV scans of isolated peaks from HPLC of 60% fraction of the DCM partition (90 µg/mL).

The compound present in B5(6:4)b produced the largest AUC indicating this had the largest concentration of possible antioxidant compounds.

Sea algae, in particular brown seaweed, have been shown to be a rich source of bioactive compounds such as alginates, fucans and laminarins [Heo et al., 2005]. These compounds are also known to be potent antioxidant compounds [Ahn *et al.*, 2004].

Scheme 7.1 summarises the $E_{1/2}$ (biological oxidation potential), the OP and the AUC obtained for the partitions, fractions and pure compounds.

Sargassum elegans

Solvent partitioning

Sample Code	$E_{1/2}$(V)	Potential (V)	AUC (µC)
A (Hexane)	-0.14	-0.09	0.63
	0.25	0.3	0.07
B (DCM)	-0.05	0.01	1.24
C (EtOAc)	-0.05	-0.02	0.04
	0.06	0.1	0.54
	0.4	0.49	0.93
D (Aqua)		No peak	

Silica column chromatography

Sample Code	$E_{1/2}$(V)	Potential(E)	AUC(µC)
A1 (10H:0E)		No peak	
A2 (9:1)		No peak	
A3 (8:2)		No peak	
A4 (7:3)		No peak	
A5 (6:4)	-0.13	-0.09	0.28
	0.23	0.3	0.46
A6 (4:6)	-0.12	-0.09	0.15
	0.25	0.3	0.00
A7 (2:8)	-0.11	-0.09	0.07
A8 (0:10)		No peak	
A9 (1E:1M)		No peak	

Sample Code	$E_{1/2}$(V)	Potential(E)	AUC(µC)
B1 (10H:0E)		No peak	
B2 (9:1)		No peak	
B3 (8:2)		No peak	
B4 (7:3)	-0.15	-0.1	0.59
B5 (6:4)	-0.14	-0.08	2.84
	0.28	0.33	0.10
B6 (4:6)	-0.14	-0.08	2.40
B7 (2:8)	-0.11	-0.08	0.27
B8 (0:10)	-0.11	-0.08	0.57
B9 (1E:1M)		No peak	

Sample Code	$E_{1/2}$(V)	Potential(E)	AUC (µC)
C1 (10H:0E)		No peak	
C2 (9:1)		No peak	
C3 (8:2)		No peak	
C4 (7:3)		No peak	
C5 (6:4)	-0.15	-0.1	0.06
C6 (4:6)		No peak	
C7 (2:8)		No peak	
C8 (0:10)	-0.13	-0.1	0.04
C9 (1E:1M)		No peak	

HPLC Chromatography

Sample Code	$E_{1/2}$ (V)	Potential(E)	AUC (µC)
B5(6:4)a		No peak	
B5(6:4)b	**-0.15**	**-0.09**	**1.99**
B5(6:4)c	**-0.15**	**-0.09**	**2.06**
	0.27	0.31	0.178
B5(6:4)d	**-0.14**	**-0.09**	**0.974**
	0.26	0.31	0.0771

Schematic 7.1: Summary of the $E_{1/2}$, oxidation potentials and AUC of the partitions and individual fractions after each stage of purification.

7.8.4. Structure elucidation of isolated compounds

The structures of four individual compounds were elucidated during NMR (section 7.6.4). The ^{1}H NMR data (Figures 7.12 – 7.15) are identical to those previously reported for sargaquinoic acid [Blunt *et al.*, 2007], sargahydroquinoic acid [Faulkner, 1987], sargaquinal [Buckingham, 1993] and fucoxanthin [Sies and Stahl, 1995], respectively. SWV has been used as an analytical tool for the determination of quinone structured compounds [Rizk *et al.*, 2000; Trindade *et al.*, 2007].

Quinone compounds have been previously determined using electrochemical methods [Rich, 2004]. These compounds have the ability to donate and accept electrons which allow for their electrochemical detection [Budnikov *et al.*, 2004]. The structure of quinones enables the delocalisation of electrons over the entire molecule after the abstraction of an electron by a ROS [de Abreu *et al.*, 2002]. Sargaquinoic acid and sargahydroquinoic acid have previously been isolated from *Roldana barba-johannis* and were shown to be potent antioxidant compounds [Perez-Castorena *et al.*, 2001].

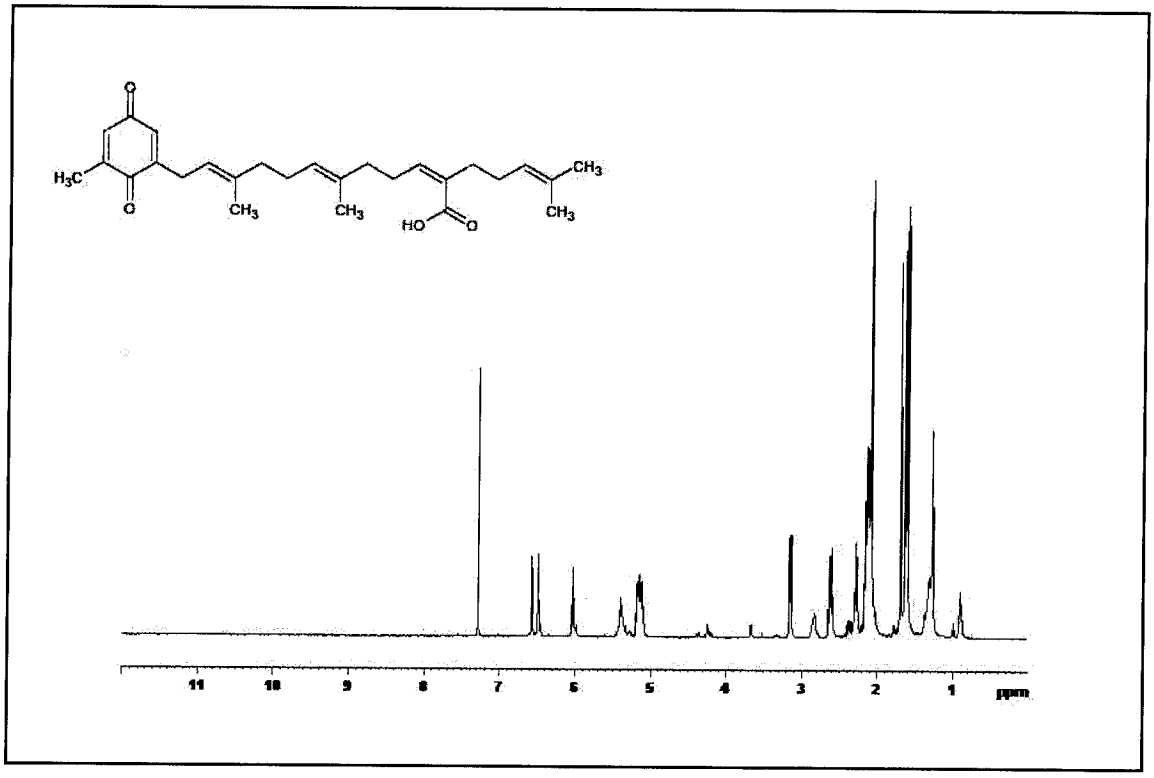

Figure 7.12: ^{1}H NMR of Sargaquinoic acid (B5(6:4)b).

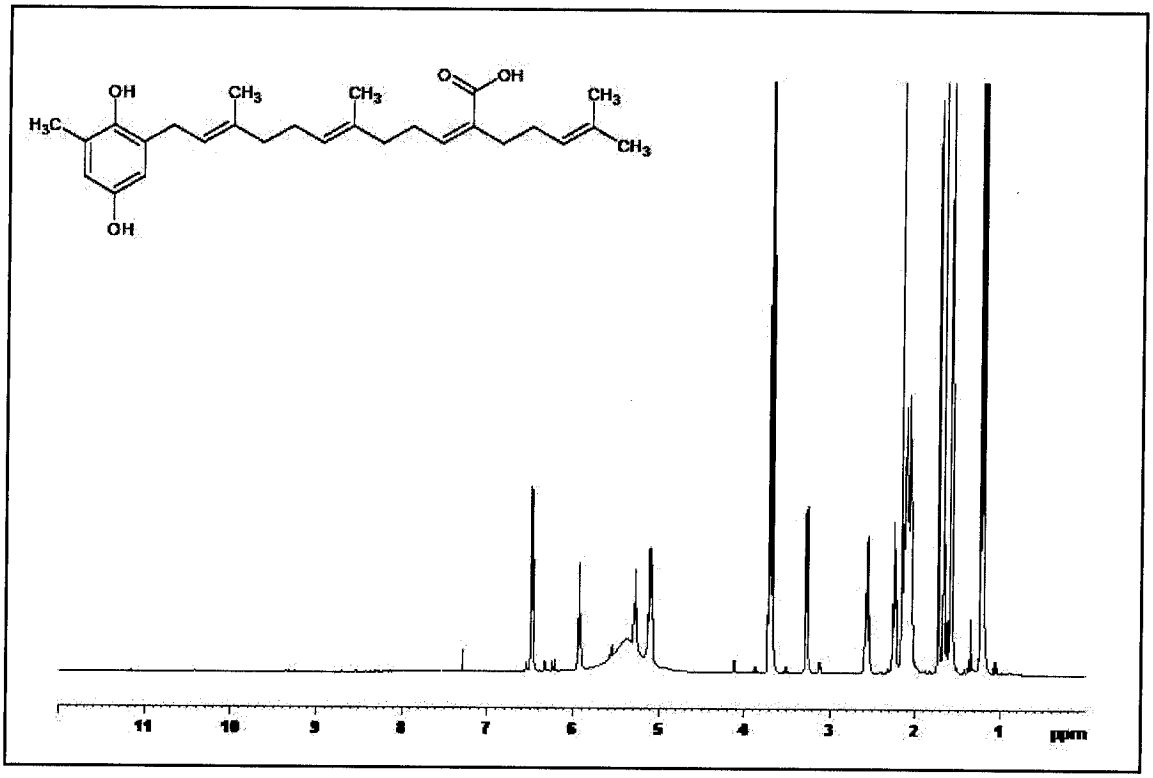

Figure 7.13: ^{1}H NMR of Sargahydroquinoic acid (B5(6:4)c).

