SPECTROELECTROCHEMICAL DETERMINATION OF THE ANTIOXIDANT PROPERTIES OF CARPOBROTUS MELLEI AND CARPOBROTUS QUADRIFIDUS NATURAL PRODUCTS

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor Philosophiae in the CHEMISTRY DEPARTMENT, SENSORLAB FACULTY OF NATURAL SCIENCES UNIVERSITY OF THE WESTERN CAPE

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KEYWORDS

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Square Wave Voltammetry

Superoxide Dismutase (SOD) Biosensor
ABSTRACT

Spectroelectrochemical determination of the antioxidant properties of *Carpobrotus mellei* and *Carpobrotus quadrifidus* natural products.

M.S. Maoela

PhD Thesis, Department of Chemistry, University of the Western Cape

South African *Carpobrotus* species have been found to contain hydrolysable tannins, various flavonoids e.g. rutin and hyperoside, phytosterols and aromatic acids which have a diverse range of pharmacological properties including antimicrobial and, antioxidant activities. The main aim of the thesis was to determine the natural products in *C. mellei* and *C. quadrifidus* using chromatographic techniques and electrochemical analysis. The antioxidant activity of both *Carpobrotus* species was determined by using a superoxide dismutase (SOD) biosensor. ESI-LC-MS was used to separate and determine flavonoids in *C. mellei* and *C. quadrifidus*. 8 flavonoid compounds: catechin, epicatechin, epicatechin-epicatechin, coumarylquinic acid, isorhamnetin, quercetin-hexose (hyperoside), rutin and myricetin-deoxyhexose were identified. Cyclic and square wave voltammetry were used to detect flavonoids from *C. mellei* and *C. quadrifidus*. Catechin was detected in the ethyl acetate extract of *C. mellei* and *C. quadrifidus*. The oxidation potential of the plant extracts were observed at +150.6 mV to +1072.6 mV. The oxidation mechanism proceeds in sequential steps, related to the catechol moiety, -OH groups in C ring and the resorcinol group. The oxidation process of the catechol moiety involves a two electron - two proton reversible reaction and forms o-quinone. This occurs first at low potential and is a reversible reaction. The hydroxyl group in the C ring and resorcinol group oxidise there after and undergo an irreversible reaction. UV-vis and FTIR spectroscopy were
used to confirm the presence of catechin in the ethyl acetate extract of both plants. UV-visible spectroelectrochemistry confirmed the oxidation process of catechin at constant potential. Since *C. mellei* and *C. quadrifidus* were confirmed to contain flavonoids by ESI-LC-MS and electrochemical analysis, the antioxidant activity was further investigated using a SOD biosensor. The superoxide dismutase (SOD) enzyme was immobilised with 1% Nafion on a platinum electrode. Detection limit and sensitivity of the SOD biosensor were found to be $0.03918 \, \mu \text{mol L}^{-1}$ and $1.44 \, \mu \text{A} \, (\mu \text{mol L}^{-1})^{-1}$, respectively. The results showed that *C. mellei* and *C. quadrifidus* have antioxidant activity, with relative antioxidant capacity (RAC) of 24% and 42%, respectively.
DECLARATION

I declare that “Spectroelectrochemical determination of the antioxidant properties of Carpobrotus mellei and Carpobrotus quadrifidus natural products” is my work, it has not been submitted for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Manki Sarah Maoela

May 2009

Signed: ..................................
DEDICATION

To my dearest parents Thabo J. Maoela and Molehali A. Maoela, my sisters Tshedid Maoela and Palesa Sekhejane, my favourite grandmother in the world Delia Maoela, my uncle and aunt Mr and Mrs Sekhejane.
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Abbreviations

CV: Cyclic Voltammetry
DPPH: 2,2-diphenyl-1-picrylhydrazyl
ESI: Electrospray Ionization
FRAP: Ferric Reducing Antioxidant Power
GCE: Glassy Carbon Electrode
Ip: Peak height
LC-MS: Liquid Chromatography-Mass Spectroscopy
MS: Mass Spectroscopy
ORAC: Oxygen Radical Absorbance Capacity
ROS: Reactive Oxygen Species
SOD: Superoxide Dismutase
SWV: Square Wave Voltammetry
TLC: Thin Layer Chromatography
tR: Retention time
UV-vis: Ultra-violet visible
XOD: Xanthine Oxidase
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Introduction

Medicinal plants, since ancient time, have been used in virtually all cultures as a source of medicine. The widespread use of herbal remedies and healthcare preparations obtained from commonly used traditional herbs and medicinal plants has been traced to the occurrence of natural products with medicinal properties. The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka and Thailand. In China about 40% of the total medicinal consumption is attributed to traditional tribal medicines. In Thailand, herbal medicines make use of legumes encountered in the *Caesalpiniaceae, Fabaceae,* and *Mimosaceae.* In the mid-90s, it was estimated that receipts of more than US$2.5 billion have resulted from the sales of herbal medicines. And, in Japan, herbal medicinal preparations are more in demand than mainstream pharmaceutical products. Africa is a rich source of medicinal plants. Perhaps, the best known species is *Phytolacca dodecandra.* Extracts of the plant are used as an effective molluscicide to control schistosomiasis (Lemma, 1991). Other notable examples are *Catharanthus roseus,* which yields anti-tumour agents such as vinblastine and vincristine; and *Ricinus communis,* which yields the laxative castor oil. In Botswana, Lesotho, Namibia and South Africa, *Harpagophytum procumbens* is produced as a crude drug for export. *Hibiscus sabdariffa* is exported from Sudan and Egypt, *Pausinystalia yohimbe* from Cameroon, Nigeria and Rwanda, which yields yohimbine; and *Rauwolfia vomitoria* from Madagascar, Mozambique and Zaire, which is exploited to yield reserpine and ajmaline (Cunningham, 1993; Cunningham, 1997; De Smet Peter, 1999).
Developed countries, in recent times, are turning to the use of traditional medicinal systems that involve the use of herbal drugs and remedies. According to a recent survey in Member States of the European Union, about 1400 herbal preparations are used widely. Currently, the major pharmaceutical companies have demonstrated renewed interest in investigating higher plants as sources for new lead structures and also for the development of standardized phytotherapeutic agents with proved efficacy, safety and quality. Herbal preparations are popular and are of significance in primary healthcare in Belgium, France, Germany and the Netherlands. Such popularity of healthcare plant-derived products has been traced to their increasing acceptance and use in the cosmetic industry as well as to increasing public costs in the daily maintenance of personal health and well being. Examples of such beauty-oriented therapeuticals are skin tissue regenerators, anti-wrinkling agents and anti-age creams. Most dermaceuticals are derived from algal extracts that are rich in minerals and the vitamin B group. Limitations of synthesized compounds in the treatment of chronic diseases and the potential of plant-based medicine as a more effective and cheaper alternative were probably responsible for the fast growing industry of herbal medicine (Kerwegi, 2001).

Despite the increasing use of medicinal plants, their future is being threatened by complacency concerning their conservation. Reserves of herbs and stocks of medicinal plants in developing countries are diminishing and in danger of extinction as a result of growing trade demands for cheaper healthcare products and new plant-based therapeutic markets in preference to more expensive target-specific drugs and biopharmaceuticals. Such concern has stimulated positive legal and economic interest. Genetic biodiversity of traditional medicinal herbs and plants is continuously under
the threat of extinction as a result of growth-exploitation, environment-unfriendly harvesting techniques, and loss of growth habitats and unmonitored trade of medicinal plants. So in order to discover new natural medicinal agents, a thorough investigation of in-depth cultural knowledge, and research into the nature of the drug and chemical analysis as well as pharmacotoxicological tests need to be done (Bowie, 2002).

Aim

The main aim of this thesis is to investigate the natural products (flavonoids) from *Carpobrotus* species (*C. mellei* and *C. quadrifidus*) using techniques such as chromatography, electrochemical and spectroelectrochemical analysis. The antioxidant activity of the species will be determined using a biosensor.

Objectives

- Solvent extraction of plant material from *C. mellei* and *C. quadrifidus*
- Identification of flavonoids using chromatography
- Identification of flavonoids using electrochemistry and spectroelectrochemistry
- Determination of antioxidant activity using biosensor

Thesis Statement

The genus *Carpobrotus* have been used for medicinal purposes over many generations and this is partly due to the presence of natural products with antioxidant properties. The antioxidant activity of *C. mellei* and *C. quadrifidus* was evaluated.
Overview of the Thesis Structure

- **Chapter one**: The literature review focuses on the chemistry, health benefits and biosynthesis of the flavonoids. The background of *Carpobrotus* species will be discussed in this chapter.

- **Chapter two**: Literature review about the determination of flavonoids using chromatographic studies and electrochemical analysis. The literature review focuses on discussing the sample preparation for flavonoids and different chromatographic techniques used for determining or identifying flavonoids. For electrochemical analysis, a detail background about the electrodes, supporting electrolyte, types of voltammetric techniques and electrochemistry of flavonoids will be discussed.

- **Chapter three**: In this chapter, all the procedures for chromatographic techniques (thin layer chromatography and ESI-LC-MS) and voltammetric analysis (cyclic and square wave voltammetry) will be discussed in detail.

- **Chapter four**: This chapter opens with the establishment of testing protocol for flavonoids products from *C. mellei* and *C. quadrifidus* and then determining which subgroups of flavonoid are present in the plant extracts. Then followed by an electrochemical characterization of quercetin, rutin, catechin, luteolin and apigenin using cyclic and square wave voltammetry.
• **Chapter Five**: In this chapter a literature review about determination antioxidant activity using biosensor is discussed. The experimental, results and discussion about spectrophometric methods such as DPPH’, ORAC, FRAP assays and biosensor with superoxide dismutase (SOD) enzyme will be discussed.

• **Chapter Six**: The study is concluded with a concise discussion of the flavonoids found in *C. mellei* and *C. quadrifidus* using chromatographic techniques and voltammetry. The recommendations will be mentioned.
CHAPTER 1

LITERATURE REVIEW OF FLAVONOIDS

1.1 Introduction

Flavonoids are receiving considerable attention in the literature because they are of biological and physiological importance and are widely distributed in plants fulfilling many functions including producing pigmentation in flowers and protection from attack by microbes and insects. However, their occurrence is not restricted to flowers but includes all parts of the plant. Flavonoids play an important role in plants as defence and signalling compounds in reproduction, pathogenesis and symbiosis (Maxwell and Philips, 1990; Barz et al., 1990). Plant flavonoids are involved in response mechanisms against stress caused by elevated UV-B radiation (Rosenberg et al., 2002; Olsson et al., 1998; Stack and Heldt, 1997), infection by microorganisms (Middleton and Teramura, 1993) or herbivore attack (Wang et al., 1998). Flavonoids affect human and animal health because of their role in diet, which is ascribed to their antioxidant properties (Rice-Evans et al., 1997), wide range of antimicrobial and pharmacological activities (Weidenborner and Jha, 1994). Many enzymes involved in intracellular signalling may be affected by flavonoids. The effects of flavonoids on protein kinesis are of importance since they directly influence immune functions in the host (Middleton and Kandaswami, 1992). Apart from the purely academic study of their natural occurrence, distribution, biosynthesis, metabolism and function in plants, flavonoids are becoming of increasing importance in applied science. Consumers and food manufacturers have become interested in flavonoids for their medicinal properties and their potential role in the prevention of cancers and
cardiovascular disease. Flavonoids are commonly referred to as bioflavonoids. The term refers to a class of plant secondary metabolites. According to the IUPAC nomenclature, they can be classified into:

- Flavonoids, derived from 2-phenylchromen-4-one structure
- Isoflavonoids, derived from 3-phenylchromen-4-one structure
- Neoflavonoids, derived from 4-phenylcoumarine structure

1.2 Chemistry of flavonoids

Flavonoids are polyphenolic compounds possessing 15 carbon atoms; two rings joined by a linear three carbon chain.

The chemical structure of flavonoids is based on a C\textsubscript{15} skeleton with a chromane ring bearing a second aromatic ring B in position 2, 3 or 4.
In a few cases, the six-membered heterocyclic ring C may occur in an isomeric open form or be replaced by a five-membered ring. The oxygen bridge involving the central carbon atom (C-2) of the 3 carbon chain occurs in a rather limited number of cases, where the resulting heterocyclic is of the furan type.

Various subgroups of flavonoids are classified according to the substitution patterns of ring C. Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification. Examples of the subgroups are: anthocyanins, isoflavonoids, chalcones, flavanones, flavones, flavonols, flavanonols and flavanols/flavan-3-ols.
Table 1.1 Subgroups of flavonoids

<table>
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<th>Subgroup</th>
<th>Ion skeleton</th>
<th>Structural formula</th>
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<tr>
<td>Anthocyanins</td>
<td>2-phenylchromenylium</td>
<td><img src="image" alt="Anthocyanins" /></td>
</tr>
<tr>
<td>Isoflavonoids</td>
<td>3-phenylchromen-4-one</td>
<td><img src="image" alt="Isoflavonoids" /></td>
</tr>
<tr>
<td>Chalcones</td>
<td>1,3-diphenyl-2-propen-1-one</td>
<td><img src="image" alt="Chalcones" /></td>
</tr>
<tr>
<td>Flavanones</td>
<td>2,3-dihydro-2-phenylchromen-4-one</td>
<td><img src="image" alt="Flavanones" /></td>
</tr>
<tr>
<td>Flavones</td>
<td>2-phenylchromen-4-one</td>
<td><img src="image" alt="Flavones" /></td>
</tr>
<tr>
<td>Flavonols</td>
<td>3-hydroxy-2-phenylchromen-4-one</td>
<td><img src="image" alt="Flavonols" /></td>
</tr>
<tr>
<td>Flavanonols</td>
<td>3-hydroxy-2,3-dihydro-2-phenylchromen-4-one</td>
<td><img src="image" alt="Flavanonols" /></td>
</tr>
<tr>
<td>Flavanols or Flavan-3-ols</td>
<td>2-phenyl-3,4-dihydro-2H-chromen-3-ol</td>
<td><img src="image" alt="Flavanols or Flavan-3-ols" /></td>
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Flavonoids are often hydroxylated in positions 3, 5, 7, 3', 4' and 5' and frequently these hydroxyl groups are methylated, acetylated, prenylated or sulphated. Flavonoid glycosides are found in plants and are present as O- or C- glycosides; O bonding occurs more frequently than C bonding. The O-glycosides have sugar substituents bound to a hydroxyl group located at position 3 or 7 of the aglycone, whereas the C-glycosides have sugar groups bound to carbon 6 or 8 of the aglycone. The most common sugars are rhamnose, glucose, galactose and arabinose. Flavonoid-diglycosides are also found and the disaccharides contain glucose and rhamnose, for example 1 → 6 linked in neohesperidose and 1 → 2 linked in rutinose. The sugars are sometimes further substituted by acyl residues (malonate and acetate) (Stumpf and Conn, 1981).

Anthocyanins are glucosides of anthocyanidins. The anthocyanidins use the flavylium (2-phenylchromenylium) ion skeleton. Examples are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin. Anthocyanins are water-soluble vacuolar pigments that may appear red, purple or blue depending on the prevailing pH. They occur in all tissues of higher plants, including leaves, stems, roots, flowers and fruits. Anthoxanthins are their clear, white to yellow counterparts occurring in plants. Anthocyanins have been shown to act as a “sunscreen”, protecting cells from high-light damage by absorbing blue-green and UV light. Plants rich in anthocyanins include Vaccinium species, such as blueberry, cranberry and bilberry, cherry and red grape. Anthocyanins are also important aesthetically and economically, since their stability is of significance in the marketability of the plant products (Wu et al., 2004).
The isoflavonoids are colourless and they have a limited taxonomic distribution within the *Leguminosae*. It has been reported that in terms of biosynthetic precursors acetate gives rise to ring A while phenylalanine, cinnamate and cinnamate derivates are incorporated into ring B and the 3 carbon atoms of the heterocyclic ring. Since chalcones and flavanones are efficient precursors of isoflavonoids, the required aryl migration of ring B from position 2 to position 3 of the phenylpropanoid precursor, must take place after formation of the basic C15 skeleton.

Chalcone is an aromatic ketone that forms the central core for a variety of important biological compounds, which are known as chalcones and has a backbone of 1,3-diphenyl-2-propen-1-one (Table 1.1). It is known also by names such as chalkone, benzyldeneacetophenone and phenyl styryl ketone. Chalcones are prepared by an aldol condensation between benzaldehyde and acetophenone in the presence of sodium hydroxide as a catalyst (Toda *et al*., 1990).
Flavones have a backbone 2-phenylchromen-4-one (Table 1.1). Natural flavones are apigenin, luteolin and tangeritin, while diosmin and flavoxate are synthetic flavones. Synthesis of flavones involves several methods such as the Allan-Robinson reaction, Auwers synthesis, Baker-Venkataraman rearrangement and Algar-Flynn-Oyamada reaction. The other method is the dehydrative cyclization of certain 1,3-diaryl diketones (Sarda et al., 2006).
Flavanones are the dominant flavonoid class in the genus citrus and 2,3-dihydro-2-phenylchromen-4-one is their skeleton. Examples are hesperetin, naringenin, eriodictyol and homoeriodictyol. 3-Hydroxy-2-phenylchromen-4-one skeleton is the skeleton of flavonols and some of the well known examples are quercetin, kaempferol, myricetin, fisetin, isorhamnetin, pachypodol and rhamnazin. Flavanonols are based on the backbone 3-hydroxy-2,3-dihydro-2-phenylchromen-4-one and common examples are taxifolin and dihydrokaempferol. Flavanols, also known as flavan-3-ols have skeleton 2-phenyl-3,4-dihydro-2H-chromen-3-ol and some of the examples are catechin, gallocatechin, gallocatechin 3-gallate and epigallocatechin (Table 1.1).

There are other groups of chromane derivatives where ring B is located at position 4, such as 4-phenyl-coumarins and they are called neoflavonoids. The isoflavonoids and neoflavonoids may be regarded as abnormal flavonoids.

4-phenyl coumarin
1.3 Health benefits of flavonoids

Flavonoids are most commonly known for their antioxidant activity. The widespread distribution of flavonoids, their variety and relatively low toxicity unlike other active plant compounds e.g. alkaloids, means that animals and humans may ingest significant quantities in their diet. They have been referred to as “nature’s biological response modifiers” because of the strong experimental evidence of their inherent ability to modify the body’s reaction to allergens, viruses and carcinogens. Flavonoids have several benefits to human health, including antioxidant activities, metal chelation (Brown et al., 1998; Rice-Evans et al., 1996), and antiproliferative, anticarcinogenic, antibacterial, anti-inflammatory, antiallergic and antiviral effects (Rice-Evans et al., 1996).