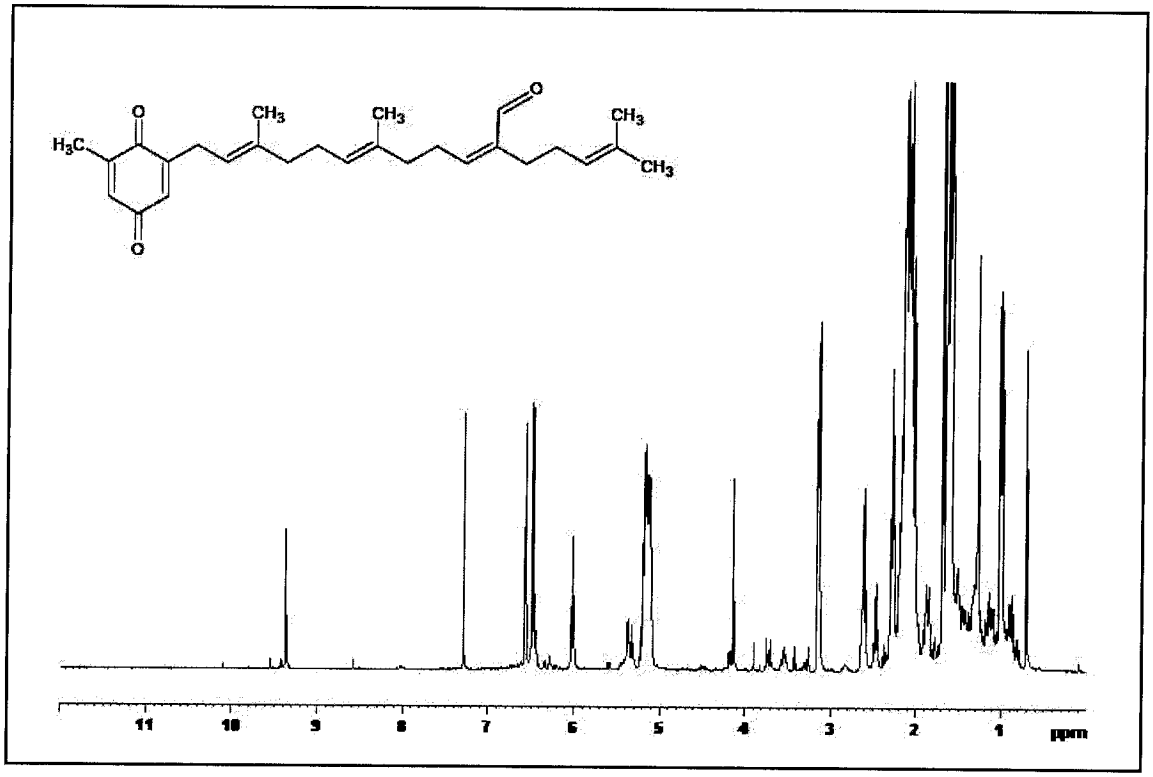

Figure 7.14: ^{1}H NMR of Sargaquinal (B5(6:4)d).

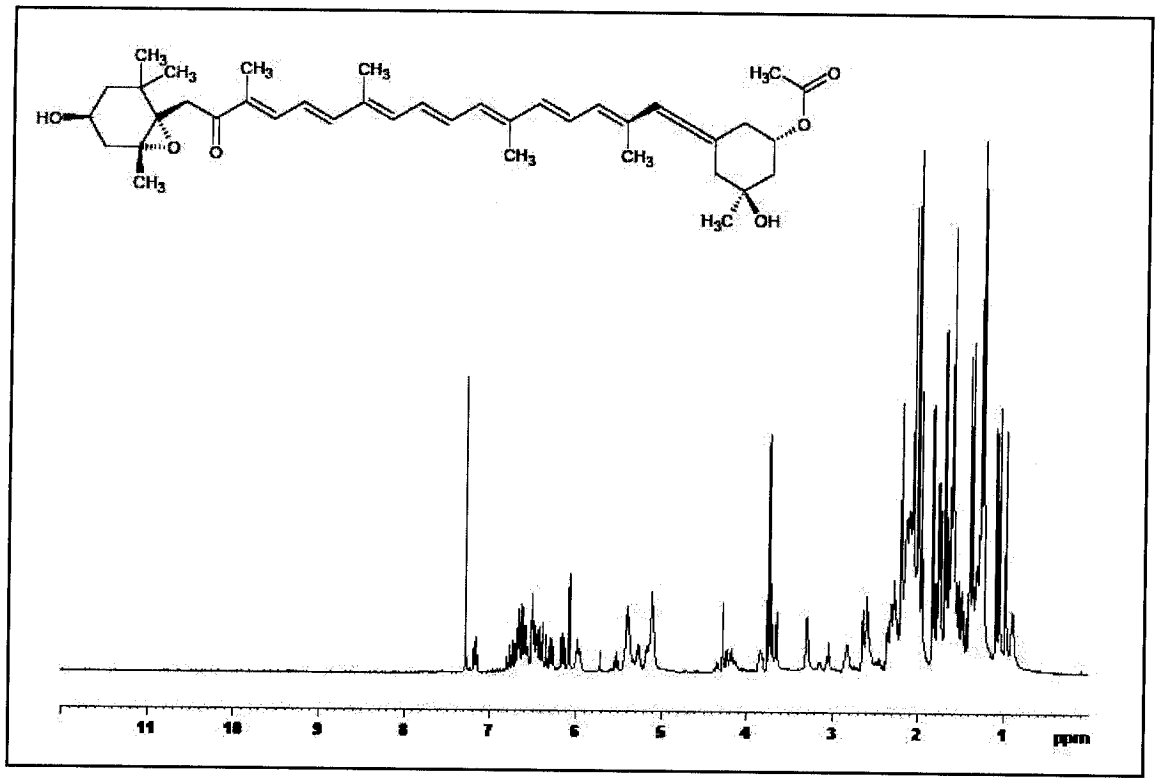

Figure 7.15: ^{1}H NMR of Fucoxanthin (B7 (2:8)).

Quinone compounds, terpenes and polyphenolic compounds have been previously isolated for the genus *Sargassum* [Blunt *et al.*, 2007; Nahas *et al.*, 2007]. These compounds have been shown to possess a variety of biological activities due to their structures. The hydroquinone and phenol structures may enable the potent antioxidant properties of these marine algae [Iwashima *et al.*, 2005]. *Sargassum ringgoldianum, Sargassum yezoense, Sargassum micracanthum, Sargassum hemiphyllum, Sargassum patene, Sargassum horneri, Sargassum ringgoldianum*, and *Sargassum confusum* have all previously shown antioxidant properties [Anggadiredja *et al.*, 1997; Nakai *et al.*, 2006]. Fucoxanthin is a well known carotenoid compound [Helmut *et al.*, 1995; Sies and Stahl, 1995] that has been identified in *Sargassum* species and its antioxidant properties have been explained. Fucoxanthin has been shown to possess some electro-active properties [Liu *et al.*, 2000]. *Sargassum* species have been documented in ancient Chinese medicine as a treatment for various ailments [Yan *et al.*, 1998].

7.7. Conclusions

Electrochemical methods allowed for the analysis of crude fractions and enabled the identification of fractions that contain potent antioxidant compounds. These compounds were shown by NMR analysis to be potent antioxidants. The antioxidant properties of these compounds have previously been indicated [Perez-Castorena *et al.*, 2001]. The marine alga *Sargassum elegans* contained compounds that may be used as antioxidants. Electrochemical methods have been shown, as suggested in this study, to be a valuable approach to aid the isolation and purification of electro-active natural products such as antioxidants as the isolation of natural products is often a costly, time-consuming procedure. Electrochemistry combined with NMR analysis enables rapid isolation and identification of crude extracts before the electro-active compounds have to be purified to completion. Electrochemical analysis also allows for lipophilic and hydrophilic electro-active compounds to be examined. This overcomes problems of solubility as compounds that have minimal solubility in aqueous solutions can also be examined, as shown in this study.

Phytochemistry has been the basis for the development of novel pharmaceutical products and the combination of electrochemical directed isolation and NMR has been shown, in this study, to be a promising hyphenated technique that enables the rapid elucidation of electro-active compounds present in crude extracts.

CHAPTER 8: SUMMARY AND GENERAL CONCLUSIONS

The overall objective of this study was to critically examine the use of electrochemistry for the evaluation of antioxidant compounds. Secondly the study aimed to apply these methods for antioxidant evaluation of plant and algal extracts.

Electrochemical methods are inherently sensitive and rapid [Wang, 1994]. Furthermore, they do not suffer from the short-comings to which the DPPH method is prone to such as steric hindrance, dependence on pH and the lowered efficacy of this assay of water soluble antioxidants [Prior *et al.*, 2005]. Electrochemical methods provide a distinct advantage over traditional antioxidant evaluation methods as they can be used to determine lipophilic and hydrophilic antioxidants. Voltammetric methods can be used to determine two of the modes of action of antioxidants. These are, the ability of antioxidants to donate an electron(s) which allows for their determination using an anodic potential. Furthermore, voltammetric methods allow for the determination of the ability of antioxidant compounds to bind to transition metals such as Fe^{3+}. Most significantly, voltammetric methods when compared to methods such as DPPH and TEAC provide not only information on electron donating abilities but also the area under the curve which provides an estimate of the total antioxidant capacity of crude plant samples. Contrasted then to non-biological antioxidant assays such as TEAC and the DPPH assay (which provide information on electron and hydrogen donating abilities), electrochemistry thus provides *both* qualitative and quantitative information.

Specifically, Chapter 3 examined the use of the electrochemical techniques of SWV and CV for the evaluation of known antioxidants. The study detailed the first electrochemical characterisation of canavanine, pinitol and extracts of the plant *Sutherlandia frutescens*. Canavanine was shown to posses the ability to donate electrons while pinitol showed no electro-activity at the surface of the bare GCE. However, its relatively high oxidation potential does not indicate that this is a potent biological electron donor. Its OP is however similar to that of the known antioxidant, melatonin.

The HW and MeOH extracts of *Sutherlandia frutescens* were shown to possess electro-active compounds with low oxidation potential and thus possess potentially potent biological electron donors. AdSV provided evidence for the role of canavanine and pinitol to bind to Fe^{3+}. The HW and MeOH extracts also indicated the presence of compounds that were able to chelate Fe^{3+}.

Chapter 4 indicated that compounds present in the HW and MeOH extracts scavenged the DPPH radical greater than 80 % at the concentrations examined, suggesting the presence of compounds that have the ability to donate electrons or hydrogen ions. The TEAC assay showed that the HW extract and canavanine had the ability to donate electrons more efficiently than the MeOH extract and pinitol. The results of the ferrozine assay indicate that the compounds examined may not have the ability to bind to Fe^{2+}. However, in light of the disadvantages of this assay (section 4.5), this assay may not be the most appropriate to indicate the metal-binding abilities of the test samples. The FC assay indicated that the HW extract possessed a greater phenolic content than the MeOH extract of *Sutherlandia frutescens*.

Chapter 5 made use of the pharmacological methods of lipid peroxidation and the superoxide anion (NBT) assay to validate the antioxidant properties of *Sutherlandia frutescens*. Canavanine and the HW extract of *Sutherlandia frutescens* significantly reduced the formation of NBD, reduced LP initiated by Fe^{2+} and QA and LP initiated by the $OH^{\cdot}$ radical at all concentrations. The MeOH extract also reduced LP initiated by Fe^{2+} and QA at all concentrations.

Chapter 4 and 5 indicated that the HW and MeOH extracts of *Sutherlandia frutescens* possessed compounds that were able to donate electrons and to quench free radicals and thus act as antioxidant compounds. Canavanine was shown to be an electron-donor with possible metal-binding abilities (chapter 3). The results from chapter 4 and 5 indicated that pinitol did not possess the ability to donate electrons or hydrogen ions, thus, supporting the results obtained from the electro-analysis of pinitol.

Chapter 6 showed that SWV can be used as a preliminary and rapid screening method of large samples of crude extracts for antioxidant compounds. The results of the DPPH and FC assays indicated that the electrochemical methods were more effective for the screening of crude extracts for electro-active compounds.

The electrochemical methods were able to detect more compounds with electron donating abilities than the DPPH assay. Electro-analysis is not affected by the problems that colorimetric assays face. Turbidity is a major problem that often affects assays such as the DPPH assay [Arnoa, 2000]. There were four extracts that exhibited total phenolic content greater than 0.05 mg/mL of sample that were not detected by electrochemical methods. This may be due to the reduction of the FC reagent by non-phenolic compounds [Singleton, and Rossi, 1965]. However, this may also suggest the presence of non-antioxidant phenolic compounds.

The use of the electrochemical methods of SWV and CV were examined in chapter 7 as SWV and CV were utilised to direct the isolation and purification of antioxidant metabolites of the marine alga *Sargassum elegans*. The use of electrochemical methods enabled the analysis of crude fractions and enabled the identification of fractions that contain potent antioxidant compounds. As electrochemistry enables rapid analysis, it can be used to determine the presence of possible antioxidant compounds and allow for their purification before these compounds are affected by oxidation. The results of the electrochemistry directed isolation were supported by the NMR analysis, which elucidated the structure of known, potent antioxidant compounds. The results of this study have shown that electrochemical methods can be used for the rapid determination of antioxidant compounds and provide information on modes of action of antioxidants.

However, electro-analysis does suffer from drawbacks such as the inability to detect antioxidants that are not able to donate electrons and the ability to determine antioxidants that have other modes of action such as hydrogen donation. Large antioxidant molecules may also not be accurately determined as the electron-donating site may not be adequately exposed to the electrode surface. Different antioxidants may have different electron donating abilities at different electrode surfaces.

8.1. Future Studies

The use of the electrochemical methods to direct the isolation and purification of electro-active compounds from *Sutherlandia frutescens*. This would allow for the identification of the compounds that impart the antioxidant properties of *Sutherlandia frutescens*.

The preliminary screening performed in chapter 6 may be used as a guide to further purify the solvent fractions that possessed electro-active compounds to determine their antioxidant properties. There were four extracts that were positive in the FC assay but were not detected electrochemically. The purification and isolation of these compounds would indicate why these extracts were not electro-active. Extracts of *Polysiphonia incompta* were also shown to possess the highest concentration of electro-active compounds (chapter 6) and the isolation and purification of these extracts would provide evidence to the structures and activities of the compounds in these extracts.

The combination of electrochemistry with NMR can be explored further as this method has been shown in this study to provide a rapid solution to identifying extracts and solvent fractions that possess electro-active compounds. The compounds isolated in this study have been shown to be potent antioxidant and the application of electrochemistry and NMR enabled the isolation and elucidation of these compounds.

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APPENDICES

Appendix A

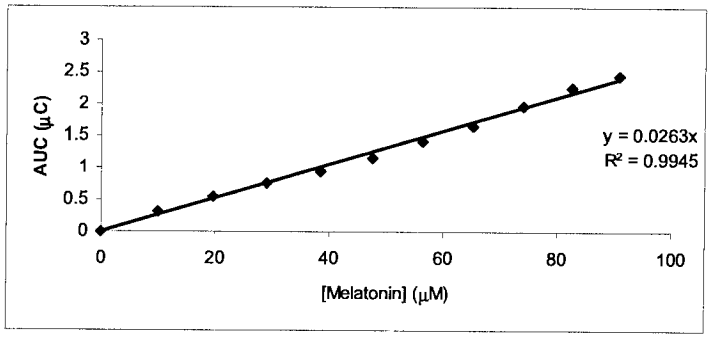


Figure. A1: Standard curve of melatonin using CV

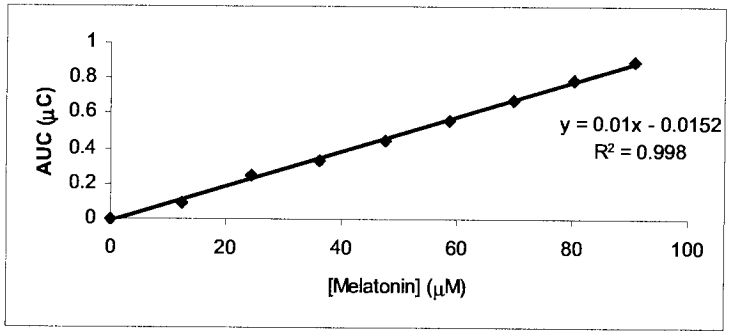


Figure. A2: Standard curve of melatonin using SWV

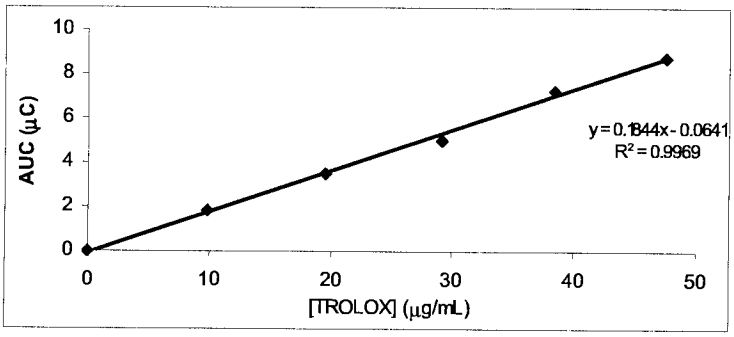


Figure. A3: Standard curve of TROLOX using CV.