Chalcones show antibacterial, antifungal, antitumor and anti-inflammatory properties. Some of them demonstrated the ability to block voltage dependent potassium channels. Quercetin has demonstrated anti-inflammatory activity because of direct inhibition of several initial processes of inflammation and also exerts potent antioxidant activity and vitamin C-sparing action. It shows anti-tumour (Paliwal et al., 2005) and antidepressant properties. Quercetin may have positive effects in helping to prevent cancer, prostatitis, heart disease, cataracts, allergies and respiratory diseases such as bronchitis and asthma. Rutin is an antioxidant and therefore plays a role in inhibiting some cancers. It strengthens the capillaries, so can reduce the symptoms of haemophilia. Rutin attaches to the iron ion Fe$^{2+}$, preventing Fe$^{2+}$ from binding to hydrogen peroxide, which would create a highly reactive free radical that may damage cells. Catechin can benefit both humans and animals. Reduction in atherosclerotic plaques was observed in animal models (Chyu, 2004) as well as
reduction in carcinogenesis *in vitro*. Green tea catechins have been shown to possess antibiotic properties due to their role in disrupting a specific stage of the bacterial DNA replication process. Luteolin is thought to play an important role in the human body as an antioxidant, a free radical scavenger, an agent in the prevention of inflammation, a promoter of carbohydrate metabolism and an immune system modulator. These characteristics of luteolin are also believed to play an important part in the prevention of cancer. Luteolin as a biochemical agent can dramatically reduce inflammation and the symptoms of septic shock.

1.4 Biosynthesis of flavonoids

In the past years, considerable progress has been made in elucidating the biosynthesis of flavonoids. The reactions of anthocyanins are still unknown, but the essential steps of the biosynthetic pathway of the main flavonoid classes are clear. 4-coumaroyl-CoA and malonyl-CoA are the direct precursors of flavonoids and both precursors are derived from carbohydrates. Malonyl-CoA is synthesized from the glycolysis intermediate acetyl-CoA and carbon dioxide, the reaction being catalysed by acetyl-CoA carboxylase. The synthesis of 4-coumaroyl-CoA involves the shikimate/arogenate pathway, which is the main route to the aromatic amino acids phenylalanine and tyrosine in the higher plants (Jensen, 1985). Transformation of phenylalanine to cinnamic acid is catalysed by phenylalanine ammonia-lyase which provides the link between primary metabolism and the phenylpropanoid pathway. Aromatic hydroxylation of cinnamate by cinnamate 4-hydroxylase leads to *p*-coumaric acid which is further transformed to 4-coumaroyl-CoA by action of 4-coumarate:CoA ligase (Scheme 1.1).
Scheme 1.1 Biosynthesis of Malonyl-CoA and 4-Coumaroyl-CoA
Flavonoid biosynthesis involves the condensation of 3 molecules of malonyl-CoA with 4-coumaroyl-CoA, which forms the C₁₅ chalcone intermediate (4, 2', 4', 6'-tetrahydroxychalcone). The reaction is catalysed by chalcone synthase. Flavonoids, aurones and other diphenylpropanoids are derived from the chalcone intermediate. The first flavonoid formed is (2S)-flavanone (naringenin) by a transformation involving the stereospecific action of chalcone isomerase. Oxidative rearrangement of the naringenin, involving a 2,3-aryl shift, yields an isoflavone (genistein) and the reaction is catalysed by 2-hydroxyisoflavanone synthase. Introduction of a double bond between C-2 and C-3 of the flavanone leads to the flavone class e.g. apigenin and two different enzymes catalyse the reaction (a dioxygenase and a mixed-function mono-oxygenase). Dihydroflavonols (e.g. dihydrokaempferol) are formed by direct hydroxylation of flavanones in position 3 and this reaction is catalysed by the dioxygenase, flavanone 3-hydroxylase (Scheme 1.2).
Scheme 1.2 Biosynthesis of Flavanone, Dihydroflavonols, Flavone and Isoflavone
Dihydroflavonols are biosynthetic intermediates towards the formation of flavonols, catechins, proanthocyanidins and anthocyanidins. Flavonols (e.g. kaempferol) are formed by introduction of a double bond between C-2 and C-3 of dihydroflavonols, the reaction being catalysed by a dioxygenase enzyme, flavonols synthase. Reduction of the carbonyl group at position 4 of dihydroflavonols yields flavan 2,3-trans-3,4-cis-diols (leucopelargonidin). These compounds are also known as leucoanthocyanidins, and are the intermediate precursors for the synthesis of catechins and proanthocyanidins. Catechins (e.g. afzelechin) are synthesized by the action of flavan-3,4-cis-diol reductase, while proanthocyanidins (propelargonidin B-3) are synthesized by a condensation of catechins with leucoanthocyanidins (Scheme 1.3).
Scheme 1.3 Biosynthesis of flavonols, Leucoanthocyanidins, Catechins and Proanthocyanidins
The reaction steps from leucoanthocyanidins to anthocyanidins (e.g. pelargonidin) are not well known so far. An obligatory reaction in the sequence of a glycosylation in position 3 of anthocyanidins leads to formation of anthocyanin (e.g. pelargonidin 3-glucoside) (Scheme 1.4).

Scheme 1.4 Biosynthesis of Anthocyanidins and Anthocyanin
As a result of extensive genetic studies in a wide variety of plants, mutants are available for each step in flavonoid biosynthesis from chalcone formation up to complex modifications of the anthocyanin molecule. Flowers of genetically defined plants have proved to be very valuable for supplementation experiments with potential precursors and for correlating single genes with particular enzymes. Such correlations have given an indication that an enzyme capable of catalysing a particular step in vitro often has the same function in vivo.

1.5 *Carpobrotus* species as sources of flavonoids

The genus *Carpobrotus* belongs to the family *Aizoaceae*, kingdom *Plantae*, phylum *Magnoliophyta*, class *Magnoliopsida* and order *Caryophyllales*. It is a genus of ground-creeping plants, with succulent leaves and large daisy-like flowers. There are 25 species in this genus, having a disperse distribution worldwide. Some of the species are *C. acinaciformis*, *C. aequilaterus*, *C. chilensis*, *C. edulis*, *C. quadrifidus*, *C. glaucescens*, *C. modestus*, *C. rossii*, *C. virescens* and many more. They are mostly South African, but there is one species in South America, another in California and Oregon, and four in Australia (Chesselet et al., 2003).
*Carpobrotus* is well known in South Africa from the names “mesembryanthemum” in English, “vygies” in Afrikaans and “iqina” in Xhosa (Watt and Breyer-Brandwijk, 1962). *Carpobrotus* species normally range from the Western Cape up to KwaZulu-Natal. *C. edulis* is presently propagated globally, while *C. acinaciformis* is more restricted to the Western Cape (Bowie, 2002). The *Aizoaceae* is considered as one of Southern African’s most diverse and abundant plant families, but also the least studied in terms of its medicinal potential. The role of many species in this family is soil stabilizers. *C. acinaciformis* is often used for ground cover due to its fast growth, ground hugging characteristics and resistance to fire. *Carpobrotus* is also drought resistant.
The fleshly fruits of *Carpobrotus* are edible. Fruits of the species are allowed to dry on the plants and then picked, and the inside thereof is eaten. Dried fruits are soaked for the preparation of a traditional Cape recipe for making a jam or to be preserved (Wisura and Glen, 1993; Watt and Breyer-Brandwijk, 1962). *C. edulis* and *C. acinaciformis* are commonly used as herbs, from which preparations are gargled to treat infections of the mouth and throat. They are also effective against toothache, earache, and oral thrush. Leaf juice of *C. edulis* is taken orally for dysentery (Forbes and Peter, 1986), digestive troubles, tuberculosis and as a diuretic and styptic (Watt and Breyer-Brandwijk, 1962). *C. edulis* is highly astringent and is applied externally to treat eczema, wounds and burns (Watt and Breyer-Brandwijk, 1962; Rood, 1994).

The boiled fruit and leaf pulp of *C. acinaciformis* are used in the treatment of pulmonary tuberculosis and other chest conditions and also fruits can be used for heart conditions, as well as diarrhoea. The leaf pulp is generally used for throat, skin and eye infections (Van Wyk *et al.*, 1997; Van Wyk and Gericke, 2000; Watt and Breyer-Brandwijk, 1962). Syrup made from the fruit is said to have laxative properties. A mixture of leaf juice, honey and olive oil in water is an old remedy for TB.

The genus contains malic acid, citric acids and calcium salts (Watt and Breyer-Brandwijk, 1962). In a recent publication by Van der Watt and Pretorius (2001), six active compounds were purified and identified as flavonoids. The flavonoids are rutin, neohesperidin, hyperoside and catechin, a phenolic compound ferulic acid and unknown flavonoid. The leaf juice of *C. edulis* is mildly antiseptic and highly astringent, so the beneficial effects are probably due to the presence of tannins (Van Wyk *et al.*, 1997).
There have been reports showing that species of *Carpobrotus* have antibacterial activity. The flavonoids investigated by Van der Watt and Pretorius (2001) from *C. edulis* showed activity against *Staphylococcus epidermis*(+), *Staphylococcus aureus*(+), *Moraxella cattharalis*(-) and *Pseudomonas aeruginosa*(-). *C. acinaciformis* showed antibacterial against *S. aureus* and less against *M. smegmatis* (Bowie, 2002). *C. mellei, C. muirrii* and *C. quadrifidus* showed antimicrobial activity against *S. aureus* and *M. smegmatis* in the disc diffusion method, where inhibition was observed as clear zones on the TLC plate (Springfield, 2001).
CHAPTER 2

LITERATURE REVIEW OF DIFFERENT TECHNIQUES FOR DETERMINATION OF FLAVONOIDS

2.1 Introduction

An important aspect of flavonoid analysis is whether to study them in their conjugated forms or as aglycones. In animal biological fluids (serum, plasma and urine) they exist as glucuronide and sulphate conjugates, whereas in plants, medicine and food they exist as intact conjugates in their natural original form (Stevens et al., 1999; Ducrey et al., 1995; Grayer et al., 2000). Due to the significant increase in the number of target analytes, analyses become more complicated and so more selective and sensitive analytical methods are required.

2.2 Sample treatment

Over the years many sample pre-treatment methods have been developed to determine flavonoids in various sample types. Three main types of flavonoid-containing matrices are plants, food and liquid samples (drinks and biological fluids). The solid samples are first homogenized, which may be preceded by freeze drying with liquid nitrogen. The next step involves isolation of the analyte(s). For this purpose, solvent extraction (SE) followed by solid-phase extraction (SPE) is the most widely used technique. This is due to its ease of use and wide-ranging applicability. Soxhlet extraction is used less frequently to isolate flavonoids from solid samples. For liquid samples, the usual approach is to first filter and centrifuge, then the sample is either directly injected into the separation system or more often, the analytes are isolated using liquid-liquid extraction (LLE) or solid-phase extraction (SPE). Examples
involving these procedures are shown in Table 2.1. For solvent extraction and Soxhlet extraction, aqueous methanol or acetonitrile is used as solvent and for liquid-liquid extraction (LLE), ethyl acetate or diethyl ether containing small amount of acid is used as extraction solvent. LLE is usually directed at the isolation of aglycones, whereas other methods may lead to the isolation of both aglycones and conjugates. If aglycones are the target analytes, chemical hydrolysis is performed with hydrochloric acid or formic acid at elevated temperatures (80 – 100 °C) or by refluxing with acid in the presence of ethanol, although enzymatic hydrolysis with β-glucuronidase or β-glucosidase may also be used (Nystrom et al., 1954; Zhang and Brodbelt, 2004).
Table 2.1 Liquid-liquid extraction (LLE), Solvent extraction (SE) and Soxhlet extraction procedures for flavonoids

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Method</th>
<th>Solvent</th>
<th>Sample</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzein and Genistein</td>
<td>Soxhlet</td>
<td>Methanol:water</td>
<td>Soybean milk, farina and meat</td>
<td>Klejduš et al., 2004</td>
</tr>
<tr>
<td>Epicatechin gallate and Epigallocatechin</td>
<td>LLE</td>
<td>Ethyl acetate:water</td>
<td>Green tea</td>
<td>Baumann et al., 2001</td>
</tr>
<tr>
<td>Epicatechin, catechin, rutin, apigenin, luteolin and quercetin</td>
<td>SE</td>
<td>Methanol</td>
<td><em>Ginkgo biloba</em> leaves</td>
<td>Cao et al., 2002</td>
</tr>
</tbody>
</table>
Other sample treatments such as solid-phase extraction (SPE), matrix solid-phase dispersion (MSPD) and solid-phase micro-extraction (SPME) are also used for isolation of analytes. Compared to the traditional extraction methods, these techniques can be easily automated; solvent consumption is low and analysis times are short. For solid-phase extraction (SPE), alkyl-bonded silica or copolymer sorbents are used for analyte extraction and enrichment from aqueous samples and sample extracts, primarily in environmental, pharmaceutical and biomedical analysis. The sorbent is often C18 bonded silica and the sample solution and solvents are usually slightly acidified to prevent ionization of the flavonoids, which will reduce their retention. A new SPE method uses a molecularly imprinted polymer as sorbent. The molecularly imprinted polymer is highly selective for the target analyte and has good mechanical and thermal stability (Molinelli et al., 2002; Ramström et al., 2001). The disadvantage of the molecularly imprinted polymer in SPE is that a newly designed polymer is required for each application; as no single polymer is compatible with all analytes. Matrix solid-phase dispersion (MSPD) enables the extraction of analytes from samples homogeneously dispersed in a solid support; usually a C18 or C8 bonded silica. The sample extraction and clean up are carried out simultaneously with good recoveries and precision. MSPD has been used to determine pesticides in fruits, vegetables, beverages and foods (Fernández et al., 2000; Kristenson et al., 2001). In solid-phase micro-extraction (SPME) a fused silica fibre coated with polyacrylate or polydimethylsiloxane as a stationary phase is used to extract analytes from a liquid or gaseous sample, or from the headspace above a liquid sample. Organic solvent consumption in SPME is less than that in solid-phase extraction and is a simple straightforward technique. SPME is generally combined with gas chromatography (GC) analysis for the extraction of semi-volatile organic compounds from
environmental, biological and food samples (Pawliszyn, 1999; Kataoka et al., 2000).

For analysis of non-volatile and polar compounds, SPME is coupled with liquid chromatography (LC) (Pawliszyn, 1999). Examples of these procedures are shown in Table 2.2.
Table 2.2 Solid-phase extraction (SPE), Matrix solid-phase dispersion (MSPD) and Solid-phase micro-extraction (SPME) procedures for flavonoids

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Method</th>
<th>Sample</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzein and Genistein</td>
<td>SPE</td>
<td>Plasma</td>
<td>Grayer et al., 2000, Wähälä et al., 2002, Yang et al., 2004</td>
</tr>
<tr>
<td>Isoflavone</td>
<td>MSPD</td>
<td><em>Radix astragali</em> and <em>Astragalus membranaceus</em></td>
<td>Xiao et al., 2004</td>
</tr>
<tr>
<td>Genistein and Daidzein</td>
<td>SPME</td>
<td>Human urine</td>
<td>Wähälä et al., 2002</td>
</tr>
</tbody>
</table>
2.3 Chromatography

2.3.1 Thin layer chromatography (TLC)

Historically, paper chromatography (PC) has been the preferred method for flavonoid analysis and relative mobility data are available for a large variety of compounds (Markham and Wilson, 1989). Not all laboratories prefer PC, and hence thin layer chromatography (TLC) is used as an alternative. Since the early 1960s, TLC has been used in flavonoid analysis. It is useful for quick analyses of plant or medicinal extracts for pharmacologically active compounds before they are analyzed by instrumental techniques. In most cases silica gel is used as stationary phase and plates are developed with either a combination of 2-(diphenylboryoxo) ethylamine and polyethylene glycol or with AlCl₃. Detection is usually performed using UV light at 350 – 365 nm or 250 – 260 nm or with densitometry at the same wavelengths. The relative mobility and appearance of spots under UV, before and after spraying with various reagents enable a good approximation of structural type. The solvent system comprising of ethyl acetate: formic acid: acetic acid: water gives a good range of mobility for flavone and flavonol glycosides, R_f: diglycosides < monoglycosides < aglycones. However, anthocyanins are best analyzed by TLC using a more acidic solvent system. Examples of studies of flavonoids by TLC are shown in Table 2.3. Soczewinski et al. (2004) used double-development TLC to separate a flavonoid mixture containing nine glucosides and seven aglycones. The more polar glycosides were separated using an eluent with high solvent strength, and after solvent evaporation, the aglycones were separated in the same direction using an eluent with relatively weak solvent strength.
Table 2.3 Examples of studies of flavonoids by TLC, HPTLC and 2D-TLC

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Method</th>
<th>Stationary phase</th>
<th>Sample</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luteolin</td>
<td>TLC</td>
<td>Silica</td>
<td><em>Bacopa-monniera, Cuminum cuminum fruit, Achillea millefolium flower</em></td>
<td>Srinivas et al., 2004</td>
</tr>
<tr>
<td>Orientin and Isoorientin</td>
<td>HPTLC</td>
<td>Silica</td>
<td><em>Passiflora leaves</em></td>
<td>Pereira et al., 2004</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>2D-TLC</td>
<td>Cyanopropyl-bonded silica</td>
<td><em>Sambucus nigra</em></td>
<td>Hawryl et al., 2002</td>
</tr>
</tbody>
</table>
Quantification is usually not the main goal of TLC studies; however, densitometry has been used in several studies to achieve this goal, (Jamshidi et al., 2000; Janeczko et al., 2004). For example, in propolis, several flavonols, flavanones and phenolic acids were quantified using two-dimensional TLC followed by densitometry at 254 and 366 (Medic-Saric et al., 2004). 2D-TLC in cyanopropyl-bonded silica was used to separate 8 flavonoids and 3 phenolic acids in *Flos sambuci* (Hawryl et al., 2002).