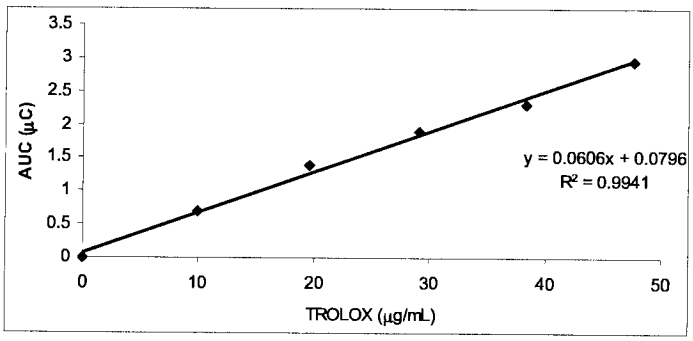


Figure. A4: Standard curve of TROLOX using SWV

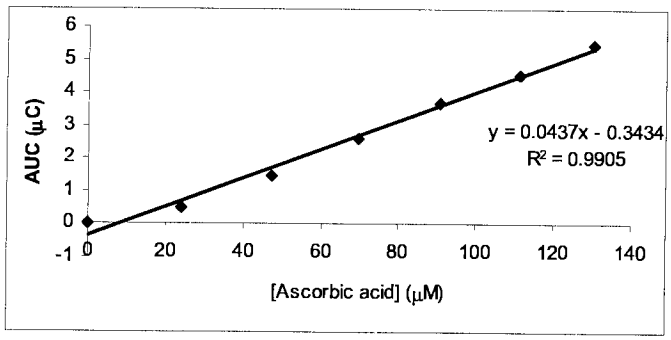


Figure. A5: Standard curve of ascorbic acid using CV

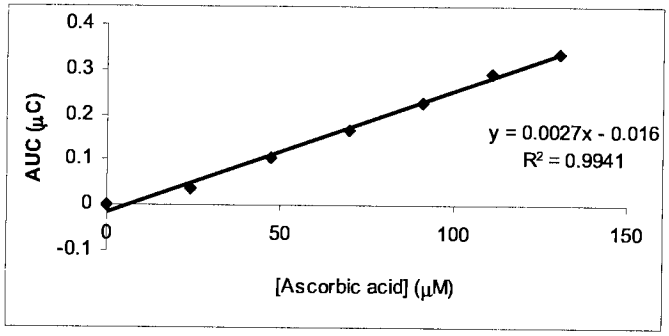


Figure. A6: Standard curve of ascorbic acid using SWV

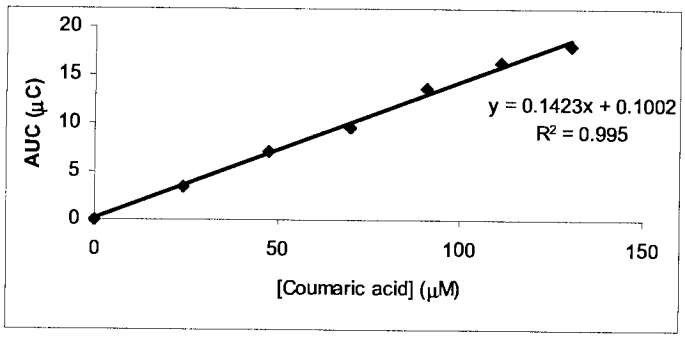


Figure. A7: Standard curve of p-coumaric acid using CV.

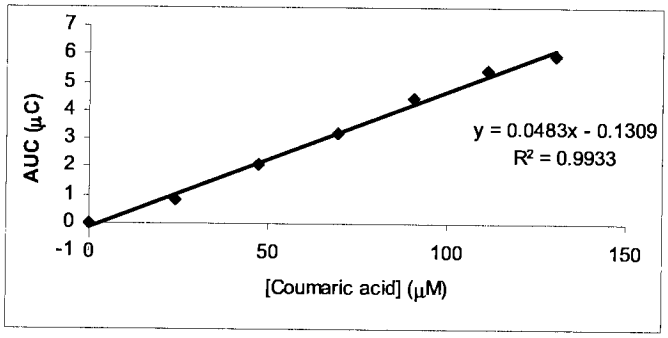


Figure. A8: Standard curve of p-coumaric acid using SWV

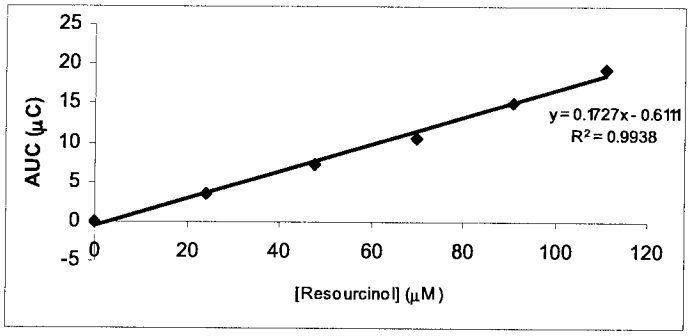


Figure. A9: Standard curve of resorcinol using CV.

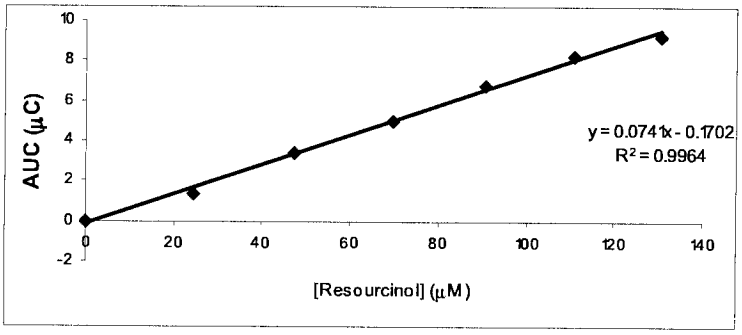


Figure. A10: Standard curve of resorcinol using SWV.

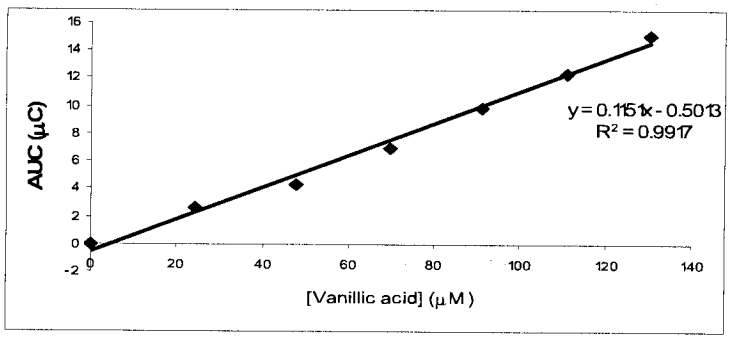


Figure. A11: Standard curve of vanillic acid using CV.