### 2.3.2 Liquid chromatography (LC)

The distinctive UV-Vis spectra of most flavonoids, the widespread availability of high performance liquid chromatography (HPLC) systems with multiwavelength capacity, and the ability to record on-line spectra, has made HPLC the method of choice for flavonoid analysis (Vande Casteele et al., 1982; Pietrogrande and Kahie, 1994). Liquid chromatography (LC) of flavonoids is usually carried out in the reversed-phase (RP) mode on C8 or C18 bonded silica columns, however, other phases such as silica, Sephadex and polyamide have also been used. Gradient elution is often used, starting with a predominantly aqueous phase such as water containing acetate or formate buffer and then introducing an increasing proportion of an organic solvent such as methanol or acetonitrile. As most flavonoids are ionisable, some acid is usually added to the mobile phase to control the pH. The order of elution is usually from most polar through to least polar; thus triglycosides and higher glycosides are eluted early, along with most anthocyanin glycosides, followed by di- and monoglycosides and then acylated or alkylated glycosides and aglycones. Phosphate buffers are no longer used, because of the dreaded contamination of ion sources when mass spectroscopic detection is used. LC is generally performed at room temperature,
but temperatures up to 40 °C are recommended to reduce the time of analysis and because thermostated columns give more reproducible elution times.

2.3.2.1 Detectors in liquid chromatography

2.3.2.1.1 Ultra-violet (UV) detector

All flavonoid aglycones contain at least one aromatic ring and consequently, absorb UV light efficiently. The first maximum in the range 240 – 285 nm is due to the A-ring and the second maximum which is in the 300 – 550 nm is due to the substitution pattern and conjugation of the C-ring (Mabry et al., 1970). Substituents such as methyl, methoxy and non-dissociated hydroxyl groups effect only minor changes in the position of the absorption maxima. UV spectrophotometry was a popular technique to detect and quantify flavonoid aglycones. Recently, UV detection became the preferred tool in LC-based analyses and LC with multiple wavelengths or diode-array UV detection is a fully satisfactory tool in studies dealing with screening, quantification, or a provisional sub-group classification of the main aglycones. Another advantage the analyst has in flavonoid analysis is the distinctive UV-vis spectra of these compounds where minor differences in structure are often observed as significant differences in their UV spectra. Modern instrumental techniques enable researchers to gain much more information regarding the mass and UV-Vis spectra of individual components in a complex mixture. A combination of some more traditional analytical techniques combined with these modern techniques enables at least a partial identification of most flavonoid components without large-scale purification of the individual compounds.
2.3.2.1.2 Fluorescence and Electrochemical detectors

Fluorescence detection is only used occasionally because the number of flavonoids that exhibit native fluorescence is limited. Subgroups that show native fluorescence include the isoflavones (De Rijke et al., 2002), flavonoids with OH group in position 3 (Sengupta and Kasha, 1979; Stoggl et al., 2004) and methoxylated flavones (Huck and Bonn, 2001). Among isoflavones, only those that do not have an OH group in position 5, show strong native fluorescence. Some flavonols show native fluorescence, where the OH group in position 3 is involved in excited state intramolecular proton transfer, which causes solvent dependent dual emission, that is, two emission bands show up the ratio thereof depending on the solvent composition (Sengupta and Kasha, 1979; Wolfbeis et al., 1983; Bader et al., 2004). Electrochemical detection can also be used, because most flavonoids are electroactive due to the presence of phenolic groups. Compared to fluorescence detection, the limit of detection is lower (Zhong et al., 2003). Reverse phase liquid chromatography electrochemical detection was used to evaluate the antioxidant activity of phenolic compounds and flavonoids by measuring the accelerated auto-oxidation of methyl linoleate in anhydrous dodecane under strong oxidizing conditions (Peyrat-Maillard et al., 2000).
2.3.2.2 Liquid Chromatography-Mass Spectroscopy (LC-MS)

Single-stage mass spectroscopy is used in combination with ultraviolet detection to facilitate the confirmation of the identity of flavonoids in a sample, using standards and reference data, while for the identification of unknowns, tandem mass spectrometry (MS/MS or (MS<sup>n</sup>) is used. In the LC-MS analysis of flavonoids, interfaces such as atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), electron ionization (EI) (Harborne, 1989), chemical ionization (CI) (Barbuch et al., 1989), fast atom bombardment (FAB) (Ma et al., 2000) and matrix-assisted laser desorption ionization (MALDI) (Wang and Sporns, 1999; Wang and Sporns, 2000) have been used. For APCI and ESI both positive and negative ionization are applied. ESI is more frequently used in flavonoid analysis, but APCI is starting to be used more frequently and in some cases a better response is obtained (De Rijke et al., 2003; Justesen et al., 1998; Boué et al., 2003). The pH of the LC eluent is influenced by composition and the nature of the buffer components added. The most common additives are acetic acid, formic acid, ammonium-acetate and ammonium-formate (Andlauer et al., 1999; Hansen et al., 1999; De Rijke et al., 2003). Trifluoroacetic acid has been used, although it suppresses the ionization due to ion-pairing and surface-tension effects (Da Costa et al., 2000). Mass analysers such as quadrupole and ion-trap gave mass spectra for flavonoids which are closely similar, even though relative abundances of fragment ions and adducts do show differences. The advantage of ion-trap is the possibility to perform MS<sup>n</sup> experiments (De Rijke et al., 2003).
2.3.3 Gas Chromatography (GC) and Capillary electrophoresis (CE)

Gas chromatography (GC) is used less frequently than liquid chromatography in flavonoid analysis. Analysis of flavonoids using GC started in the early 1960s, and the first published paper, described how derivatized flavonoids were separated on a semi-preparative scale using SE-30 silicone polymer column with subsequent thermal conductivity detection. Fractions were collected for ultraviolet-visible and infrared spectroscopy (Narasimhachari and Rudloff, 1962). GC methods provide high resolution and low detection limit, but they are labour intensive because derivatization towards the formation of trimethylsilyl ether (TMS) derivatives is unavoidable, as this is necessary to increase the volatility of the flavonoids and to improve their thermal stability. For flavonoids containing more than one hydroxyl substituent, methylation may yield several derivatives which make quantification difficult. The conditions for GC have not changed much since the 1960s, except that fused silica capillary columns are now used. The capillary electrophoresis (CE) modes primarily used capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) with phosphate or borate buffer, capillaries of 50 - 100 μm I.D., voltages of 10 - 20 kV and 10 - 50 nL injection volumes. Detection can be performed with UV, fluorescence (Baumann et al., 2001) and MS detectors (Aramendia et al., 1995). CE was used for analysis of plants (Baumann et al., 2001; Baggett et al., 2002; Mellenthin and Galensa, 1999), vegetables (Dadakova et al., 2001), herbs (Chen et al., 2000) and plant or fruit derived products. CZE is only applicable to charged analytes and the charge-to-size ratios determine the electrophoretic migration times, while MEKC distinguishes between neutral and charged analytes. Capillary electrochromatography (CEC) has also been used for flavonoid analysis. CEC was compared with LC for analysis of hop acids and prenylated hop flavonoids (Vanhoenacker et al., 2001); the
capillary CEC column was packed with C18 bonded silica and acetonitrile-TRIS buffer was used for separation with UV detection. There are few reports about CE-MS for the determination of flavonoids and phenolic compounds (Lafont et al., 1999). Lafont et al (1999) analyzed a standard mixture of 8 phenolic compounds using CE-ESI (-)-MS.

2.4 Electrochemical Analysis

The branch of electrochemistry called voltammetry developed from the discovery of polarography in 1922 by the Czechoslovakian chemist Jaroslav Heyrovsky (1890-1967), for which he was awarded the Noble Prize in Chemistry in 1959. In the 1960s and 1970s significant advances were made in all areas of voltammetry (theory, methodology and instrumentation), which enhanced the sensitivity and expanded the repertoire of analytical methods. The coincidence of these advances with the advent of low-cost operational amplifiers also facilitated the rapid commercial development of relatively inexpensive instrumentation (Barek et al., 2001).

Voltammetry is an electrochemical method in which current is measured as a function of the applied potential. It is a branch of electrochemistry in which the electrode potential or the Faradaic current or both are changed with time. The principle of this technique is a measurement of the diffusion controlled current flowing in an electrolysis cell in which one is polarisable (Fifield and Kealey, 2000). In this technique a time dependent potential is applied to an electrochemical cell and then the current flowing through the cell is measured as a function of that potential. A plot of current which is directly proportional to the concentration of an electroactive species versus the applied potential is called voltammogram. The voltammogram provides
quantitative and qualitative information about the species involved in the oxidation or reduction reaction at the working electrode. The analytical advantages of the various voltammetry techniques includes excellent sensitivity with a very large useful linear concentration range for both inorganic and organic species, different used of solvents and electrolytes, a wide range of temperature, simultaneous determination of several analytes, the ability to determine kinetic and mechanistic parameters, a well developed theory and thus the ability to reasonably estimate the values of unknown parameters and the ease with which different potential waveforms can be generated and small currents measured (Fifefield and Kealey, 2000).

Analytical chemists routinely use voltammetric techniques for the quantitative determination of a variety of dissolved inorganic and organic substances. Inorganic, physical and biological chemists widely use voltammetric techniques for a variety of purposes, including fundamental studies of oxidation and reduction processes in various media, adsorption processes of surfaces, electron transfer and reaction mechanisms, kinetics of electron transfer processes and transport, speciation and thermodynamic properties of solvated species. Voltammetric methods are also applied to the determination of compounds of pharmaceutical interest and when couple with high pressure liquid chromatography (HPLC), they are effective tools for the analysis of complex mixtures (Zhang et al., 2002; Yardimer and Ozaltin, 2001; Volkoiv and Wesigwa, 2001; Woolever and Dewald, 2001). Since then many different forms of voltammetry have been developed, such as direct current polarography (DCP), normal pulse polarography (NPP), differential pulse polarography (DPP), square wave polarography (SWP), alternating current polarography (ACP), cyclic voltammetry
(CV), stripping voltammetry (SV), adsorptive stripping voltammetry (AdSV) and adsorptive catalytic stripping voltammetry (AdCSV) techniques.

2.4.1 Voltammetric measurements

Voltammetry technique makes use of a three electrode system: working electrode (WE), reference electrode (RE) and auxiliary electrode (AE). The whole system consists of a voltammetric cell with a various volume capacity, magnetic stirrer and gas line for purging and blanketing the electrolyte solution. A pontentiastat monitors the voltage over the working and an auxiliary electrode which is automatically adjusted to give the correct applied potential. A typical arrangement for a voltammetric electrochemical cell is shown in Figure 2.1.
2.4.1.1 Working, auxiliary and reference electrodes

The working electrode (WE) is the electrode where the redox reaction of electroactive species takes place and where the charge transfer occurs. It is potentiostatically controlled and can minimise errors from cell resistance. It is made of several different materials such as mercury, platinum, gold, silver, carbon, chemically modified and screen printed electrode. The ideal characteristics of the electrode are wide potential range, low resistance, reproducible surface and able to provide a high signal-to-noise response. The working electrode must be made of a material that will not react with the solvent or any component of the solution over as wide a potential range as possible (Wang, 2000).
Mercury has been used as the working electrode in the earlier voltammetry techniques. The advantages of the electrode include high over potential for the reduction of hydronium ion to hydrogen gas, new drops or new thin mercury films can be readily formed and the cleaning process removes problems that could be caused by contamination. The other advantage is the possibility to achieve a state of pseudostationary for linear sweep voltammetry (LSV) using higher scan rate. Miniaturised and compressible mercury electrode offer new possibilities in voltammetry especially for determination of biologically active species and surfactant (Dahmen, 1986). The disadvantages of the use of mercury electrode are limited anodic range which is due to the oxidation of mercury, and toxicity. There are three main types of mercury electrode used in voltammetry techniques; they are the hanging mercury drop electrode (HMDE), dropping mercury drop electrode (DME) and static mercury drop electrode (SMDE) (Metrohm, 2005). Flavonoids were determined using mercury electrode (Reichert and Obendorf, 1998; Ensafi and Hajian, 2006; Wu et al., 2008).

Other solid or metal electrodes are commonly used as working electrode are carbon, platinum, gold, graphite and diamond (Figure 2.2). Electrode based on carbon are currently in widespread use in voltammetric technique, primarily because of their broad potential window, low background current, rich surface chemistry, low cost, chemical inertness and suitability for various sensing and detection application. It includes glassy carbon (GCE), carbon paste electrode (CPE), chemically modified electrode (CME) and screen-printed electrode (SPE). The glassy carbon electrode is the most commonly used carbon electrode in electro analytical application. It is particularly useful for anodic studies, modified electrode and for stripping analysis.
(Zoulis and Efstathiou, 1996; Volikakis and Efstathiou, 2000; Zeng et al., 2006; Blasco et al., 2004; Ghica and Brett, 2005).

Figure 2.2 Carbon, platinum, gold, graphite and diamond electrodes

Voltammetric techniques also utilize microelectrodes with the size of electrode radius much smaller than the diffusion layer thickness as the working electrode. It is constructed from 5 different materials such as platinum, gold, palladium, silver and iridium. The diameter of microelectrodes is smaller to enhance mass transport of analyte to the electrode surface due to smaller electrode than the diffusion layer. Hence, increasing signal-to-noise ratio and measurement can be made in highly resistive media due to decrease of the ohmic drop that results when the electrode size reduced (Andrieux et al., 1990a; Souza et al., 2006; Lafleur et al., 1990).
The second electrode used in the voltammetric system is auxiliary electrode (AE). The AE is made of an inert conducting material typically a platinum electrode wire. It provides a surface for a redox reaction to balance the process that occurred at the surface of the working electrode. In order to support the current generated at the working electrode, the surface of the auxiliary electrode must be equal to or larger than of the working electrode. The function of the auxiliary electrode is to complete the circuit allowing charge to flow through the cell (Fifield and Haines, 2000).

The third electrode used in the voltammetric technique is reference electrode (RE). The RE provides a stable potential so that any change in cell potential is attributed to the working electrode. The major requirement for reference electrode is that the potential does not change during the recording voltammetric curve at different applied voltage (Heyrovsky and Zuman, 1968). The most common reference electrodes are the standard hydrogen electrode (SHE), calomel electrode (SCE) and silver/silver chloride electrode (Ag/AgCl). The SHE is rarely used because it is difficult to prepare and inconvenient to use. The Ag/AgCl electrode is the most common reference electrode, because it can be used at higher temperature (Harvey, 2000).
2.4.1.2 Solvent and supporting electrolyte

Electrochemical measurements are commonly carried out in a medium which consists of solvent containing a supporting electrolyte. Supporting electrolyte has to be added to dissolve sample in an attempt to achieve the following (Zutshi, 2006):

- To make solution conductive
- To control the pH value so that organic substances are reduced in a given potential range and inorganic substances are not hydrolysed
- To ensure the formation of such complexes that give well developed and well separated waves
- To shift the hydrogen evaluation towards more negative potentials and to eliminate catalytic effects on hydrogen evolution
- To suppress migration by addition of surface active substances to the supporting electrolyte

2.4.1.3 Current in voltammetry

When an analyte is oxidised at the working electrode, a current passes electrons through the external electric circuit to the auxiliary electrode, where reduction of the solvent or other components of the solution matrix occurs. Reducing an analyte at the working electrode requires a source of electrons, generating a current that flows from the auxiliary electrode to the cathode. A current resulting from redox reaction at the working electrode and auxiliary electrode is called a Faradaic current. A current due to the analyte reduction is called cathodic current and the anodic current is due to oxidation reaction. The magnitude of the Faradaic current is determined by the rate of the resulting oxidation or reduction reaction at the electrode surface. Two factors contribute to the rate of the electrochemical reaction: the rate at which the reactants
and products are transported to and from the electrode and the rate at which electron pass between the electrode and the reactants and products in solution (Harvey, 2000).

There are three modes of mass transport that influence the rate at which reactant and products are transported to and from the electrode surface, they are diffusion, migration and convection. The rate of mass transport is one factor influencing the current in voltammetry experiment. When electron transfer kinetics is fast, the redox reaction is in equilibrium and the concentrations of reactants and products at the electrode are those specified by Nernst equation. Such systems are considered electrochemically reversible. When the transfer kinetics is sufficiently slow, the concentration of reactants and products at the electrode surface and the current differ from that predicted by the Nernst equation, in this case the system is electrochemically irreversible (Harvey, 2000).

2.4.2 Types of voltammetric techniques

2.4.2.1 Polarography

Polarography is a subclass of voltammetry in which the working electrode is the dropping mercury electrode (DME). The technique has been widely used for the determination of many important reducible species since the DME has special properties particularly its renewable surface and wide cathodic potential range. Polarography is used extensively in the analysis of metal ion, inorganic anions and organic compounds containing easily reducible or oxidizable functional group (Dean, 1995) and also analyse antioxidant and eicosanoid enzyme inhibition properties of pomegranate seed oil and fermented juice flavonoids (Schubert et al., 1999).
The current for the polarographic plateau can be predicted by the Ilkovic equation (Ilkovic, 1934; Ewing, 1997; Heyrosky and Zuman, 1968):

\[ i_d = 708nD^{1/2}m^{2/3}t^{1/6}c^0 \]

where:
- \( n \): number of electrons transferred in the redox reaction
- \( D \): analyte diffusion coefficient (cm\(^2\) sec\(^{-1}\))
- \( m \): the rate of flow of the Hg through the capillary (g sec\(^{-1}\))
- \( t \): drop time (sec)
- \( c^0 \): the bulk analyte concentration (mol L\(^{-1}\))

### 2.4.2.2 Cyclic Voltammetry (CV)

Cyclic voltammetry (CV) is a potential controlled reversal electrochemical experiment. A cyclic potential sweep is imposed on an electrode and the current respond is observed. CV is an extension of linear sweep voltammetry in that the direction of the potential scan is reversed at the end of the first scan and the potential range is scanned again in the reverse direction. The potential can be cycled between the two switching potential for several cycles before the experiment is ended at the final potential (Gosser, 1993). Cyclic voltammetry has become an important and the most widely used electroanalytical technique in many areas of chemistry. It is rarely used for quantitative determinations, but it is widely used for the study of redox processes, for understanding reaction intermediates, and for obtaining stability of reaction products. The resulting plot of current versus potential is called a cyclic voltammogram (Figure 2.3). The important parameters in a cyclic voltammogram are
the peak potential \( E_{pc}, E_{pa} \) and peak currents \( i_{pc}, i_{pa} \) of the cathodic and anodic peaks, respectively. Reports have shown that cyclic voltammetry can be used to determine the electrochemical properties of flavonoids (Brett and Ghica, 2003; Ghica and Brett, 2005; Janiero and Brett, 2004; Chen et al., 2000; Rapta et al., 1995) and antioxidant activities of flavonoids (Cosio et al., 2006; Zielinska et al., 2008; He et al., 2007; Teixeira et al., 2005)

![Figure 2.3 A typical cyclic voltammogram](image)

**2.4.2.3 Pulse methods**

Pulse methods are techniques that use pulse waveform in recording its voltammetry which offers enhanced sensitivity and resolution. The advantages of pulse techniques is the waveform is designed so as to discriminate against non-faradic current, hence increase sensitivity (Wang, 2000). Differential pulse and square wave voltammetric techniques are the most commonly used pulse method.
2.4.2.3.1 Normal Pulse Voltammetry (NPV)

This technique uses a series of potential pulse of increasing amplitude. The current measurement is made near the end of each pulse, which allows time for the charging current to decay. It is usually carried out in an unstirred solution at dropping mercury electrode (DME), called normal pulse polarography or solid electrodes.

2.4.2.3.2 Differential Pulse Voltammetry (DPV)

This technique is comparable to normal pulse voltammetry in that the potential is also scanned with a series of pulse. However, it differs from normal pulse voltammetry because each potential pulse is fixed and is superimposed on a slowly changing base potential. Current is measured at two points for each pulse: (1) just before the application of the pulse and (2) at the end of the pulse. Subtraction of the first current sampled from the second provides a stepped peak shape derivative voltammogram (Figure 2.4). Differential pulse voltammetry was used for electrochemical analysis of flavonoids (Brett and Ghica, 2003; Ghica and Brett, 2005; Janiero and Brett, 2004).
2.4.2.3.3 Square Wave Voltammetry (SWV)

Square wave voltammetry is a large amplitude differential technique in which a waveform composed of a symmetrical square wave pulse of amplitude $E_{SW}$, superimposed on a base staircase potential of step height $\Delta E$, where the forward pulse of the square wave coincides with the staircase step, is applied to the working electrode. The current is sampled twice during each square wave cycle, once at the end of forward pulse and another at the end of the reverse pulse. Since the square wave modulation amplitude is very large, the reverse pulses cause the reverse reaction of the product of the forward pulse (Figure 2.5). The advantage of square wave voltammetry is that a response can be found at a high effective scan rate, thus reducing the scan time (Arranz et al., 1999; Ghoneim and Tawfik, 2004). Also it is excellent sensitivity and the rejection of background currents. There are several reports for determination of setraline in commercial products and ketorolac in human serum (Nouws et al., 2005; Radi et al., 2001) and electrochemical analysis of flavonoids (Brett and Ghica, 2003; Ghica and Brett, 2005; Janiero and Brett, 2004).

Figure 2.4 A typical differential pulse voltammogram
2.4.3 Electrochemical analysis of flavonoids

The flavonoids are a family of phenolic compounds that occur naturally in fruits, vegetables, legumes, nuts and seeds of vascular plants. Because of interdisciplinary interest on class of flavonoids, many authoritative books devoted to its isolation, analysis, identification and general chemistry and biochemistry have been published (Williams, 1986; Seikel, 1962; Markham 1994). Methods have been used to do this, such as high pressure liquid chromatography (HPLC) (Wang et al., 1998), reverse-phase (RP)-HPLC (Lu et al., 1999), liquid chromatography mass spectroscopy (LC-MS) (Heinig and Henion, 1999) and spectrophotometry (Deng et al., 1998). Investigations and applications of flavonoids in electroanalytical chemistry are rarely used (Volikakis and Efstathiou, 2000; Lunte, 1987). The method is found to be simple, convenient, reliable, reagent saving.
Phenolic compounds and flavonoids have one or more hydroxyl groups at various positions of the aromatic backbone; hence they can be electrochemically oxidizable. The oxidation reaction of flavonoids is strongly related to their structure, which contains several free phenolic hydroxyl groups, particularly $o$-phenolic ones (Ghica and Brett, 2005; Brett and Ghica, 2003; Kang et al., 2002). Flavonoids with $3',4',5'$-trihydroxy (pyrogallol type) and $3',4'$-dihydroxy (catechol type) substitution patterns are most easily oxidized, while flavonoids with trihydroxy type and phenol type substitution patterns are much harder to oxidize. The electrochemical oxidation of phenolic compounds is generally followed by chemical reactions such as a coupling reaction (Papouchdo et al., 1975; Waters, 1971; Richards et al., 1975; Bailey et al., 1983; Bailey and Ritchie, 1985; Ryan et al., 1980; Proudfoot and Ritchie, 1983; Golabi and Nematollahi, 1997; Zare and Golabi, 1999). Several mechanisms of phenolic oxidation have been suggested. There is mechanism involving one electron in the first oxidation step and this happens for compounds such as 4-hydroxybenzoic acid and ortho-, para- and meta-coumaric acids (Scheme 2.1). The other mechanism is a two electron oxidation and this happens for (-)-epicatechol and caffeic acid (Scheme 2.2). The reported mechanism of electrooxidation for quercetin, rutin, catechin and others involves a predissociation of a proton to give the monoanoionic species followed by a one electron one proton oxidation of the monoanoionic species to form a radical anion. Then the radical anion undergoes a second reversible one electron oxidation to form dehydro-flavonoid. The latter species is rapidly protonated and then dehydrated to yield the final product of $3',4'$-diquinone. The mechanism is similar to the reported oxidative mechanism of ascorbic acid on glassy carbon electrode (Kang et al., 2002; Dryhurst et al., 1982). For synapic acid, the mechanism involves
oxidation of the hydroxyl group yielding electrochemically active compounds, followed by dimerization and polymerization (Yakovleva et al., 2007).

Scheme 2.1 One electron transfer for 4-hydroxybenzoic acid

Scheme 2.2 Two electron transfer for caffeic acid
Coulometric measurements showed that when some catechols were oxidized at higher pHs, they exhibited more electrons involved in their oxidation than those expected from their structure (i.e. the number of OH moieties) (Ryan et al., 1980). The catechols with two OH moieties, number of electrons are 2 at pH < 7, but increase at pH > 7 (Hotta et al., 2001).

The electrochemical behaviour of flavonoids was studied by cyclic voltammetry (CV) and this technique is one of the most used technique (Cosio et al., 2006; Chevion et al., 2000; Hotta et al., 2001; Kilmartin and Hsu, 2003; Sousa et al., 2004). Other voltammetric techniques were also used for the investigation of electrochemical behaviour of quercetin, such as differential pulse voltammetry (DPV) and adsorptive stripping voltammetry (ASV) (Brett and Ghica, 2003; Vestergaard et al., 2005; He et al., 2005). CV and DPV have been used most often for analyzing flavonoids on glassy carbon electrodes (Brett and Ghica, 2003; Hotta et al., 2001; Blasco et al., 2004; Janeiro and Brett, 2004; Corduneanu et al., 2006). Kang et al (2002) quantified rutin using differential pulse voltammetry in several samples of Chinese medicines, while Hua et al (2008) used square wave stripping voltammetry for determining rutin in tablets. Malagutti et al (2006) reported a comparative study at glassy carbon electrode and rigid graphite-polyurethane composite electrode. Phosphate buffer was used as solvent electrolyte.
CHAPTER 3

EXPERIMENTAL

3.1 Chromatographic Studies

3.1.1 Apparatus and Reagents

Thin layer chromatography (TLC) was performed on aluminium plates coated with normal phase Merck Kieselgel 60 F254. The materials were weighed using Mettler Toledo AB104 analytical scale and all aqueous solutions were made up with high-purity water from a Millipore Milli-Q system (resistivity greater than or equal to 18 mΩ cm). Methanol, ethyl acetate and butanol were used for extraction and formic acid was purchased from Kimix (South Africa) and the extraction solvents were distilled before use except butanol. Quercetin, rutin, catechin, luteolin, apigenin, naringenin, and gallic acid were purchased from Sigma-Aldrich (South Africa). Chemicals such as potassium metabisulfite (K2S2O5), potassium chloride (KCl) and sodium acetate (CH3CO2Na) were purchased from Sigma-Aldrich (South Africa), while Folin-Ciocalteus phenol reagent, 4-(dimethylamino)-cinnamaldehyde (DMACA) were purchased from Merck (South Africa). Methanol, ethanol and hydrochloric acid (HCl) were purchased from Saarchem. 2,4-dinitrophenylhydrazine (DNPH), and sodium carbonate (Na2CO3) were purchased from Sigma-Aldrich (South Africa). Ultraviolet (UV) absorbance was measured using the instruments: Multiskan spectrum, Nicolet evolution 300 and Fluoroskan ascent were from thermo Electron Corporation. Liquid chromatography-mass spectrometer (LC-MS) analyses were performed using Waters API Q-TOF Ultima with negative electrospray ionization source (ESI - ).
3.1.2 Plant Materials

The fresh leaves of *C. quadrifidus* were collected on the 30\(^{th}\) November 2004 from Saldanha (Western Cape Province, South Africa), while those of *C. mellei* were collected from Montague (Western Cape Province, South Africa).

3.1.2.1 Preparation of plant materials

The leaves of *C. quadrifidus* and *C. mellei* were washed with distilled water and air dried for 10 minutes. The leaves were weighed, obtaining 506.53 g for *C. quadrifidus* and 570.19 g for *C. mellei*. The leaves were homogenized in methanol (MeOH) (300 mL) using a domestic blender. Additional methanol (500 mL) was added and the suspension was stirred for 2 days at room temperature. The suspension was filtered through muslin cloth and centrifuged (1500 R.P.M) for 20 minutes. The supernatant was removed and then evaporated to dryness under vacuum at 45 °C. The methanol extract for both plants gave a reddish brown colour.
Yield of crude methanol extract for *C. quadrifidus* = 28.54 g

Yield of crude methanol extract for *C. mellei* = 36.90 g

The crude methanol extract, as suspension in a mixture of water and methanol 3:1, v/v (100 mL), was successively partitioned between chloroform (CHCl₃), ethyl acetate (EtOAc) and then butanol (BuOH) (100 mL each). The partition was repeated twice for all the organic solvents. The chloroform extracts for both plant materials gave a green colour.

Yield of chloroform extract for *C. quadrifidus* = 1.77 g

Yield of chloroform extract for *C. mellei* = 1.30 g

The colour of the ethyl acetate extract for *C. quadrifidus* was light yellow and for *C. mellei* it was greenish yellow.

Yield of ethyl acetate extract for *C. quadrifidus* = 1.00 g

Yield of ethyl acetate extract for *C. mellei* = 950.00 mg

Butanol extracts for *C. quadrifidus* and *C. mellei* gave a reddish brown colour.

Yield of butanol extract for *C. quadrifidus* = 9.69 g

Yield of butanol extract for *C. mellei* = 8.90 g
3.1.2.2 Removal of tannins

3.1.2.2.1 Preparation of Sephadex LH-20 column

The method of Van der Watt and Pretorius (2001) was followed. Lipophilic sephadex LH-20 (20 g) was suspended in 200 mL of 80% ethanol. The suspension was stirred and poured into a coarse sintered glass funnel. Using gentle suction, the ethanol was slowly removed from the sephadex beads under vacuum.

3.1.2.2.2 Removal of tannins from plant extracts

Ethyl acetate and butanol extracts for both *C. quadrifidus* and *C. mellei* were dissolved in 25 mL of 80% ethanol and then poured over the sephadex bed. All the compounds, except the tannins were removed by washing gently with absolute ethanol (500 mL). Tannins were removed from the column by washing with 1 L of 50% acetone. The ethyl acetate extract gave a goldish colour, while the butanol extract gave a brick red colour.

Yield of ethyl acetate extract for *C. quadrifidus* = 410 mg

Yield of ethyl acetate extract for *C. mellei* = 230 mg

Yield of butanol extract for *C. quadrifidus* = 4.18 g

Yield of butanol extract for *C. mellei* = 3.87 g
FLOW CHART

Fresh Leaves

Methanol

Methanol Extract

Partition Exercise

Chloroform Extract

Ethyl Acetate Extract

Butanol Extract

Remove tannins

Ethyl Acetate Extract

Butanol Extract

Remove tannins
3.1.3 Chromatography

3.1.3.1 Sample preparation

A flavonoid standards cocktail containing quercetin, rutin, catechin, luteolin and apigenin with concentration of 200 μg mL⁻¹ were combined. The ethyl acetate and butanol extracts with a concentration of 5 mg mL⁻¹ were prepared for *C. mellei* and *C. quadrifidus*. All samples were dissolved in methanol and the sample solutions were filtered prior to injection/analysis.

3.1.3.2 Thin Layer Chromatography (TLC)

The ethyl acetate and butanol extracts of *C. mellei* and *C. quadrifidus* were analysed on normal phase TLC plates using Ethyl Acetate (EtOAc): Methanol (MeOH): Water (12:3:2, v/v/v) as eluent. The dried TLC plates were first viewed under UV light, then sprayed with vanillin/H₂SO₄ (25:1, v/v) spray reagent (Wagner and Bladt, 1996) and then heated on a hot plate. Quercetin, rutin, catechin, luteolin and apigenin were used as reference compounds.

3.1.3.3 Liquid Chromatography-Mass Spectroscopy (LC-MS)

A reverse phase column: Zorbax Eclipse XDB C18 with 5 μm particles size and dimensions 4.6 x 150 mm was used at room temperature. The mobile phase was 0.1% acetic acid in water (Solvent A) and 0.1% acetic acid in acetonitrile (Solvent B). Column elution was according to the following gradient: increasing the organic phase (Solvent B) to 100% over 28 minutes and finally decreased to 0% B over 7 minutes, the running time was 35 minutes with flow rate of 300 μL min⁻¹. The ultraviolet (UV) detector was set at 254 nm wavelength and 5 μL of sample was injected. Electrospray ionization was used as ion source and negative mode was applied. The capillary
temperature was 200 °C and the capillary voltage 3.5 kV. The desolvation temperature was 350 °C with desolvation gas at flow rate of 350 L h⁻¹. All data were collected as negative-ion spectra and MS was calibrated for a full mass scan over the 100-1000 range.

3.1.4 Determination of phenolic compounds

The methanol extracts of *C. mellei* and *C. quadrifidus* were used for these tests. The extracts were dissolved in methanol to make different concentration. The following concentrations were prepared: 5.39, 5.64 and 6.14 mg mL⁻¹ from the methanol extract of *C. mellei* and 5.27, 5.71 and 7.88 mg mL⁻¹ from *C. quadrifidus*.

3.1.4.1 Total polyphenolic content

The content of total polyphenolic compounds in methanolic plant extracts was determined by the Folin-Ciocalteu method (Folin and Ciocalteu, 1927). Gallic acid was used as a standard for the test. For the preparation of calibration curve, the following concentrations 0, 20, 50, 100, 250 and 500 mg L⁻¹ of ethanolic gallic acid solutions were used. Folin-Ciocalteu’s phenol reagent and the methanolic plant extract were diluted ten-fold. A solution of Na₂CO₃ (7.5%) was prepared by dissolving the salt in distilled H₂O. Aliquots of ethanolic gallic acid solutions (25 μL) were mixed with Folin-Ciocalteu’s phenol reagent (125 μL) and Na₂CO₃ (7.5%) solution (100 mL). A blue colour was observed and the mixtures were left at room temperature for 2 hours. The absorbance was measured at 765 nm and a calibration curve was drawn. Methanolic plant extracts were treated similarly and the absorbance was measured for the determination of total polyphenolic content. All determinations were performed in triplicate.
3.1.4.2 Flavanone content

Naringenin was used as a standard, at the following concentrations in methanol: 0.2, 0.5, 1.0, 1.5 and 2.0 mg mL\(^{-1}\). A solution of DNPH (0.1%) was prepared by dissolving 0.05 g of solid in 50 mL of methanol. Methanol (70 mL) and H\(_2\)O (30 mL) were added to 1 g of KOH to make a 1% KOH solution. Methanolic naringenin solutions (100 \(\mu\)L) and DNPH (200 \(\mu\)L) were mixed well and then heated in a waterbath (50 \(^{\circ}\)C) for 50 minutes. The mixtures were left standing at room temperature to cool down before 700 \(\mu\)L of 1% KOH were added. The resulting mixture was centrifuged (14000 R.P.M) for 1 minute and the supernatant (30 \(\mu\)L) was added to 270 \(\mu\)L of methanol. The absorbance was measured at 495 nm and then the calibration curve was drawn. The methanolic plant extracts were treated similarly and the absorbance was measured to determine the content of flavanones. All determinations were performed in triplicate.

3.1.4.3 Anthocyanin content

A KCl buffer solution (0.025 mol L\(^{-1}\)) was prepared in H\(_2\)O and the pH was adjusted to 1.0 by adding concentrated HCl. A CH\(_3\)CO\(_2\)Na buffer solution (0.4 mol L\(^{-1}\)) was prepared in H\(_2\)O and concentrated HCl was added to adjust the pH to 4.5. Two dilutions of methanolic plant extracts were prepared, one with KCl buffer and the other with CH\(_3\)CO\(_2\)Na buffer. The absorbance of each dilution was measured at wavelength of 520 and 700 nm using a Nicolet Evolution 300 UV model and with water as blank.
3.1.4.4 Flavonol content

Quercetin was used as a standard for flavonols test and the concentrations: 0, 5, 10, 20, 40 and 80 mg L$^{-1}$ were prepared in 95% ethanol. A solution of HCl (0.1%) in 95% ethanol and another (2%) in H$_2$O were prepared. A mixture of 2% HCl (225 μL), 0.1% HCl (12.5 μL) and the ethanolic quercetin solution (12.5 μL) was prepared. The mixture was left to stand for 20 minutes at room temperature and then the absorbance was measured at 360 nm using a Multiskan spectrophotometer UV model. The calibration curve was drawn. The methanolic plant extracts were treated similarly and the absorbance was measured to determine the content of flavonols. All determinations were performed in triplicate.