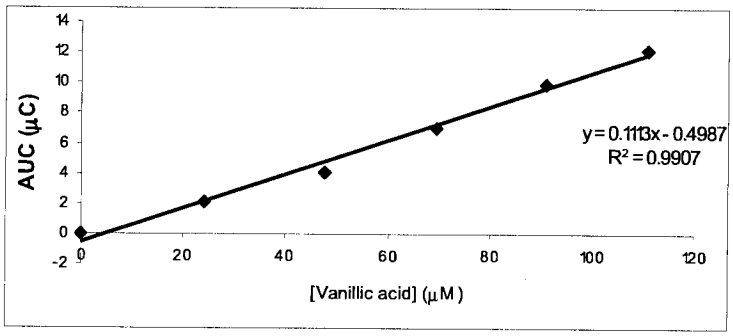


Figure. A12: Standard curve of vanillic acid using SWV

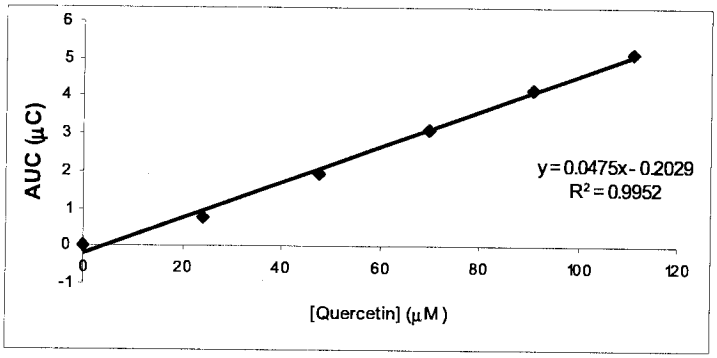


Figure. A13: Standard curve of quercetin using SWV

Appendix B

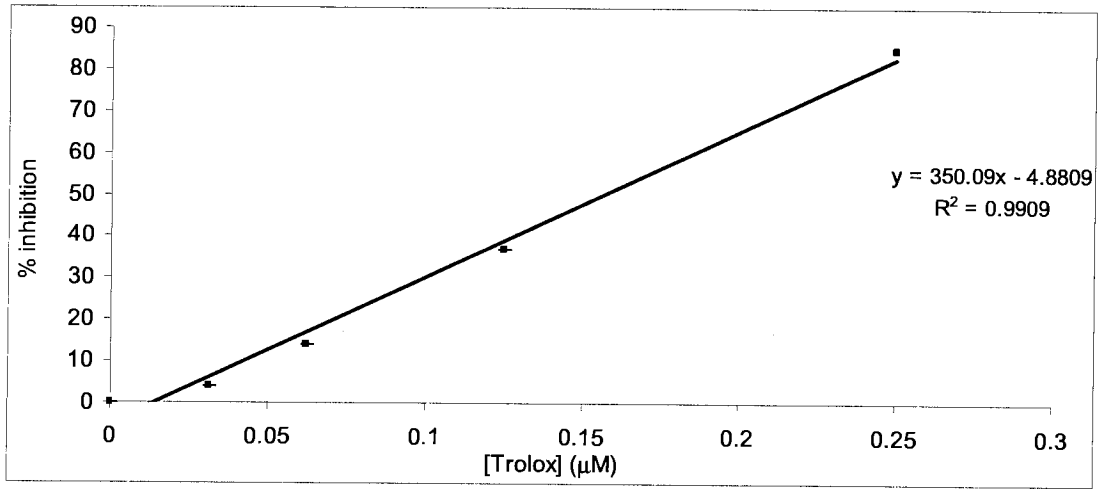


Figure B1: Standard curve of TROLOX at increasing concentrations of 0.03125, 0.0625, 0.125, 0.25 µM.

Gallic acid standard curve:

A stock solution of 0.5 mg/mL of Gallic acid was made and then serial dilutions made to 0.0078 mg/mL. Then the FC assay was followed and standard curve obtained. The phenol concentration was expressed as Gallic acid equivalence (GAE).

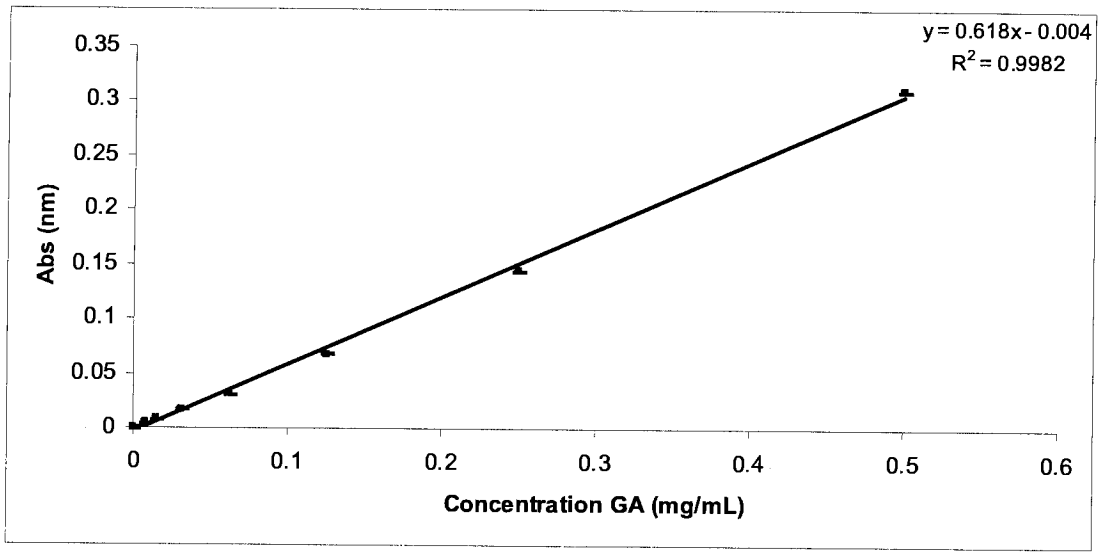


Figure B2: Standard curve of gallic acid at increasing concentrations

Appendix C

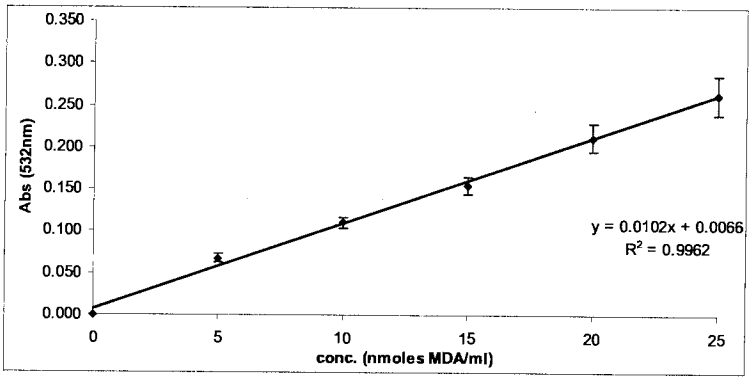


Figure C1. Standard curve of increasing concentrations of MDA.

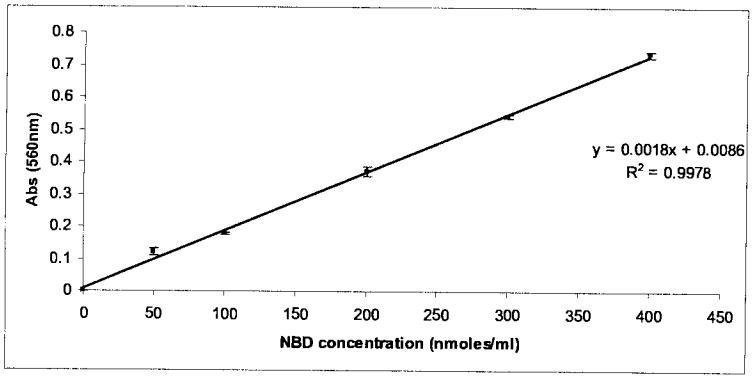
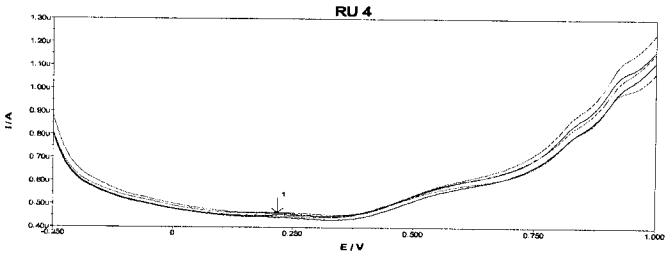
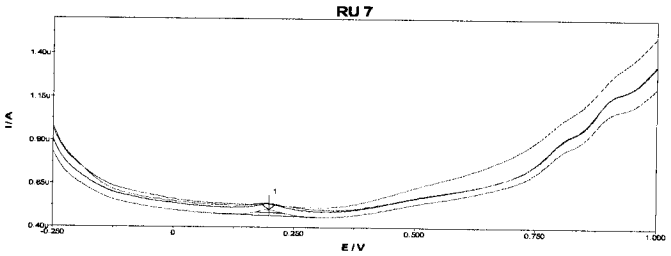


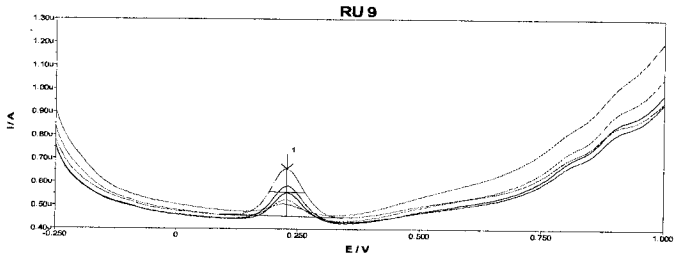
Figure C2.: Standard curve of NBD at increasing concentrations

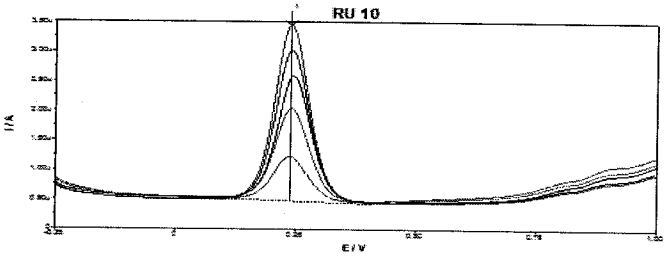
Appendix D

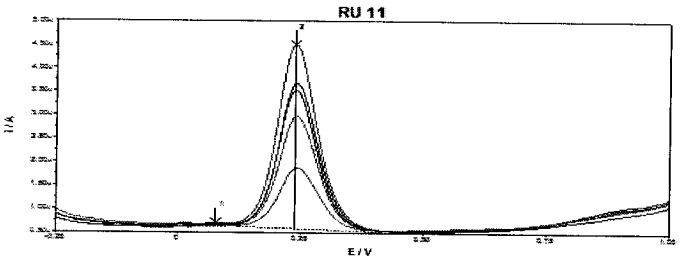
Appendix D1: SWV of algal extracts that provided anodic potentials (24.4-90.9 µg/mL)