3.1.4.5 Flavanol content

Catechin was used as a standard at the following concentrations: 0, 1.36, 2.72, 6.80, 13.60 and 27.20 mg L$^{-1}$ in methanol. A mixture of HCl-methanol was prepared by adding 250 mL concentrated HCl to 750 mL methanol. The HCl-methanol mixture (500 mL) was added to 250 mg of 4-(dimethylamino)-cinnamaldehyde (DMACA). Aliquots of the methanolic catechin solution (50 μL) and the DMACA solution (250 μL) were mixed, and a green colour was observed. The absorbance was measured at 640 nm and then the calibration curve was drawn. The methanolic plant extracts were treated similarly and the absorbance was measured to determine the content of flavanols. All determinations were performed in triplicate.
Table 3.1 Chemical properties of quercetin, rutin, catechin, luteolin and apigenin

<table>
<thead>
<tr>
<th></th>
<th>Quercetin</th>
<th>Rutin</th>
<th>(+)-catechin</th>
<th>Luteolin</th>
<th>Apigenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C₁₅H₁₀O₇</td>
<td>C₂₇H₃₀O₁₆</td>
<td>C₁₅H₁₄O₆</td>
<td>C₁₅H₁₀O₆</td>
<td>C₁₅H₁₀O₅</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>302.236</td>
<td>610.52</td>
<td>290.30</td>
<td>286.24</td>
<td>270.23</td>
</tr>
<tr>
<td>Molecular Structure</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>
3.2 Electrochemical Analysis

3.2.1 Apparatus and reagents

A BAS 100 electrochemical analyser (Bioanalytical Systems, West Lafayette, Indiana) equipped with a three electrode system and a 20 mL capacity cell were used for all the voltammetric measurements. Glassy carbon electrode of 3 mm diameter was used as working electrode, as a reference and counter electrode; a silver-silver chloride (Ag/AgCl) and platinum wire were used, respectively. All aqueous solutions were made up with high-purity water from a Millipore Milli-Q system (resistivity greater than or equal to 18 MΩ cm). The pH of the phosphate buffer was measured using a pH meter (Hanna instrument, HI 221 Calibration Check Microprocessor pH meter with accuracy of ± 0.05). UV-spectrochemical experiments were carried out with Nicolet Evolution 100 which was supplied by Thermo Electron Corporation (South Africa) and the infrared (IR) spectra were obtained with a Spectrum 100 FT-IR spectrometer supplied by PerkinElmer (South Africa) using KBr pellets. MOD 7050 potentiostat was used to apply constant potential. The chemicals were purchased from Sigma-Aldrich (South Africa) and organic solvents were of analytical reagent quality grade. Quercetin, rutin, catechin, apigenin and luteolin were purchased from Sigma-Aldrich (South Africa).

3.2.2 Preparation of flavonoids stock and plant extract solutions

Stock solutions of quercetin, rutin, catechin, apigenin and luteolin with concentration of 0.2 mg mL⁻¹ each were prepared by dissolving 10 mg in 50 mL methanol. Ethyl acetate and butanol extracts of both C. mellei and C. quadrifidus were prepared as stock solutions with 0.2 mg mL⁻¹ by dissolving 2 mg with 10 mL methanol. All the stock solutions were kept in the refrigerator at a temperature of 4°C.
3.2.3 Preparation of 0.05 mol L\(^{-1}\) phosphate buffer

Phosphate buffer solutions were prepared by mixing appropriate volumes of 0.05 mol L\(^{-1}\) potassium dihydrogen phosphate (KH\(_2\)PO\(_4\)) and 0.05 mol L\(^{-1}\) of sodium hydrogen phosphate (Na\(_2\)HPO\(_4\)) to set the desired pH.

3.2.4 Procedure for voltammetry analysis

A 3 mL aliquot of buffer solution was placed in a cell and the required flavonoid stock solution was added using a micropipette. The electrochemical cell was a three-electrodes cell consisting of a glassy carbon working electrode (GCE), a platinum wire auxiliary electrode, and an Ag/AgCl (3 mol L\(^{-1}\) NaCl) reference electrode. Before each experiment the GCE was polished with 0.05 μm and 0.3 μm alumina on a microcloth pad and then rinsed with ultra pure water. The platinum wire, auxiliary electrode was always cleaned by fire polishing after rinsing with water. The solution was stirred using a stirring bar and also purged with argon gas for 5 minutes and 10 seconds were allowed to elapse so to allow the solution to become quiescent and then carry out voltammetric measurements. When further volume of flavonoids added to the cell, the solution was purged with argon for 10 minutes before carrying out further voltammetric measurements.

3.2.5 Cyclic Voltammetry (CV)

Unless otherwise indicated, the initial parameters were as follows; initial potential (E\(_i\)) = -1500 mV, high potential (E\(_H\)) = 1500 mV, low potential (E\(_L\)) = -1500 mV, scan rate (\(\nu\)) = 200 mV s\(^{-1}\), number of segments = 2, quiet time = 2 s and sensitivity = 10 μA V\(^{-1}\).
3.2.5.1 Repetitive Cyclic Voltammetry

Repetitive cyclic voltammetry were run using the same parameters as mentioned above with 10 and 20 cyclic numbers. Flavonoids with concentration of 16.67 μg mL⁻¹ were used for this experiment.

3.2.5.2 Effect of scan rate (υ)

Cyclic voltammetry was run for flavonoids at different scan rate (υ) from 20 mV s⁻¹ to 200 mV s⁻¹ while other parameters are kept the same. The applied υ were 20, 50, 100 and 200 mV s⁻¹. The concentration of flavonoids was 16.67 μg mL⁻¹.

3.2.5.3 Effect of pH

Cyclic voltammetry was repeated for flavonoids (16.67 μg mL⁻¹) with series of phosphate buffer from pH 2.36 to 12, while other parameters are kept the same. The pH were as follows; 2.36, 4.03, 6.50 which were prepared by adding 0.1 M HCl to Na₂HPO₄, and pH 8.00, 10.40 and 12 were prepared by adding 0.1 M NaOH to KH₂PO₄

3.2.6 Square Wave Voltammetry (SWV)

The parameters for square wave voltammetry were as follows; initial potential (Eᵢ) = -1500 mV, final potential (Eᵣ) = 1500 mV, sensitivity = 10 μA V⁻¹, step potential = 4 mV, amplitude = 25 mV, frequency = 10 Hz and quit time = 2 s. The concentration of flavonoid used for this experiment was fixed at 16.67 μg mL⁻¹.
3.2.6.1 Effect of pH

Square wave voltammetry was repeated for flavonoids (16.67 μg mL⁻¹) with series of phosphate buffer from pH 2.36 to 12, while other parameters are kept the same. The pH were as follows; 2.36, 4.03, 6.50, 8.00, 10.40 and 12.

3.2.7 Uv-vis spectroelectrochemical analysis

UV-Spectrochemical experiments were carried out using a three electrode modified UV cell for a spectral range of 200 to 450 nm using 0.8 nm intervals. Platinum electrode was used as working electrode, as a reference and counter electrode; a silver-silver chloride (Ag/AgCl) (3 mol L⁻¹ NaCl) and platinum were used, respectively. The platinum electrode and wire were purchased from BAS Inc. Phosphate buffer pH 7.5 (2.5 mL) and 250 μL of sample were poured into UV cell and then the solution was purged with argon gas. A constant potential (200 mV) was applied and all experiments were carried out at room temperature.
CHAPTER 4

RESULTS AND DISCUSSION FOR CHROMATOGRAPHIC, ELECTROCHEMICAL AND SPECTROELECTROCHEMICAL STUDIES

4.1 Chromatographic Studies

4.1.1 Thin Layer Chromatography (TLC)

Phytochemical screening of the ethyl acetate and butanol extracts of *C. mellei* and *C. quadrifidus* was performed on TLC plates with EtOAc : MeOH : H₂O (12:3:2, v/v/v) as solvent system. The TLC results showed both that ethyl acetate and butanol extracts of *C. mellei* and *C. quadrifidus* gave pinkish colour spots when sprayed with vanillin/H₂SO₄ (Figure 4.1 and 4.2). The colour spot of catechin have the same colour as to that of ethyl acetate and butanol extracts for both species. Their Rₖ values were as followS: for ethyl acetate extract, 0.88 and butanol extracts, 0.90. The same colours were observed with flavonoids from different plant species (Wagner and Bladt, 1996). From the TLC results, it was suggested that the plant extracts of *C. mellei* and *C. quadrifidus* may contain flavonoids.
Figure 4.1 TLC of ethyl acetate extracts 

$E$: extract, $Q$: quercetin, $R$: rutin, $C$: catechin
and $A$: apigenin
C. quadrifidus

4.1.2 The content of polyphenolic, flavanones, anthocyanins, flavonols and flavanols in C. mellei and C. quadrifidus

Since the phytochemical screening analysis suggested the presence of flavonoids, it was reasonable to determine their total amount in C. mellei and C. quadrifidus. The results for total contents of polyphenolic compounds, flavanone, anthocyanin, flavonol and flavanol for C. mellei and C. quadrifidus are presented in Table 4.1. The results clearly show that the plants are rich in polyphenolic compounds. The content of polyphenolic compounds (mg g⁻¹) in methanolic extracts of C. mellei and C. quadrifidus, determined from regression equation of calibration curve (y = 0.0067x +
0.0228, $R^2 = 0.999$) (Appendix A) and expressed in gallic acid equivalents (GAE), varied between 252.3 and 292.8 mg g$^{-1}$. Higher amounts were found in the methanolic extract of *C. mellei* than in *C. quadrifidus*. The content of flavonols (mg g$^{-1}$) in quercetin equivalents (QE) from regression equation ($y = 0.0020x - 0.0019$, $R^2 = 0.999$) (Appendix A) varied between 7.6 and 10.3 mg g$^{-1}$; with *C. mellei* containing the highest amounts. The ratio of total flavonols/polyphenolic was determined and must be close to 1. The ratio of total flavonols/polyphenolic for *C. mellei* and *C. quadrifidus* were 0.04 and 0.03, respectively (Table 4.2). Flavanols content in mg g$^{-1}$ of the methanolic plant extract, in catechin equivalents, regression equation ($y = 0.0301x - 0.0009$, $R^2 = 0.999$) (Appendix A) varied from 40.3 to 44.4 mg g$^{-1}$ and the highest amounts was found in the methanolic plant extract of *C. mellei*. The ratio of total flavanols/polyphenolic was 0.15 for *C. mellei* and 0.16 for *C. quadrifidus* (Table 4.2). This shows that the methanolic plant extracts of *C. mellei* and *C. quadrifidus* contain more flavanols than other polyphenolic compounds. Anthocyanins and flavanones were not detected in the plant extracts for both species. It has been reported that *C. mellei* and *C. quadrifidus* have antimicrobial activity. The dried leaves showed antibacterial activity against *Staphylococcus aureus* and *Mycobacterium smegmatis* (Springfield and Weitz, 2006; Springfield *et al.*, 2003). It is possible that the high content of polyphenolic compounds may be the sole contributor to the bioactivity in *C. mellei* and *C. quadrifidus*. 
Table 4.1 Total content of polyphenolic, flavanones, anthocyanins, flavonols and flavanols

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total polyphenolic compounds, mg g⁻¹ plant extract (in GAE)</th>
<th>Total flavanone</th>
<th>Total anthocyanins</th>
<th>Total flavonols, mg g⁻¹ plant extract (in QE)</th>
<th>Total flavanols, mg g⁻¹ plant extract (in CE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. mellei</td>
<td>292.8 ± 10.08</td>
<td>nd</td>
<td>nd</td>
<td>10.3 ± 1.12</td>
<td>44.4 ± 3.55</td>
</tr>
<tr>
<td>C. quadrifidus</td>
<td>252.3 ± 12.76</td>
<td>nd</td>
<td>nd</td>
<td>7.6 ± 0.46</td>
<td>40.3 ± 2.22</td>
</tr>
</tbody>
</table>

nd: not detected

Table 4.2 Ratio of total flavonols/polyphenolic and flavanols/polyphenolic compounds

<table>
<thead>
<tr>
<th>Sample</th>
<th>Flavonols/Polyphenolic</th>
<th>Flavanols/Polyphenolic</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. mellei</td>
<td>0.04</td>
<td>0.15</td>
</tr>
<tr>
<td>C. quadrifidus</td>
<td>0.03</td>
<td>0.16</td>
</tr>
</tbody>
</table>
4.1.3 Liquid Chromatography-Mass Spectroscopy (LC-MS)

The choice of the experimental conditions was guided by the need to obtain chromatograms with a high resolution of adjacent peaks within a short analysis time. According to De Souza et al. (2002), owing to the phenolic feature of the flavonoid compounds, an acidified eluent was used which provided a satisfactory separation. To improve the peak shape, acetic acid was added as a mobile phase modifier to inhibit the dissociation of the phenolic hydroxyl groups of flavonoids. Preliminary studies indicated that acetic acid provided an efficient separation (De Souza et al., 2008; Liu et al., 2008). There have been other reports showing that other acids such as formic acid and trifluoroacetic acid can improve peak separation when added to the mobile phase (Tsimogiannis et al., 2007; Merken and Beecher, 2000; Häkkinen and Auriola, 1998; Dubber et al., 2005). Several solvent systems based on acetonitrile, methanol and water were tried, and the best results were observed using water and acetonitrile as mobile phase, both mixed with 0.1% acetic acid. Gradient elution was required for satisfactory resolution of the flavonoids. The analysis was completed within 30 min. using a Zorbax Eclipse XDB C18 column at a temperature of 25 °C. The identification of the peaks in the ethyl acetate and butanol extracts of C. mellei and C. quadrifidus were achieved by comparing the retention time (t_R) with those obtained for the flavonoid standards cocktail. Figure 4.3 shows the chromatogram of flavonoid cocktail at 254 nm and Table 4.3 shows peak identification.
Figure 4.3 Chromatogram of flavonoid standards cocktail monitored at 254 nm
Table 4.3 Retention time and deprotonated molecular masses of the flavonoid cocktail

<table>
<thead>
<tr>
<th>Peak</th>
<th>Flavonoid</th>
<th>Retention time ((t_R), \text{min})</th>
<th>([M – H] )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Catechin</td>
<td>12.02</td>
<td>289</td>
</tr>
<tr>
<td>2</td>
<td>Rutin</td>
<td>13.07</td>
<td>609</td>
</tr>
<tr>
<td>3</td>
<td>Luteolin</td>
<td>17.73</td>
<td>285</td>
</tr>
<tr>
<td>4</td>
<td>Quercetin</td>
<td>18.00</td>
<td>301</td>
</tr>
</tbody>
</table>

These separations were conducted on a reversed phase column and as expected, the most polar flavonoids (catechin) eluted early, and then followed by the flavonols (rutin and quercetin) and flavones (luteolin and apigenin). The polarity of the ring structure of flavonols and flavones is similar, so the addition of hydroxyl group(s) to the B ring results in substantial alterations in the polarity of the molecule. Apigenin has considerable electronic resonance among all three rings, and hence it will elute last, suggesting it to be the least polar of the flavonoids tested. The peak due to apigenin could not be observed in Figure 4.3, probably because the run time was too short. The same pattern of peaks separation was observed using the same column but a different solvent system (Merken and Beecher, 2000). The ethyl acetate and butanol extracts of *C. mellei* and *C. quadrifidus* were separated under the same conditions as described for the flavonoid standards cocktail. The chromatograms of the extracts are shown in Figure 4.4, 4.5, 4.6 and 4.7. It may be observed that peaks corresponding to two components of the flavonoid standards cocktail and other unknown compounds were successfully resolved. Catechin was directly identified from the ethyl acetate extract of *C. mellei* (Figure 4.4 inset) and rutin was also directly identified from the
butanol extract of *C. quadrifidus* (Figure 4.7). This was done by comparison of their retention times with those of the flavonoid standards cocktail constituents. Rutin was reported to be present in the *Carpobrotus* species (Springfield *et al.*, 2003).
Figure 4.4 Chromatogram of ethyl acetate extract of *C. mellei* monitored at 254 nm.
Figure 4.5 Chromatogram of ethyl acetate extract of *C. quadrifidus* monitored at 254 nm.
Figure 4.6 Chromatogram of butanol extract of *C. mellei* monitored at 254 nm.
Figure 4.7 Chromatogram of butanol extract of *C. quadrifidus* monitored at 254 nm
For the unknown compounds (5, 6, 7, 8, 9, and 10), peak identification was achieved using the ESI/LC-MS with an ESI ion source in negative mode. The results are illustrated in Table 4.4, structures are shown in scheme 4.1 and the full scan negative ionization mass spectra are shown in Appendix B. In the MS spectrum, these compounds observed as the deprotonated molecules ([M – H]−) at m/z 337, 577, 289, 465 and 465 for compounds 5, 6, 7, 8 and 9, respectively, and [M + Na – H]− at m/z 337 for compound 10. From the ethyl acetate extract of C. mellei and C. quadrifidus, three flavanols were detected. These were coumarylquinic acid (5), epicatechin-epicatechin (6) and epicatechin (7). Compounds 5 and 6 were unambiguously identified by comparison of their MS spectra with those from the literature (Poon, 1998; De Souza et al., 2008). Compound 5 has been detected in a tea extract (Bailey et al., 1994). For compound 6, the signal at m/z 1155 corresponds to the dimer of epicatechin-epicatechin because this compound is present at fairly high concentration in the infusion solutions. The ion found at m/z 577 for compound 6 corresponds to

### Table 4.4 Identification of compounds 5, 6, 7, 8, 9, and 10

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>Retention time (tR), min</th>
<th>[M – H]</th>
<th>Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Coumarylquinic acid</td>
<td>11.38</td>
<td>337</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Epicatechin-epicatechin</td>
<td>12.28</td>
<td>577</td>
<td>407 289</td>
</tr>
<tr>
<td>7</td>
<td>Epicatechin</td>
<td>12.81</td>
<td>289</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Quercetin-hexose</td>
<td>18.01</td>
<td>465</td>
<td>301 285</td>
</tr>
<tr>
<td>9</td>
<td>Myricetin-deoxyhexose</td>
<td>26.91</td>
<td>465</td>
<td>319</td>
</tr>
<tr>
<td>10</td>
<td>Isorhamnetin</td>
<td>21.51</td>
<td>337</td>
<td>315</td>
</tr>
</tbody>
</table>
two units of epicatechin and this is supported by the occurrence of a fragment at m/z 289, which corresponds to a single unit of epicatechin. The same compound was found to be present in the extract from leaves of *Maytenus Ilicifolia* (De Souza et al., 2008). The signals for compound 7 at m/z 579 and 865 correspond to the dimer and trimer, respectively. This suggests the presence of epicatechin in high concentration. Confirmation that compound 7 is a epicatechin rather than catechin is due to the different retention times of the two, epicatechin (12.81) and catechin (12.07) (Figure 4.4, inset).

Two flavonol glucosides, quercetin-hexose (8) and myricetin-deoxyhexose (9) were detected from the ethyl acetate extracts of *C. mellei* (Figure 4.4) and *C. quadrifidus* (Figure 4.5). Compound 8 shows a peak due to the deprotonated molecule [M – H]⁻ at m/z 465. The product-ion mass spectrum of the compound contains abundant fragment ion at m/z 301, which may be associated with loss of a 162 mass unit (u). This usually suggests loss of a hexose sugar. The hexose sugar may be galactose or glucose. The m/z 301 ion is indicative of the aglyconic component quercetin. Fragmentations at m/z 301 and 285 matched with the fragmentation pattern of quercetin. Compound 8 may possibly be hyperoside (quercetin-galactoside) or isoquercetin (quercetin-glucoside). The similar compound was identified from lingonberry with ESI-MS as hyperoside (Kühnau, 1976; Häkkinen and Auriola, 1998), while from the ethanolic extract of *Hypericum perforatum* both hyperoside and isoquercetin were detected (Silva et al., 2005). This is an agreement with the report that South African *Carpobrotus* species contain hyperoside (Springfield et al., 2003).