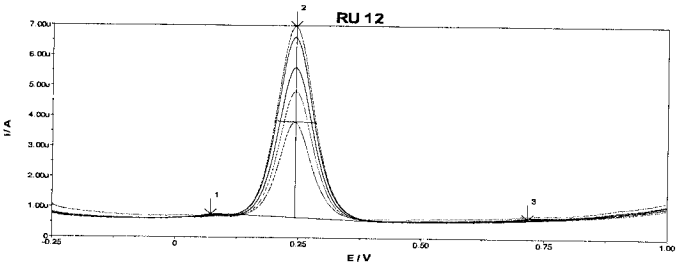


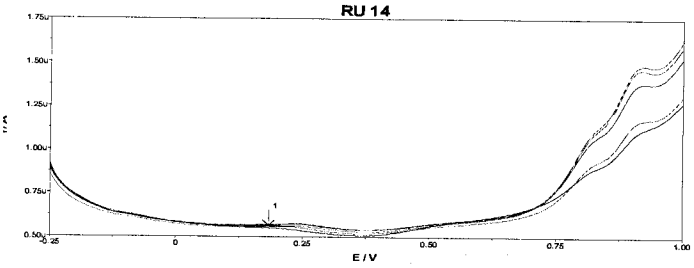


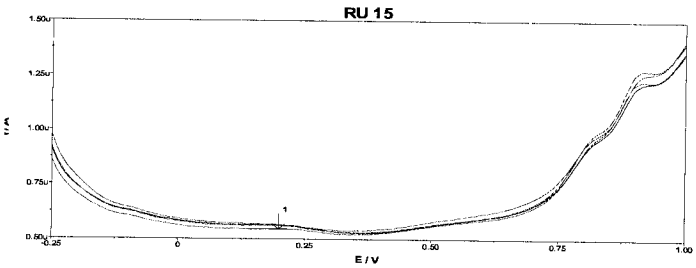


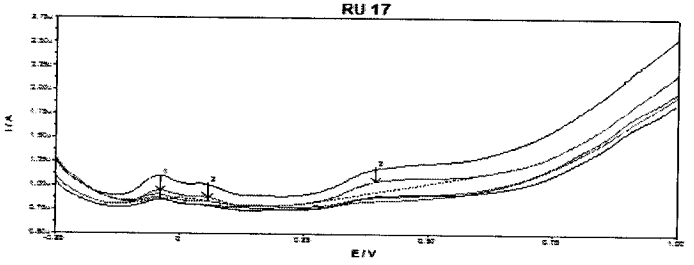


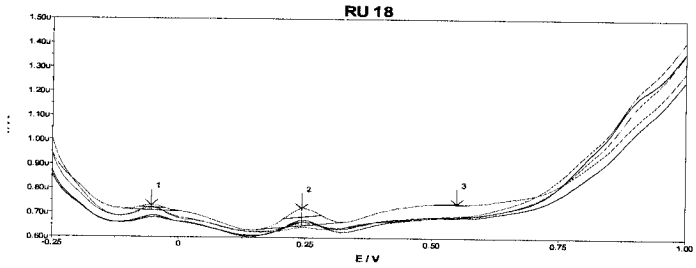


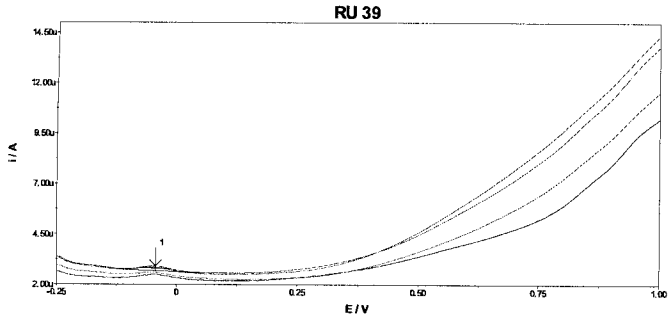


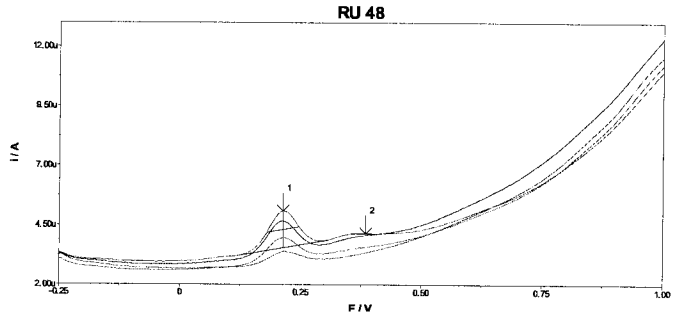









RU 39
i / A
E / V


RU 48
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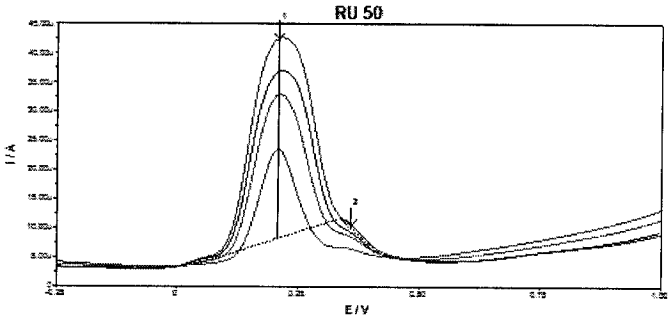
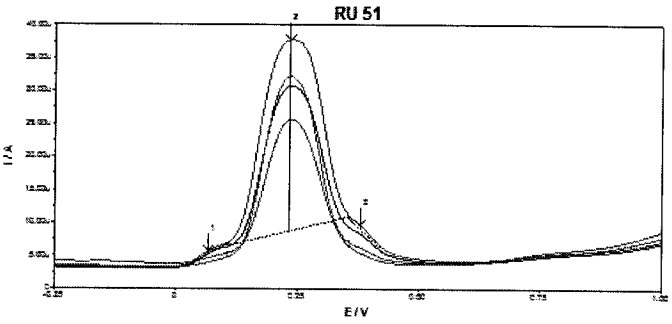
E / V


RU 50
i / A
E / V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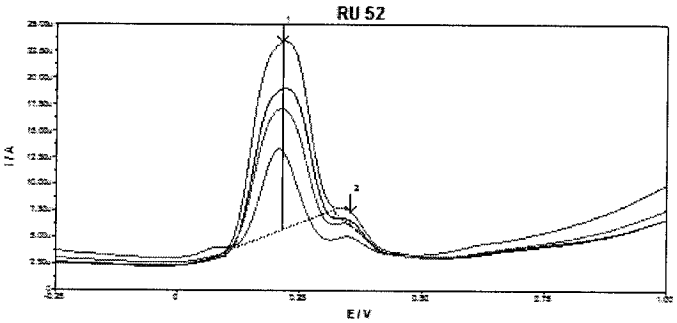
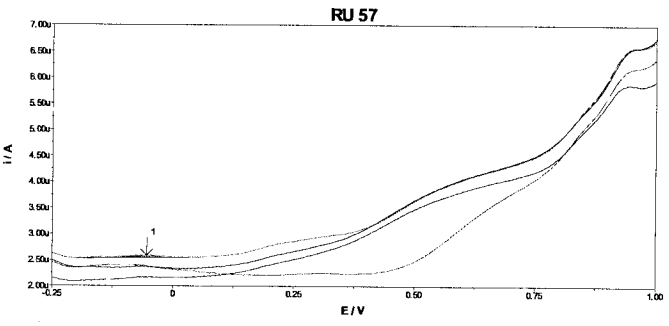
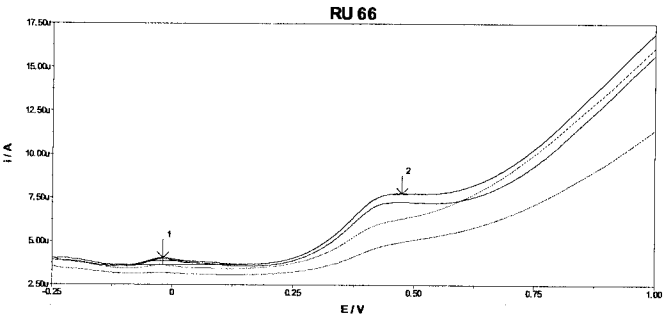
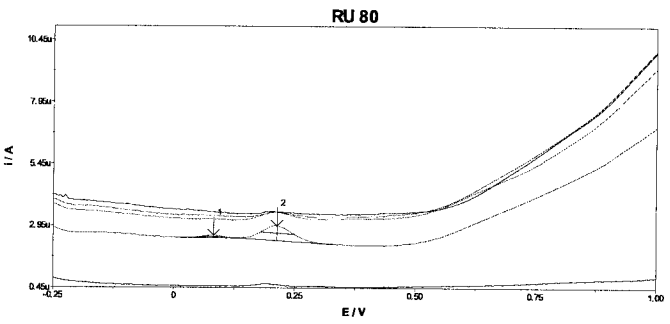
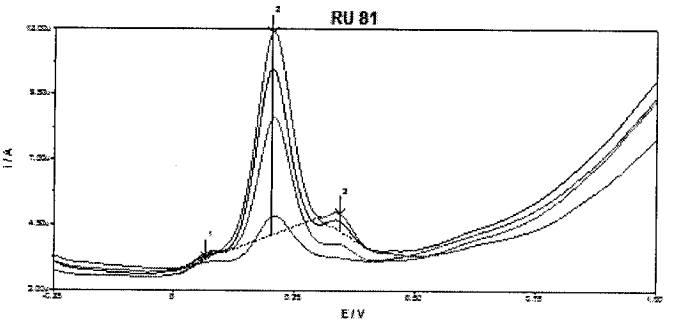
E / V

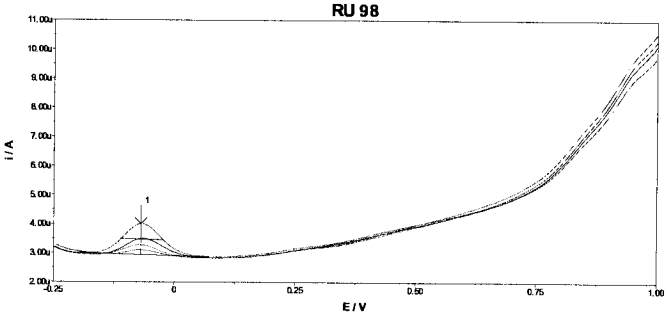

RU 52
i / A
E / V


RU 57
i / A
E / V


RU 66
i / A
E / V


RU 80
i / A
E / V


RU 81
i / A
E / V


RU 98
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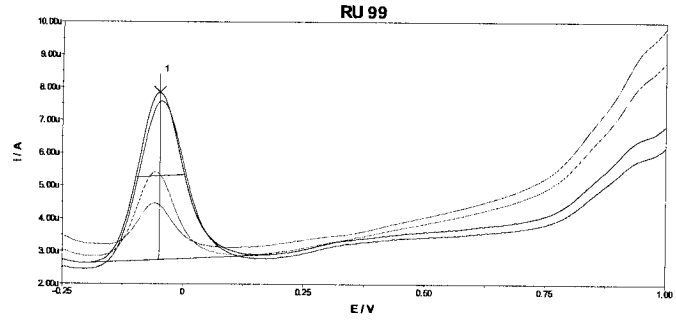
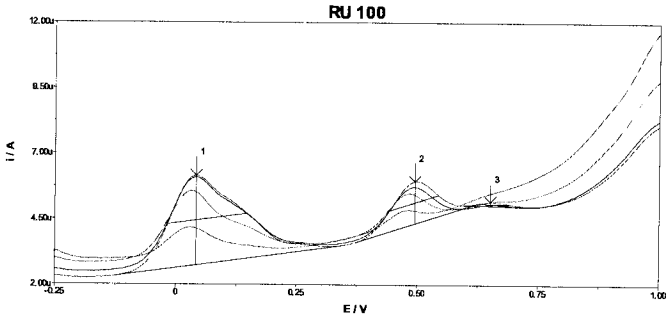
E / V


RU 99
i / A
E / V
1


RU 100
i / A
E / V
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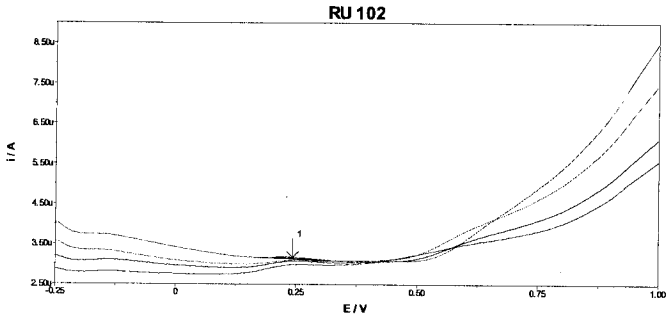
2
3


RU 102
i / A
E / V
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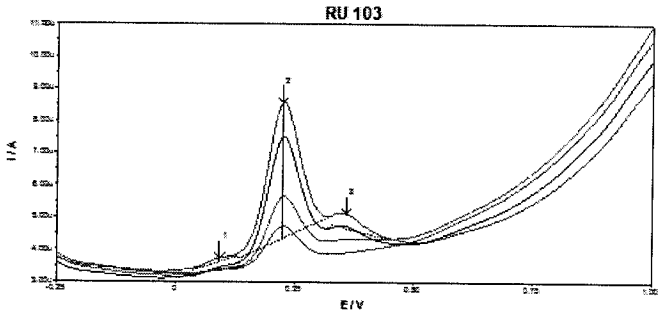
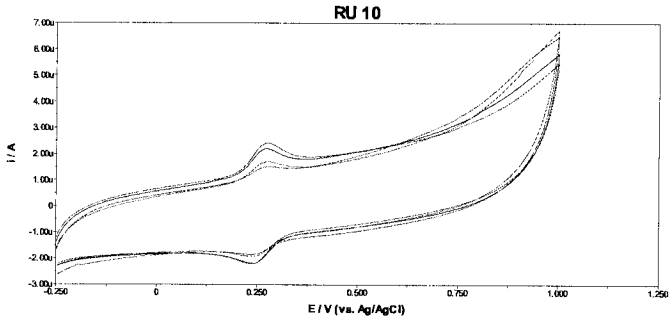
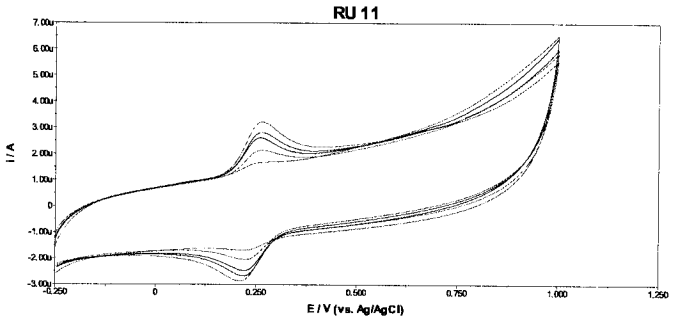
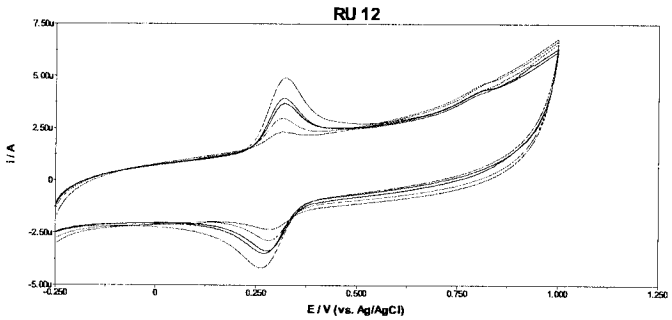
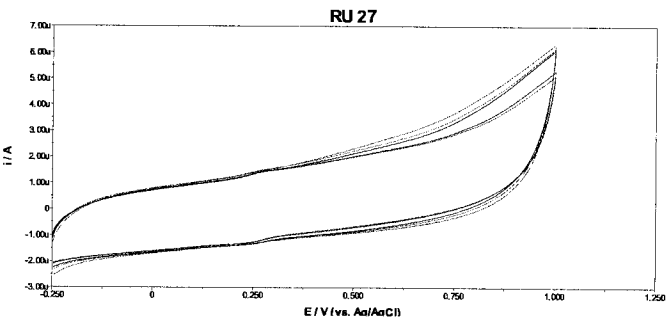

RU 103
i / A
E / V

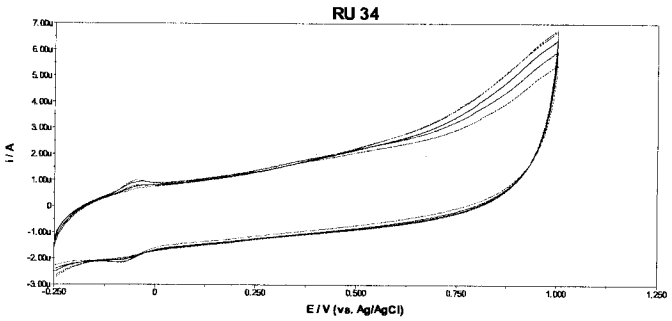
Appendix D2: Cyclic voltammetry of algal samples that were determined with SWV.