From the MS results of compound 9 (Appendix B), the compound has an [M – H]⁻ ion at m/z 465. The product-ion mass spectrum of the compound contains an abundant
fragment ion at m/z 319, which indicates a loss of a 146 u. The loss of 146 u is usually indicative of the presence of a deoxyhexose sugar. The m/z 319 ion is suggestive of the aglyconic component myricetin. Compound 9 has been detected in blackcurrant where the sugar moiety was rhamnose. It has also been found in Betula pendula and Betula pubescens leaves (Keinänen and Julkunen-Tiitto, 1998; Häkkinen and Auriola, 1998). One flavonol, isorhamnetin (10) was identified in the ethyl acetate extract of C. quadrifidus (Figure 4.5) and the butanol extract of C. mellei (Figure 4.6). Compound 10 has [M + Na – H]⁻ ion at m/z 337. The abundant fragment ion at m/z 315 suggests isorhamnetin as the base component. Compound 10 has been detected in several Ginkgo biloba solid dosage forms (Dubber et al., 2005).

![Scheme 4.1 Structures for compounds 7, 8, 9 and 10](image)
4.2 Electrochemical Analysis

The oxidation reaction of flavonoids is strongly related to their structure, which contains several free phenolic hydroxyl groups, particularly ortho-phenolic hydroxyl groups (Ghica and Brett, 2005; Brett and Ghica). The mechanism of electrooxidation of flavonoids involves, ionization, losing a proton to give the monoanionic species followed by a one electron, one proton oxidation of the monoanionic species to form a radical anion. This then undergoes a second reversible one electron oxidation to give dehydro-flavanoids. The latter species is rapidly protonated and then dehydrated to yield the final product of 3',4'-diquinone (Kang et al., 2002). General information about the electroactivity and possible surface activity of quercetin, rutin, catechin, luteolin and apigenin could be obtained from the cyclic voltammetry (CV).

4.2.1 Cyclic Voltammetry of flavonoids

4.2.1.1 Cyclic Voltammetry of quercetin

The cyclic voltammogram of quercetin at pH 7.5 is shown in Figure 4.8. The CV shows three oxidation peaks (1-3) at the potential of +97.0, +392.8 and +891.3 mV and reduction peak (1') at +88.2 mV. These oxidation peaks are associated with oxidation of the five functional OH groups of the quercetin according to literature (Zielinska et al., 2008). A reduction peak (1') corresponds to reduction of the oxidation products in peak 1. Quercetin has two different pharmacophores, the catechol moiety (3',4'-dihydroxyl group) in ring B, resorcinol group (5,7-dihydroxyl group) in ring A, also hydroxyl group in position 3 of ring C. The oxidation of the catechol moiety occurs first at a low positive potential and is associated with peak 1 in the cyclic voltammogram (Figure 4.8). The oxidation process involves a two electron - two proton reversible reactions as reported by Brett and Ghica (2003), and forms o-
quinone (Scheme 4.2). The results in this study show that quercetin has the lowest oxidation potential (+97.0 mV), since the catechol moiety is direct conjugation with the electron withdrawing carbonyl oxygen at position 4 on ring C. This results in a weakening of the bond strength between the oxygen and hydrogen atoms of the catechol moiety thus making it easier for the hydrogen to leave.

Figure 4.8 Cyclic voltammogram of 16.67 μg mL⁻¹ quercetin in phosphate buffer pH 7.5, scan rate 200 mV s⁻¹
Scheme 4.2 Oxidation of catechol moiety of quercetin to o-quinone.

The hydroxyl group at C3 in ring C of quercetin is oxidized afterwards (peak 2) and undergoes an irreversible oxidation reaction (Brett and Ghica, 2003; Vestergaard et al., 2005). The oxidation of the resorcinol group occurs at higher potential and corresponds to peak 3 for quercetin and it undergoes an irreversible oxidation reaction (Brett and Ghica, 2003; Xu et al., 2005; Zhou et al., 2007). The electrochemical studies reveal general trends in the electron donating abilities of flavonoids. It demonstrates that the catechol moiety in ring B is more easily oxidizable than the resorcinol group in A-ring. The influence of deprotonation of the catechol moiety is related to the electron/proton donating capacity.
4.2.1.2 Cyclic Voltammetry of rutin

The cyclic voltammogram of rutin (Figure 4.9), shows two oxidation peaks at about +233.8 mV and +874.9 mV associated with oxidation of hydroxyl groups as well as the reduction peak 1' appears at +225.7 mV which is due to reduction of the oxidation products in peak 1. Rutin has catechol moiety (3',4'-dihydroxyl group) in ring B and resorcinol group (5,7-dihydroxyl group) in ring A. The oxidation of the catechol moiety occurs first at a low positive potential and is associated with peak 1 in the cyclic voltammogram (Figure 4.9). The oxidation process involves a two electron - two proton reversible reactions and forms \( o \)-quinone (Scheme 4.3) (Ghica and Brett, 2005). The oxidation of the resorcinol group occurs at higher potential and corresponds to peak 2 of the cyclic voltammogram (Figure 4.9). The resorcinol group undergoes an irreversible oxidation reaction (Ghica and Brett, 2005; Xu et al., 2005).
Figure 4.9 Cyclic voltammogram of 16.67 μg mL⁻¹ rutin in phosphate buffer pH 7.5, scan rate 200 mV s⁻¹
4.2.1.3 Cyclic Voltammetry of catechin

Cyclic voltammetry of catechin shows two oxidation peaks (Figure 4.10), occurring at the potentials of +197.0 mV and +612.7 mV. These oxidation peaks are associated with oxidation of the hydroxyl groups. A reduction peak 1’ at +70.7 mV corresponds to the reduction of oxidation products in peak 1. The structure of catechin has hydroxyl groups in the ring and so can be electrochemically oxidised. This showed that the catechol moiety is more easily oxidizable than the resorcinol group (Cren-Olivé et al., 2002). Catechin contains catechol moiety namely, the 3’,4’-dihydroxyl electron-donating group at ring B, the oxidation of the catechol moiety occurs first at a low positive potential and is associated with peak 1 in the cyclic voltamagram. The
oxidation process involves a two electron - two proton reversible reactions and forms \( o \)-quinone (Scheme 4.4) (Janeiro and Brett, 2004; Vestergaard \textit{et al.}, 2005; Kilmartin \textit{et al.}, 2001).

Figure 4.10 Cyclic voltammogram of 16.67 \( \mu \)g mL\(^{-1}\) catechin in phosphate buffer pH 7.5, scan rate 200 mV s\(^{-1}\)
The hydroxyl group at C3 in ring C of catechin is oxidized (peak 2) next and undergoes an irreversible oxidation reaction. (Janeiro and Brett, 2004; Kilmartin et al., 2001). The oxidation peak for the resorcinol group was not observed using cyclic voltammetry. But it previously was reported to occur at very high positive potentials and is irreversible, this was observed using differential pulse voltammetry (Janiero and Brett, 2004).
4.2.1.4 Cyclic Voltammetry of luteolin

Cyclic voltammogram of luteolin is shown in Figure 4.11, two oxidation peaks at potential +281.8 (1) and 968.4 (2) mV and reduction peak (1') at +227.3 mV. A reduction peak (1') corresponds to reduction of the oxidation products in peak 1. Luteolin has catechol moiety (3’,4’-dihydroxyl group) in ring B and resorcinol group (5,7-dihydroxyl group) in ring A. The oxidation of the catechol moiety occurs first at a low positive potential and is associated with peak 1 in the cyclic voltammogram (Figure 4.11). The oxidation process involves a two electron - two proton reversible reactions and forms o-quinone (Scheme 4.5). The oxidation of the resorcinol group occurs next at higher potential and corresponds to peak 2 of the cyclic voltammogram (Figure 4.11). The resorcinol group undergoes an irreversible oxidation reaction (Filipiak, 2001).
Figure 4.11 Cyclic voltammogram of 16.67 μg mL⁻¹ luteolin in phosphate buffer pH 7.5, scan rate 200 mV s⁻¹
Scheme 4.5 Oxidation of catechol moiety of luteolin to $o$-quinone

4.2.1.5 Cyclic Voltammetry of apigenin

Cyclic voltammogram of apigenin shows two oxidation peaks in Figure 4.12, at potential +709.0 and +932.8 mV. Apigenin contains hydroxyl group at C4' in ring B and resorcinol group (5,7-dihydroxyl group) in ring A. The oxidation of the 4' hydroxyl group occurs at a high potential (+709.0 mV) and corresponds to peak 1 of the cyclic voltammogram (Figure 4.12). The oxidation reaction is irreversible. The oxidation of the resorcinol group occurs at higher potential and corresponds to peak 2 of the cyclic voltammogram (Figure 4.12). The resorcinol group undergoes an irreversible oxidation reaction.
Figure 4.12 Cyclic voltammogram of 16.67 μg mL⁻¹ apigenin in phosphate buffer pH 7.5, scan rate 200 mV s⁻¹

4.2.2 Square Wave Voltammetry of flavonoids

Square wave voltammetry has shown to have advantages over cyclic voltammetry. These include: a shorter analysis time, a lower consumption of the electroactive species and a decrease in problems associated with fouling of the electrode surface (Brett and Ghica, 2003). Also the great advantage of the square wave voltammetry is the possibility to see during one scan if the electron transfer reaction is reversible or not.
4.2.2.1 Square Wave Voltammetry of quercetin

The square wave voltammogram for quercetin (Figure 4.13) shows three oxidation peaks (1-3) at potential +125.0, +712.2 and +896.7 mV and the reduction peak 1' at potential +125.0 mV. The results are similar to those obtained with cyclic voltammetry. Furthermore, reversibility of the quercetin oxidation reaction is clearly enhanced in the square wave voltammetry, where the oxidation potential and reduction potential are equal. The oxidation of the catechol moiety occurs first at a low positive potential and is associated with peak 1. The oxidation process involves a two electron - two proton reversible reactions as reported by Brett and Ghica, 2003, and forms o-quinone. The 3-hydroxyl group at ring C of quercetin is oxidized afterwards (peak 2) and undergoes an irreversible oxidation reaction (Brett and Ghica, 2003; Vestergaard et al., 2005). The oxidation of the resorcinol group occurs at higher potential and corresponds to peak 3 for quercetin and it undergoes an irreversible oxidation reaction (Brett and Ghica, 2003; Xu et al., 2005).
4.2.2.2 Square Wave Voltammetry of rutin

The square wave voltammogram (Figure 4.14) of rutin shows two oxidation potentials at potential +217.0 and +842.1 mV and reduction peak 1' at potential +217.0 mV. The results are similar to those obtained with cyclic voltammetry and reversibility of the rutin oxidation reaction is clearly enhanced. The oxidation of the catechol moiety occurs first at a low positive potential and is associated with peak 1. The oxidation process involves a two electron - two proton reversible reactions and forms o-quinone (Ghica and Brett, 2005). The oxidation of the resorcinol group occurs at higher potential and corresponds to peak 2 and the resorcinol group undergoes an irreversible oxidation reaction (Ghica and Brett, 2005; Xu et al., 2005).
4.2.2.3 Square Wave Voltammetry of catechin

The square wave voltammogram (Figure 4.15) shows two oxidation peaks (1-2) for catechin at potential +171.0 and +631.6 mV and the reduction peak 1' at potential +171.0 mV. The result is similar to that obtained with cyclic voltammetry and reversibility of the catechin oxidation reaction is clearly enhanced in the square wave voltammetry. The oxidation of the catechol moiety occurs first at a low positive potential and is associated with peak 1 in the cyclic. The oxidation process involves a two electron - two proton reversible reactions and forms o-quinone (Janeiro and Brett, 2004; Vestergaard et al., 2005; Kilmartin et al., 2001). The 3-hydroxyl group at ring C of catechin is oxidized (peak 2) next and undergoes an irreversible oxidation reaction. (Janeiro and Brett, 2004; Kilmartin et al., 2001). The oxidation peak for the resorcinol group was not observed, but it was reported to occur at very high positive
potentials and irreversible, this was observed using differential pulse voltammetry (Janiero and Brett, 2004).

Figure 4.15 Square Wave Voltammogram of 16.67 μg mL⁻¹ of catechin in phosphate buffer pH 7.5, frequency 10 Hz.
4.2.2.4 Square Wave Voltammetry of luteolin

The square wave voltammogram for luteolin (Figure 4.16) shows two oxidation peaks (1-2) at potential +250.4 and +948.7 mV and the reduction peak 1' at potential +250.4 mV. The results are similar to those obtained with cyclic voltammetry. Furthermore, reversibility of the luteolin oxidation reaction is clearly enhanced in the square wave voltammetry. The oxidation of the catechol moiety occurs first at a low positive potential and is associated with peak 1 and the oxidation process involves a two electron - two proton reversible reactions and forms $o$-quinone. The oxidation of the resorcinol group occurs next at higher potential and corresponds to peak 2 and undergoes an irreversible oxidation reaction.

![Square Wave Voltammogram](image)

Figure 4.16 Square Wave Voltammogram of 16.67 μg mL$^{-1}$ luteolin in phosphate buffer pH 7.5, frequency 10 Hz
4.2.2.5 Square Wave Voltammetry of apigenin

The square wave voltammogram (Figure 4.17) shows two oxidation peaks (1-2) for apigenin at potential +634.5 and +861.3 mV. The result is similar to that obtained with cyclic voltammetry. The oxidation of the 4’ hydroxyl group occurs at a high potential and corresponds to peak 1. The oxidation reaction is irreversible. The oxidation of the resorcinol group occurs next at higher potential and corresponds to peak 2 and undergoes an irreversible oxidation reaction.

![Square Wave Voltammogram of 16.67 μg mL⁻¹ apigenin in phosphate buffer pH 7.5, frequency 10 Hz](image)

Figure 4.17 Square Wave Voltammogram of 16.67 μg mL⁻¹ apigenin in phosphate buffer pH 7.5, frequency 10 Hz
4.2.3 Effects of pH

For more insight into the number of electrons and protons involved in the electrochemistry of flavonoids, effect of variation in pH on its voltammetric response was studied in 0.05 mol L\(^{-1}\) phosphate buffer, in the pH range from 2 to 10. Information on the mechanism of polyphenol oxidation was provided by comparing first oxidation potentials peak at different pH values. The square wave voltammograms of the net peak for quercetin, rutin and catechin are shown in Figure 4.18. It is observed that an increase of pH the first oxidation potentials peak are shifted toward less positive values and at high pH the reduction peak almost disappeared. From this, it is concluded that at high pH o-quinones generated in the reaction are unstable. There is an enhancement of the peak currents in basic region. The peak potentials were plotted against the pH and are shown in Figure 4.19. The 28 mV per pH unit, which is close to the Nernstian value of 29.5 mV (59/n for n = 2), which is consistent with the expected two-electron two-proton oxidation of the catechol 3',4'-dihydroxyl group in flavonoid molecules to the quinine form (Zare et al., 2005; Malagutti et al., 2006; Bao et al., 2001; Kang et al., 2002; Janiero and Brett, 2004; Brett and Ghica, 2003; Ferreira et al., 2006). A two step electron transfer reaction associated with the loss of two protons occurs at pH 2 to 8. At pH 10 only one proton is lost resulting in a lower current (Van Acker et al., 1996; Janeiro and Brett, 2004; Zhou et al., 2007; Ghica and Brett, 2005; Brett and Ghica, 2003).
Figure 4.18 Square wave voltammograms of 16.67 μg mL⁻¹ quercetin, rutin and catechin as a function of pH, scan rate 200 mV s⁻¹
Figure 4.18 (continue) Square wave voltammograms of 16.67 μg mL⁻¹ quercetin, rutin and catechin as a function of pH, scan rate 200 mV s⁻¹.
Figure 4.19 Plot of peak potential versus pH for 16.67 μg mL⁻¹ quercetin, rutin and catechin in phosphate buffer
Figure 4.19 (continue) Plot of peak potential versus pH for 16.67 μg mL⁻¹ quercetin, rutin and catechin in phosphate buffer.

\[ y = -54.486x + 578.036 \]

\[ R^2 = 0.9987 \]
4.2.4 Effect of scan rate ($\nu$)

The effect of increasing scan rate ($\nu$) from 20 to 200 mV s$^{-1}$) on Ip of flavonoids (quercetin, rutin, catechin, luteolin and apigenin) were observed and the cyclic voltammograms are shown in Figure 4.20. The results show that the oxidation and reduction peaks increase with the increase scan rate with no shift in potential. The lower current signal at lower scan rate could indicate a rapid formation of a non-electro-active film on the electrode surface (Vestergaard et al., 2005; Zare et al., 2005; Malagutii et al., 2006). The faster the scan rate, the lesser time the film is allowed to deposit on the electrode surface and the higher the current signal as can be seen in Figure 4.21, the anodic peak currents increased linearly with the square root of $\nu$ for quercetin, rutin, catechin, luteolin and apigenin with correlation coefficient of 0.9993, 0.9982, 0.9875, 0.9942 and 0.9931, respectively. The change in response due to changes in scan rates suggests that the electrode response was controlled by semi-finite diffusion. As the scan rate was increased, there was less time for the diffusion layer to extend into the bulk of the solution, causing a large diffusion gradient and a higher peak current (Bard and Faulkner, 2001).
Figure 4.20 Cyclic voltammograms of 16.67 μg mL\(^{-1}\) flavonoids in phosphate buffer pH 7.5 at different scan rates
Figure 4.20 (continue) Cyclic voltammograms of 16.67 μg mL⁻¹ flavonoids in phosphate buffer pH 7.5 at different scan rates
Figure 4.20 (continue) Cyclic voltammograms of 16.67 μg mL⁻¹ flavonoids in phosphate buffer pH 7.5 at different scan rates.
Figure 4.21 Plot of Ip versus the square root of scan rate for five flavonoids in phosphate buffer pH 7.5
Figure 4.21 (continue) Plot of \( I_p \) versus the square root of scan rate for five flavonoids in phosphate buffer pH 7.5

- **Catechin**
  
  \[
  y = 4.5645 \times 10^7 x - 1.0396 \times 10^6 \\
  R^2 = 0.9875
  \]

- **Luteolin**
  
  \[
  y = 2.0785 \times 10^7 x - 8.4303 \times 10^8 \\
  R^2 = 0.9942
  \]
4.2.5 Repetitive Cyclic Voltammetry

Repetitive cyclic voltammetry studies for flavonoids (quercetin, rutin, catechin, luteolin and apigenin) were carried out in order to observe whether any adsorption phenomenon took place at the surface of the electrode. The cyclic voltammograms are shown in Figure 4.22. The results show that all the flavonoids adsorb strongly on the electrode surface and the final oxidation product blocks the electrode surface. This is shown by the rapid decrease of the first oxidation peak and been constant for the rest of the repeated cyclic. This was observed also when using differential pulse voltammetry (DPV) for flavonoids (Ghica and Brett, 2005; Janeiro and Brett, 2004; Brett and Ghica, 2003).
Figure 4.22 Repetitive cyclic voltammograms of 16.67 $\mu$g mL$^{-1}$ flavonoids in phosphate buffer pH 7.5
Figure 4.22 (continue) Repetitive cyclic voltammograms of 16.67 μg mL⁻¹ flavonoids in phosphate buffer pH 7.5
4.2.6 Effect of concentration

The effect of increasing concentration on peak current (Ip) of flavonoids (quercetin, rutin, catechin, luteolin and apigenin) were observed and the cyclic voltammograms are shown in Figure 4.23. As can be seen the peak current increased with the increase of concentration and reached a maximum value, and then the peak current decreased. This indicated that the adsorption of quercetin, rutin, catechin, luteolin and apigenin on the glassy carbon electrode belongs to monomolecular layer. The same was observed for rutin (Kang et al., 2002). The plots of Ip versus concentration for quercetin, rutin, catechin, luteolin and apigenin are shown in appendix C.
Figure 4.23 Cyclic Voltammograms of flavonoids with different concentration in phosphate buffer pH 7.5 at scan rate 200 mV s⁻¹
Figure 4.23 (continue) Cyclic Voltammograms of flavonoids with different concentration in phosphate buffer pH 7.5 at scan rate 200 mV s$^{-1}$
4.2.7 UV-Vis Spectroelectrochemical Analysis

The UV-vis absorption spectra of quercetin, rutin, catechin, luteolin and apigenin over the wavelength range 600-200 nm are shown in Figure 4.24. The flavonoids showed two bands; an intense band at around $\lambda$ 218 nm (Band II) and less intense band at around $\lambda$ 330 nm (Band I). In general terms the band II absorption may be considered as having originated from the A ring benzoyl system and band I from the B ring cinnamoyl system (Markham, 1982). Quercetin, rutin, catechin, luteolin and apigenin showed band II at 210, 202, 218, 202 and 210 nm, respectively. Band I of quercetin, rutin, catechin, luteolin and apigenin shown at 370, 362, 282, 346 and 338 nm, respectively. Quercetin, rutin, luteolin and apigenin, all exhibited an extra weak band at 258, 258, 258 and 266 nm, respectively. The results are in agreement with that from literature (Bao et al., 2001; Zhou and Sadik,; Hassan et al., 1999; Jiménez-Atiénezar et al, 2004).
Figure 4.24 UV-vis spectra for 25 μg mL\(^{-1}\) flavonoids in methanol
Figure 4.24 (continue) UV-vis spectra for 25 μg mL⁻¹ flavonoids in methanol
The UV-vis spectra of quercetin, rutin, catechin and luteolin in 0.05 mol L\(^{-1}\) phosphate buffer pH 7.5 after the oxidation processes are illustrated in Figure 4.25 by applying a constant potential of +220 mV. After oxidation process band I and II have shifted (hipsochromic shift) and the intensity has decreased from 7 AU to 3.5 AU during electro-oxidation at potential +220 mV. This supports the carbonylation of the 3',4'-OH groups at B ring, which causes loss of the electron donating ability of B ring. This is indicative that the quinonic structure is the chromophore present in the final species. The observation was reported for other quinones like catechin, 3,5-diteritary butyl-o-benzoquinone and quercetin, rutin (Bodini et al., 2001; Bodini et al., 1999; He et al., 2007; Petrucci et al., 2007, Ramos-Tejada et al., 2002).
Figure 4.25 UV-vis spectra of 25 μg mL⁻¹ flavonoids in phosphate buffer pH 7.5 at potential +220 mV
Figure 4.25 (continue) UV-vis spectra of 25 μg mL⁻¹ flavonoids in phosphate buffer pH 7.5 at potential +220 mV
4.2.8 Cyclic and Square Wave Voltammetry of plant extracts

4.2.8.1 Cyclic and Square Wave Voltammetry of ethyl acetate extracts

All ethyl acetate and butanol extracts of *C. mellei* and *C. quadrifidus* were analysed using cyclic and square wave voltammetry methods. The cyclic and square wave voltammograms of 20 μg mL\(^{-1}\) ethyl acetate extracts from *C. mellei* and *C. quadrifidus* are shown at Figure 4.26 and 4.27. The cyclic voltammogram of ethyl acetate extract of *C. mellei* shows two oxidation peaks at potential +201.5 mV and +500.0 mV and a reduction peak 1' at potential +37.3 mV which correspond to reduction of the oxidation products presented in peak 1. The cyclic voltammogram of the ethyl acetate extract of *C. quadrifidus* also shows two oxidation peaks at potential +182.2 mV and +507.0 mV. Furthermore, reduction peak 1' at +70.1 mV which corresponds to the reduction of oxidation products formed at peak 1 is also present (Figure 4.26 and Table 4.5). Square wave voltammogram shows three oxidation peaks 1-3 for *C. mellei* and four oxidation peaks 1-4 for *C. quadrifidus* and reduction peak 1'. The reversible oxidation reaction of the ethyl acetate extracts of *C. mellei* and *C. quadrifidus* can be clearly identified (Figure 4.27 and Table 4.6). The first oxidation peak (peak 1) in the cyclic and square wave voltammograms of ethyl acetate extracts of *C. mellei* and *C. quadrifidus* is associated with that of catechol moiety, 3',4'-dihydroxyl group in ring B of markers and is a two electron, two proton reversible reaction. The oxidation potential closely corresponds with that of catechin (Table 4.5 and 4.6). The oxidation potential of *C. mellei* (peak 2) and *C. quadrifidus* (peak 3) corresponds to that of catechin which involves the oxidations of 3-hydroxyl group in the C ring (Table 4.6).
Figure 4.26 Cyclic voltammograms of 20 μg mL⁻¹ ethyl acetate extracts in phosphate buffer pH 7.5, scan rate 200 mV s⁻¹
Figure 4.27 Square wave voltammograms of 20 μg mL\(^{-1}\) ethyl acetate extracts in phosphate buffer pH 7.5, frequency 10 Hz
Table 4.5 Peak Potentials from Cyclic Voltammetry of ethyl acetate extract

<table>
<thead>
<tr>
<th>Samples</th>
<th>Peak 1 (mV)</th>
<th>Peak 2 (mV)</th>
<th>Peak 3 (mV)</th>
<th>Peak 1' (mV)</th>
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<tr>
<td>Quercetin</td>
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<td>+891.3</td>
<td>+88.2</td>
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</tr>
<tr>
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<tr>
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<tr>
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<td>+507.0</td>
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<td>+70.1</td>
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</tbody>
</table>

Table 4.6 Peak Potentials from Square Wave Voltammetry of ethyl acetate extract

<table>
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<th>Samples</th>
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<th>Peak 2 (mV)</th>
<th>Peak 3 (mV)</th>
<th>Peak 4 (mV)</th>
<th>Peak 1' (mV)</th>
</tr>
</thead>
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<td>+712.2</td>
<td>+896.7</td>
<td>+125.0</td>
<td></td>
</tr>
<tr>
<td>Rutin</td>
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<td></td>
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<tr>
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<tr>
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<tr>
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</tr>
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4.2.8.2 Square Wave Voltammetry of butanol extracts

The square wave voltammograms of 20 μg mL⁻¹ butanol extracts from *C. mellei* and *C. quadrifidus* are shown at Figure 4.28. Square wave voltammetry shows five oxidation peaks 1-5 for *C. mellei* and three oxidation peaks 1-3 for *C. quadrifidus* (Figure 4.28 and Table 4.7). The oxidation peaks of butanol extract does not correspond with that of quercetin, rutin, catechin, luteolin and apigenin.
Figure 4.28 Square wave voltammograms 20 μg mL\(^{-1}\) butanol extracts in phosphate buffer pH 7.5, frequency 10 Hz
Table 4.7 Peak Potentials from Square Wave Voltammetry of butanol extract

<table>
<thead>
<tr>
<th>Samples</th>
<th>Peak 1 (mV)</th>
<th>Peak 2 (mV)</th>
<th>Peak 3 (mV)</th>
<th>Peak 4 (mV)</th>
<th>Peak 5 (mV)</th>
<th>Peak 1' (mV)</th>
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<tr>
<td>Quercetin</td>
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<tr>
<td>Catechin</td>
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</tr>
<tr>
<td>Luteolin</td>
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<td></td>
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<td></td>
<td>+250.4</td>
</tr>
<tr>
<td>Apigenin</td>
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<td>+861.3</td>
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<td>C. quadrifidus</td>
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<td>+248.9</td>
<td>+908.7</td>
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</table>

4.2.8.3 Analysis of catechin in plant extract

The plant extracts of *C. mellei* and *C. quadrifidus* were analysed using cyclic and square wave voltammetry. The ethyl acetate extracts shown to contain catechin. The cyclic and square wave voltammograms of 16.67 μg mL⁻¹ ethyl acetate extracts with added 6.67 μg mL⁻¹ catechin are shown in Figure 4.29. It shows that by addition of catechin in the extracts, the first oxidation peak increases. This shows the presences of catechin in the ethyl acetate.
Figure 4.29 Cyclic voltammograms of (a) ethyl acetate extract, (b) with the addition of 6.67 \( \mu \text{g mL}^{-1} \) catechin and extract in phosphate buffer pH 7.5, scan rate 200 mV s\(^{-1}\)
Ultraviolet-visible (UV-Vis) and infrared (IR) spectroscopy was used to confirm the presence of catechin in ethyl acetate extracts of *C. mellei* and *C. quadrifidus* as was reported using electrochemical analysis. The FT-IR absorption spectra of catechin and ethyl acetate extracts are overlapping; confirming the presences of catechin in the plant extracts (Figure 4.30). The spectra show the characteristic absorption regions for O-H group (3400 – 3100 cm⁻¹), C = C group around 1600 cm⁻¹, as well as C – O group (1150 – 1010 cm⁻¹). The UV-vis absorption spectra of *C. mellei* and *C. quadrifidus* shows two absorption band, strong one at 218 nm (Band II) and weak one at 282 nm (Band I) (Figure 4.31). In general terms the band II absorption may be considered as having originated from the A ring benzoyl system and band I from the B ring cinnamoyl system (Markham, 1982). The overlapping of the plant extracts with catechin shows the presence of catechin.
Figure 4.30 FT-IR spectra of catechin, *C. mellei* and *C. quadrifidus*
The UV-vis spectra of catechin in 0.05 mol L⁻¹ phosphate buffer pH 7.5 before and after the oxidation processes are illustrated in Figure 4.32. After oxidation process of catechin three characteristic absorption bands at 214 nm (Band II), 278 nm (Band I) and 362 nm were exhibited. Figure 4.32 shows that band I and II have shifted and the intensity has decreased during electro-oxidation at potential +220 mV. This supports the carbonylation of the 3',4'-OH groups at B ring, which causes loss of the electron donating ability of B ring. This is indicative that the quinonic structure is the chromophore present in the final species. The same pattern was observed by other quinones like catechin, 3,5-ditertbuthyl-o-benzoquinone and quercetin in DMSO (Bodini et al., 2001; Bodini et al., 1999). Figure 4.33 shows the UV-visible spectra of ethyl acetate extracts from C. mellei and C. quadrifidus after oxidation at potential +220 mV. It shows that the behaviour is the same as that of catechin. This suggests that catechin is present in the ethyl acetate extracts of both species.
Figure 4.32 UV-vis spectra of catechin in 0.05 mol L$^{-1}$ phosphate buffer pH 7.5 before and after oxidation processes at potential +220 mV.
Figure 4.33 UV-vis spectra of ethyl acetate extracts from *C. mellei* and *C. quadridifus* in 0.05 mol L$^{-1}$ phosphate buffer pH 7.5 after oxidation processes at potential +220 mV
4.3 Conclusion

This is the first work to identify the natural products in *C. mellei* and *C. quadrifidus*. The study demonstrates that ESI-HPLC-MS is a valuable method in separating and determining flavonoids from *C. mellei* and *C. quadrifidus*. The system is based on separation on a reversed phase column (Zorbax Eclipse XDB C18) and gradient mobile phase consisting of water and acetonitrile. All solvents contained 0.1% acetic acid to reduce peak tailing. The results showed that flavonols and flavanols are the major polyphenolic compounds in the plants. 8 flavonoids were identified from the ethyl acetate and butanol extracts of *C. mellei* and *C. quadrifidus*. The 8 flavonoids identified were catechin, rutin, coumarylquinic acid, epicatechin, epicatechin-epicatechin, quercetin-hexose, myricetin-deoxyhexose and isorhamnetin. The observed levels of the polyphenols confirm the importance of *Carpobrotus* species as excellent sources of plant antioxidants.

This study revealed for the first time the identification of flavonoids in *C. mellei* and *C. quadrifidus* using cyclic and square wave voltammetry. The oxidation reaction of flavonoids is strongly related to their structure, which contains several free phenolic OH groups. The oxidation process of flavonoids showed a reversible reaction corresponding to oxidation of the catechol moiety, namely 3’,4’-dihydroxyl group in B ring and then followed by irreversible reaction due to the oxidation of the 3-hydroxyl group and 5,7-dihydroxyl group in ring A. Oxidation potentials for both *Carpobrotus* species correspond to that of catechin. The oxidation potentials are not exactly the same as that of catechin because of other constituents in the ethyl acetate extract. UV-
vis and FT-IR spectroscopy confirm the presence of catechin in the ethyl acetate extracts of *C. mellei* and *C. quadrifidus*. 
CHAPTER 5
DETERMINATION OF ANTIOXIDANT ACTIVITY

5.1. Introduction

The flavonoids are a naturally occurring as phenolic compounds, which have been estimated to be present in human diet and occur naturally in fruits, vegetables, legumes, nuts and seeds of vascular plants. Recently flavonoids have been studied extensively for their vast antioxidant properties (Pietta, 2000). The antioxidant activity of flavonoids involves efficient lipid peroxidation inhibition (Nardini et al., 1995; Graf, 1992), peroxynitrite-induced oxidation (Ketsawatsakul et al., 2000), and antiradical activity (Rice-Evans et al., 1996; Madsen et al., 2000; Cotelle et al., 1996). The antioxidant capacity of human cells is determined by enzymatic antioxidant mechanisms, while the capacity of blood plasma which is greatly contributed by plant and animal antioxidants. A supply of natural antioxidants is important for supporting the antioxidant protection of the human body. This fact draws the attention of scientists to phenolic acids and flavonoids in the context of their application in the medical and food industries. The search for efficient and non-toxic natural antioxidants demands the development of new methods and improvement of existing ones for analysis of antioxidant activity or capacity in cases of compound mixtures.
5.2. Literature Review

5.2.1 Free radicals and Antioxidants

Oxygen is essential for all human beings. However, stress, ultraviolet rays, cigarette smoke and vigorous workouts tend to cause oxygen to change into free radicals. Free radical can kill germs once they enter the body. However, they can also join readily with other compounds to attack cells and cause a great deal of damage to the body. Free radicals are unstable molecules that include the hydrogen atom, nitric oxide (NO) and reactive oxygen species (ROS). ROS is consists of superoxide anion radical (O$_2^-_{}$), hydroxyl radicals (HO$^-$) and other non-radical oxygen derivatives, e.g. hydrogen peroxide (H$_2$O$_2$) and oxygen (O$_2$). A free radical can be defined as any species containing one or more unpaired electrons capable of independent existence (Halliwell, 1996).

A pathological increase of reactive oxygen species (ROS) generation has been recognized in human and animal diseases including cancer, cardiovascular disease, diabetes mellitus, male infertility, renal disease and dialysis, cataracts and neurological, liver, periodontal, lung and inflammatory diseases. ROS circulate free in the body with access to all organs and tissues. They cause tissue damage by a variety of different mechanisms including lipid peroxidation (by activation of cyclooxygenases and lipoxygenases), damage to proteins and DNA, oxidation of important enzymes, e.g. anti-proteases such as $\alpha_1$-antitrypsin, and stimulation of pro-inflammatory cytokine release by monocytes and macrophages (Halliwell, 1996).
It is not surprising that all oxygen-consuming organisms have a complex antioxidant system to counteract reactive oxygen species (ROS) and to reduce their damage (Halliwell, 1996; Cao et al., 1995). Antioxidants are compounds of different chemical nature that can retard or eliminate the non-enzymatic free radical oxidation of organic compounds by different forms of oxygen. The most efficient and widespread antioxidants are vitamin E, some phenols (eugenol and its derivatives) and polyphenols (conidendrin, pyrocatechol and derivatives of gallic acid), flavonoids, ubiquinones, some steroid hormones and phospholipids including lecithin and cephalin. This group of compounds includes ascorbic, citric, nicotinic, dehydrocaffeic, and benzoic acids and their salts; sulphur containing amino acids such as cysteine and glutathione; serotonin; adrenaline; bilirubin and blood proteins (Yankovskii, 2000; Diplock, 1994). Two classes of antioxidant are known, the low molecular (LMW) compounds (tocopherols (Vitamin E), ascorbate, β-carotene, glutathione, uric acid, bilirubin and many more) and the proteins (albumin, tranferrin, ceruloplasmin, ferritin, superoxide dismutase, catalase, glutathione peroxidase and many more). LMW antioxidants penetrate specific locations in the cell in which oxidative stress may occur and protect against ROS. Superoxide Dismutase changes the structure of oxidants and breaks them down into hydrogen peroxide. Catalase in turn, breaks down hydrogen peroxide into water and oxygen particles or gasses. Glutathione is a detoxifying agent, which binds with different toxins to change their form so that they are able to leave the body as waste. The biological activity of antioxidants is due to the stereoelectronic effects of the aromatic and chromane rings, the ortho and para position of hydroxyl groups, their tert-butyl screening, the formation of semiquinone forms, thiol-containing compounds, the chelation of variable-valence metals, receptor interactions with the cell membrane and many more.
Four primary protective systems of an organism are distinguished, in which the following compounds are “working” (Yankovskii, 2000; Diplock, 1994):

- Antioxidant enzymes
- Metal chelates retarding their oxidation and reduction
- Low-molecular compounds interacting with radicals to form lower active compounds
- High-molecular compounds (proteins) in the extracellular medium exhibiting antioxidant properties

Bioantioxidants suppress free radical autooxidation and control the effect of oxidation on the majority of metabolic processes. The action of antioxidants is to maintain the normal growth of cells and tissues. Bioantioxidants play an important role in protecting biosubstrates such as readily oxidizable lipids and fats and fatty acids in the membrane mass of the cell from autooxidation. Bioantioxidants are commonly polyfunctional compounds with the autooxidation function expressed to a variable degree (Yankovskii, 2000; Diplock, 1994).
5.2.2 Detection of free radicals and antioxidant

Free radicals and antioxidant have widely been investigated in the food technology and human health fields. Several methods have been proposed for the detection so far. The methods of detection of antioxidants in vivo were summarized in a recent review by Prior and Cao (1999). For in vitro characterization, photometric, fluorimetric, chromatographic and electrochemical methods have been developed. The photometric methods are based on the detection of absorption of radical scavengers such as cytochrome c or artificial radicals. An example is the stable free radical 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid), which is used to compare antioxidant activities with the standard antioxidant Trolox, to calculate the TEAC value (Trolox equivalent antioxidant activity) (Miller et al., 1993). Chemiluminescence and fluorescence methods have been used to study the antioxidant properties of substances by inhibition of luminal activity (Popov and Lewin, 1994; Van Dyke et al., 2000). Gas chromatographic (GC) and high performance liquid chromatographic (HPLC) assays have been described for the detection of oxyradical scavenging capacity of antioxidants and biological fluids with antioxidant properties (Winstin et al., 1998).
Recently, electrochemical approaches have intensively been used for antioxidant detection. The behaviour of antioxidants was investigated by the electroanalytical techniques such as differential pulse voltammetry (DPV) and square wave voltammetry (SWV) employing cylindrical carbon fibre microelectrodes (Agui et al., 1995). Cyclic voltammetry (CV) has also been used for the evaluation of the total antioxidant capacity (Chevion et al., 2000). A poly-(vinyl chloride)-composite electrode is described for the determination of antioxidants in the processed food (Luque et al., 1999).