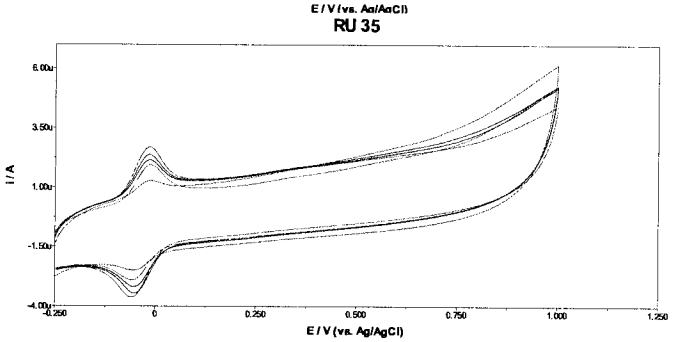


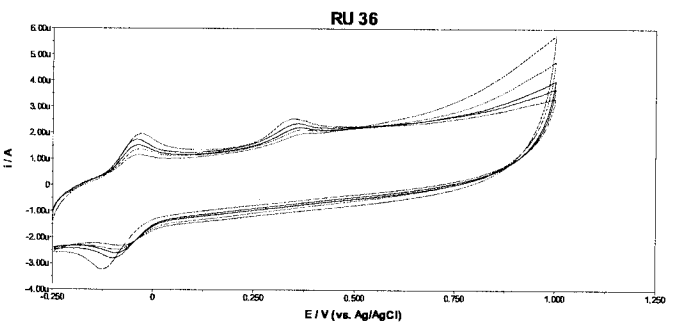


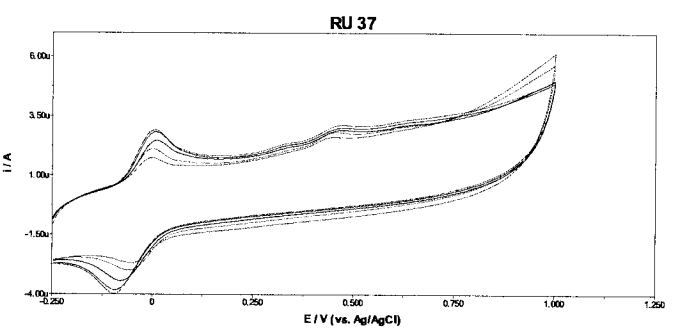


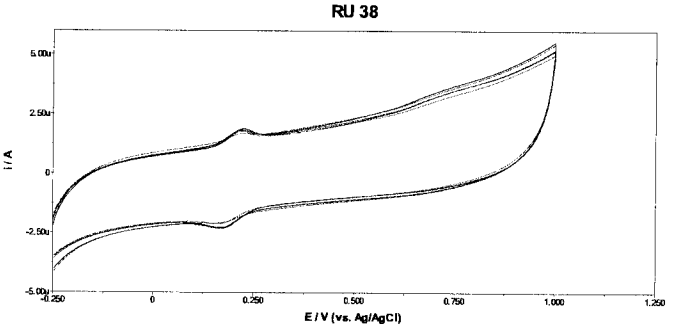


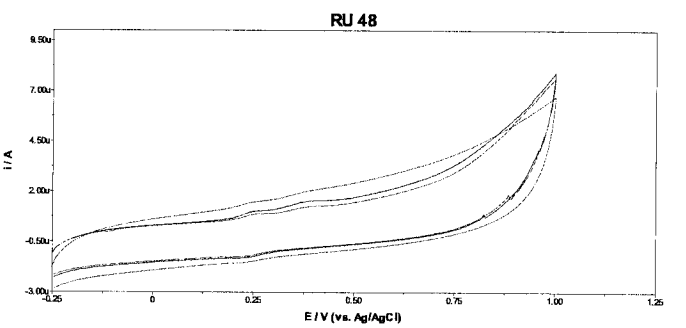




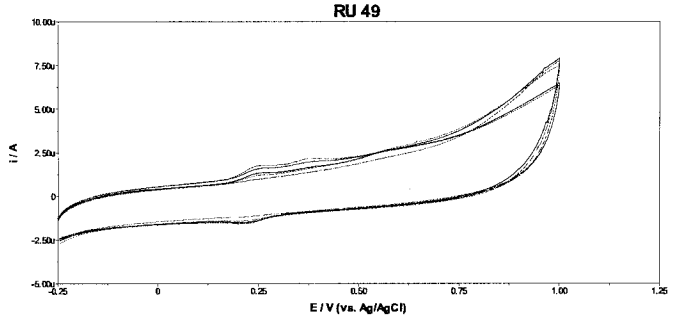




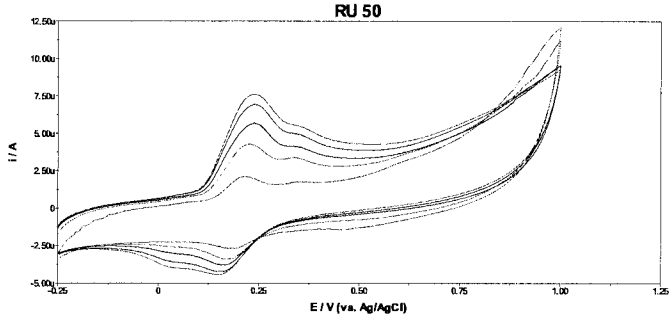




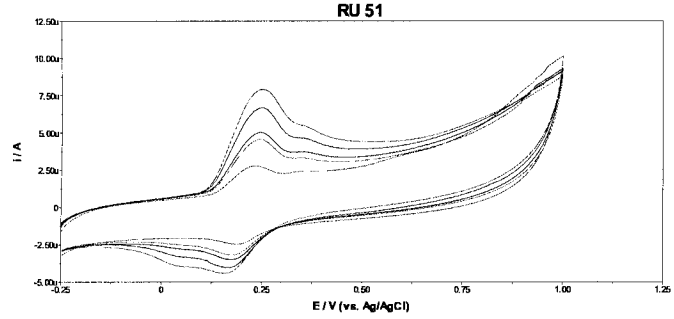
RU 49



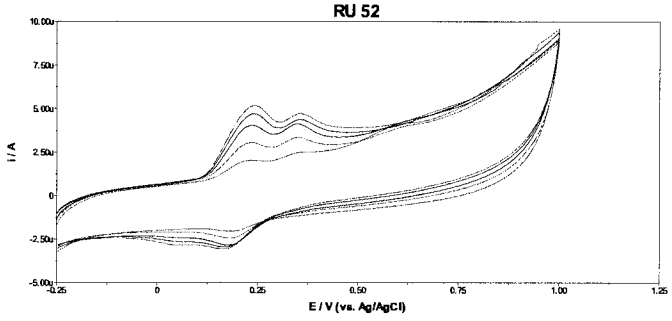
RU 50



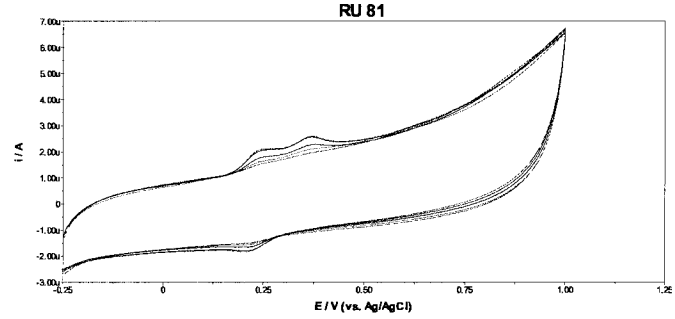
RU 51



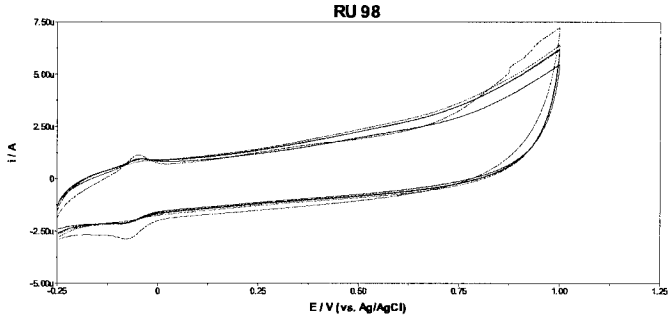
RU 52



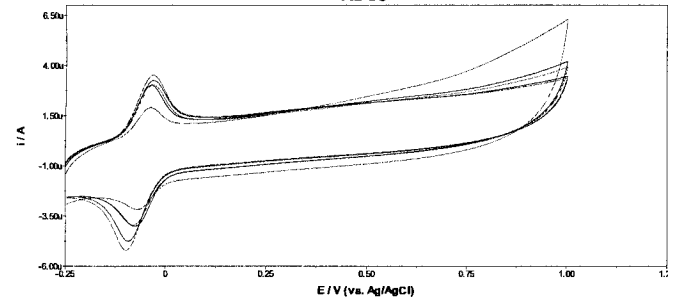
RU 81



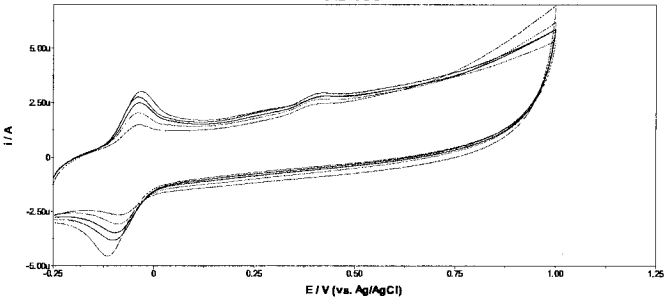
RU 98



RU 99



RU 100



RU 103