Biosensors have also been employed for the detection of antioxidants: biosensors such as cytochrome c (cyt c) sensors and DNA sensors were used. In cytochrome c sensors, the detection of these biosensors is based on the redox reaction of cyt c. The immobilized cyt c is reduced by $\text{O}_2^{\cdot-}$ and immediately regenerated at the surface of the electrode polarized at the oxidation potential. The current generated due to the electron transfer from the radical, via cyt c, to the electrode is proportional to the radical concentration (Tammeveski et al., 1998). To avoid the interference from $\text{H}_2\text{O}_2$, catalyse enzyme is added to the reaction media. Addition of antioxidant reduces the radical concentration and the oxidation current, allowing the quantification of the antioxidant capacity. Gold electrodes are commonly used for these biosensors and are the formation of self-assembled monolayers (SAMs). Short-chain alkanethiols show a high efficiency of communication between cyt c and the electrode (Tammeveski et al., 1998; Manning et al., 1998). They do not effectively block the electrode from interfering substances. To eliminate the interference, cyt c has been immobilized on long-chain thiol (mercaptoundecanoic acid) modified electrodes. Ignatov et al. (2002) evaluated the antioxidant capacity of flavonoids and following trend was established:
flavanols > flavonols > flavones > flavonones > isoflavonones. Some authors tried to simultaneously detect both $O_2^{•−}$ and $H_2O_2$ produced in the course of its spontaneous dismutation (Shipovskov et al., 2004; Krylov et al., 2004). SAM-modified electrodes showed that the sensitivity of the sensor was directly proportional to the amount of immobilized protein (Ge and Lisdat, 2002). With the aim of increasing the amount of immobilized biomolecules, multilayer structures of cyt $c$ and polyanilinesulfonic acid (PASA) on long-chain mixed SAM-modified electrodes were constructed. These multilayer electrodes were successfully applied for the quantitative detection of $O_2^{•−}$ and much more sensitive than monolayer electrodes (Guo et al., 2005; Dronov et al., 2007). Cyt $c$-based biosensors allow the measurement of the $O_2^{•−}$ produced in vivo (during ischemia and reperfusion injury) (Scheller et al., 1999; Buttemeyer et al., 2002; Buttemeyer et al., 2003).

Reactive oxygen species (ROS) are known to damage DNA by oxidation of the bases, which results in their destruction and release or attack of the deoxyribose moieties, which results in strand breaks (Jaruga and Dizdaroglu, 1996). Taking advantage of this property, DNA-based sensors have been developed for the measurement of the antioxidant activity of different compounds. The strategy is based on the immobilisation of double stranded DNA (dsDNA) from calf thymus, on screen-printed carbon electrodes by simple adsorption. The detection of the guanine oxidation peak was between +800 and +1000 mV ($vs$ Ag/AgCl) by square wave voltammetry (Mascini et al., 2001; Mello et al., 2006). Peak current was proportional to the guanine concentration, and the immersion of the DNA-modified electrode into a Fenton solution produces a signal decreased in peak current.
The introduction of antioxidants into Fenton solution results in the scavenging of the OH’, which when immersion of the electrode into the solution, the peak current is very close to the original one, which demonstrates the DNA integrity. Another way to detect the DNA damage is using an electrochemical which is able to interact with dCDNA. This interaction is based on an intercalation phenomenon (at high ionic strength) or an electrostatic interaction (at low ionic strength). The amount of redox label bound to the DNA layer decreases proportionally to the concentration of radicals present into the sample (Labuda et al., 1999; Korbut et al., 2001). Tris-1,10-phenanthroline cobalt (III) as a redox marker, was used to evaluate the antioxidant activity of different yeast polysaccharides. Instead of using Fenton solution, methylene blue as intercalating probe was used. DNA was photo-oxidised on TiO₂-modified indium-tin oxide (ITO) electrodes. Gallic acid was found to be more antioxidant than glutathione (Liu et al., 2005). DNA sensors are promising devices to perform tests for the routine evaluation of the antioxidant activity in an easy way.
5.2.3 Biosensor Development

A serious breakthrough in analytical chemistry was announced by Professor Leland C. Clark in 1962 at a New York Academy of Sciences symposium. He described the concept of construction of electrochemical devices for the rapid, cheap, selective and sensitive glucose analysis produced by the immobilisation of the enzyme glucose oxidase in the vicinity of an electrochemical oxygen sensor (Clark and Lyons, 1962). Then, having improved the design of this sensor, Updike and Hicks (1967) reported the first functional biosensor for glucose. The first commercially available glucose biosensors, based on the amperometric detection of hydrogen peroxide generated by glucose oxidase, were delivered on the market by the Yellow Springs Instruments Company (USA) in 1975. Since then many researchers in the field of medical diagnostics (Bayer, Boehringer Mannheim, Eli Lilly, Lifescan, DKK Corporation), invested in the development and the mass scale production of biosensors, which are utilised in health care, environmental monitoring (Dennison and Turner, 1995), food and drink, the process industries (White and Turner, 1997), defence and security.

According to the definition by IUPAC, an electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element (Thevenot et al., 1999). Biosensors which do not need any additional reagents in a sample solution for the detection of an analyte, because they have all the components necessary for the reaction sequence, are called reagentless biosensors.
Traditional techniques such as spectrophotometry, fluorescence and gas or liquid chromatography (Prior et al., 2005; Roginsky and Lissi, 2005) are being replaced by other innovating technologies, such as electrochemical biosensors. Electrochemical biosensors are promising tools because they are suitable for fast analyses based on inexpensive instrumentation and simple operation protocols. Two different kinds of biosensors have been reported in the antioxidant domain. The first is that several amperometric biosensors for the detection of mono- and polyphenols have been developed on the basis of enzymes such as tyrosinase, laccase or peroxidase (Mello and Kubota, 2002). These configurations allow the evaluation of total phenol content. The second kind of biosensor is based on the free radical scavenging activity. All biosensors developed for measuring antioxidant activity are electrochemical and use reactive oxygen species (ROS) in their configurations. The first step in the development of these biosensors is the generation of ROS in vitro.

5.2.3.1 Superoxide Dismutase (SOD) Biosensor

Superoxide Dismutase (SOD) biosensors are alternative to cyt c biosensors for evaluation of the antioxidant activity. SOD enzyme is involved in cell protection mechanisms against oxidative damage from reactive oxygen species (ROS). SOD enzyme catalyses the dismutation of the \( \text{O}_2^- \) producing \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) via a cyclic oxidation/reduction electron transfer mechanism as shown in scheme 5.1.
Scheme 5.1 $\text{O}_2^{\cdot-}$ dismutation catalyzed by superoxide dismutase (SOD)

The high reactivity of $\text{O}_2^{\cdot-}$ limits its direct detection, so a more accurate measurement based on the determination of one of the enzymatically generated species, $\text{O}_2$ or $\text{H}_2\text{O}_2$, which can easily be detected using amperometric transducer, is required. That is the case of the first-generation SOD-based biosensors. Some are based on the measurement of $\text{O}_2$, but most SOD biosensors are usually based on the $\text{H}_2\text{O}_2$ oxidation at the electrode surface. High potential (> 500 mV vs Ag/AgCl) is required for this electrochemical reaction resulting in interference problems. To improve the selectivity approaches have been proposed based on the use of a $\text{H}_2\text{O}_2$-impermeable Teflon membrane (Song et al., 1995) or on the simultaneous detection of $\text{O}_2^{\cdot-}$ and $\text{H}_2\text{O}_2$ with two-channel sensor (Lvovich and Scheeline, 1997). The latter sensor is a system based on two glassy carbon working microelectrodes, one covered by an electrodeposited polypyrrole/horseradish peroxidase (PPy/HRP) membrane acting as $\text{H}_2\text{O}_2$ sensor, and another covered by a composite membrane composed of an inside layer of PPy/HRP and an outside layer of SOD used as a working electrode for $\text{O}_2^{\cdot-}$ detection. These biosensors have not been applied to the assessment of the antioxidant capacity, but can be envisaged.
There has been development of different SOD-based biosensors for assessing the antioxidant capacity of several compounds, based on the immobilization of superoxide dismutase (SOD) in a K-carrageenan gel and amperometric detection of H$_2$O$_2$ (Campanella et al., 2004b; Bonanni et al., 2007; Campanella et al., 2000; Campanella et al., 2005). The gel is sandwiched between an internal cellulose acetate membrane, which improves the selectivity of the electrode by blocking the access to possible electroactive interference and an external dialysis membrane. This biosensor can be used for evaluating red and white wines (Campanella et al., 2004a), fresh aromatic herbs, olives and fresh fruit, bulbs and vegetables, plant products sold by herbalists or pharmacies, tea (Campanella et al., 2001; Campanella et al., 2003a; Campanella et al., 2003b), phytotherapeutic diet integrators (Campanella et al., 2004b) and drugs containing mean component acetylsalicyclic acid (Campanella et al., 2004c), ascorbic acid, cysteine, melatonin and β-carotene (Campanella et al., 2000). The developed biosensor also enables the measurement of the antioxidant activity of healthy and diseased human kidney tissues in vitro (Campanella et al., 2000). Another biosensor developed by Emregül (2005) was applied to determine the antioxidant properties of acetylsalicyclic acid-based drugs and the antioxidant activity of healthy and cancerous human brain tissues. SOD immobilization on a platinum electrode surface is carried out within gelatine, which provides a biocompatible microenvironment around the enzyme and efficiently stabilizes its activity.
An amperometric enzyme electrode for O$_2^-$ determination was developed by anodic polymerization of pyrrole and concomitant incorporation of SOD on a platinum wire (Mesáros et al., 1998a; Mesáros et al., 1998b). The overoxidation of the polymer resulted in an insulating film and the generated H$_2$O$_2$ diffused into the film until the electrode surface. The selectivity of this biosensor was studied and it was concluded that the biosensor was unaffected by the presence of interfering substance and so the biosensor could be used in a blood matrix (Descroix and Bediou, 2001).

Another approach based on the electron transfer from SOD instead of the measurement of the enzymatic products, second-generation SOD-based sensors has been reported. Ohsaka et al. (1995) demonstrated the ability of methyl viologen to efficiently mediate the electron transfer between polyethylene oxide-modified SOD and electrode in dimethylsulphoxide (DMSO). The third-generation biosensor is based on rapid and direct electron transfer of SOD, without any mediator. This is the most attractive due to its simple sensor design, high sensitivity and selectivity. The required potentials are lower than those involved in the H$_2$O$_2$ oxidation, avoiding the interference of this molecule generated by spontaneous dismutation. The report suggested that electron transfer could be efficiently promoted by thiol self assemble monolayers (SAMs) formed on gold electrodes (Tian et al., 2002a; Tian et al., 2002b; Tian et al., 2004a; Tian et al., 2004b; Ohsaka et al., 2002). Cysteine SAMs act as effective promoters for the direct electron transfer of the superoxide dismutase (SOD), favouring both the oxidation of O$_2^-$ to O$_2$ and its reduction to H$_2$O$_2$. They have demonstrated the efficient electron transfer between SOD and carbon fibre microelectrodes modified with cysteine-SAM gold nanoparticles (Tian et al., 2005).

Another third-generation biosensor for O$_2^-$ was developed based on the entrapment
of SOD in a thin silica-poly(vinly alcohol) sol-gel film deposited on a gold electrode (Di and Zhang, 2004). The uniform porous structure of the film not only acts as a stabilizing matrix but also provides a fast response rate.

A hybrid ultra-sensitive electrophysiological O$_2$˙¯ sensor has been developed based on “membrane-engineered” mammalian cells immobilized in an alginate matrix (Moschopoulou and Kintzios, 2006). SOD molecules were electroinserted in cells, which acted as catalytic units able to convert O$_2$˙¯ to H$_2$O$_2$. This dismutation process induced changes to the cell membrane potential, which were then measured by appropriate microelectrodes according to the principle of the bioelectric recognition assay. It was found that the sensitivity improved one hundred-fold higher than the previously described SOD biosensors.
5.2.4 Generation of Reactive Oxygen Species (ROS)

The key factors when developing a biosensor for evaluating the antioxidant capacity is the generation of the radicals that will be subsequently scavenged by the antioxidant compounds. The following are several processes to produce ROS:

- The Fenton reaction, reduced transition metals ions (Fe (II), Cu (II) or Cr (II)) react with H$_2$O$_2$ in a one-electron redox reaction producing hydroxyl radical (OH’) and hydroxide anion. The addition of reducing agent increases the radical generation rate and also the transition metal can be reduced by the application of an appropriate electrode potential (Fojta et al., 2000).

- Radical generation by photocatalysis starts with the absorption of light of a wavelength higher than its band gap by TiO$_2$, which results in the transition of an electron from the valence band to the conduction band, leaving a hole behind. Adsorbed water or hydroxide ions are trapped by holes to produce OH’. Subsequently, electrons are trapped by the reaction with adsorbed O$_2$ to produce superoxide radical (O$_2^{•−}$), which then forms more OH’ (Nagaveni et al., 2004).

- Xanthine oxidase (XOD) catalyses the oxidation of xanthine or hypoxanthine with concomitant reduction of O$_2$ to H$_2$O$_2$; O$_2^{•−}$ is formed as an intermediate of reaction (Fridovich, 1978).
• The addition of NaOH to dimethylsulphoxide (DMSO) generates $O_2^{-}$.
  This $O_2^{-}$ production is inversely proportional to the water concentration
  in DMSO and solutions are stable for few days (Hyland and Auclair,

• Injection of KO$_2$ in aprotic organic solvents, specially DMSO results in
  $O_2^{-}$ generation by the following reaction (Lvovich and Scheeline, 1997;
  Ge and Lisdat, 2002):

  \[ \text{KO}_2 \rightarrow \text{K}^+ + O_2^{-} \]

Evaluation of the antioxidant activity of different compounds can be determined
based on the variation of the ROS concentration in the reaction medium. Evaluation
of antioxidant capacity based on the measurement of $O_2^{-}$ concentration, cytochrome
$c$ (cyt $c$) and superoxide dismutase (SOD) biosensors are used. DNA biosensors are
used to determine the antioxidant activity by measuring the damage produced to DNA
by free radicals. Presence of antioxidants involves a decrease in DNA alterations.
5.2.5 Enzymes

Enzymes are biocatalysts involved in the performance of metabolic reactions (Copeland et al., 1991). They are high molecular weight protein compounds, principally made up of amino acid chains linked together by peptide bonds. The catalytic activity of most enzymes is aided by other compounds known as cofactors. The cofactors are mainly co-enzymes, prosthetic groups or metal ion activators. A co-enzyme is a non-protein organic substance which is dialyzable, thermostable and loosely attached to the protein part. A prosthetic group is an organic substance which is dialyzable, thermostable and firmly attached to the protein portion whereas metal-ion activators include cations (K$^+$, Fe$^{2+}$, Fe$^{3+}$, Cu$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Mn$^{2+}$, Ca$^{2+}$ and Mo$^{3+}$).

5.2.5.1 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is a class of enzymes that catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide and is ubiquitous metalloenzymes in oxygen tolerant organisms. SOD enzyme is involved in cell protection mechanisms against oxidative damage from reactive oxygen species (ROS) and so it serves a key antioxidant and inflammatory role. SOD are normally homodimeric and are proteins cofactored with copper/zinc (CuZnSOD), or manganese (MnSOD), iron (FeSOD), or nickel (NiSOD) in the active sites of each subunit (Fridovich, 1978; Fridovich, 1998). CuZnSOD is the only class of SOD in which the active site contains two different metals in a histidine-rich environment and is commonly used by eukaryotes. The copper ion is responsible for the activity of the protein and the zinc ion plays a role in maintaining the protein structure and in electrostatic stabilization (Konecny, 1999). FeSOD and MnSOD are used by prokaryotes and protests, while ZnSOD is used by prokaryotes. SOD isozymes have
been localized in different cell compartments in higher plants. MnSOD is present in mitochondria and peroxisomes, FeSOD in chloroplasts and peroxisomes and CuZnSOD in cytosol, chloroplasts, peroxisomes and apoplast (Corpas et al., 2001; Corpas et al., 2006). In humans, three forms of superoxide dismutase are present. They are located in cytoplasm (SOD1), mitochondria (SOD2) and extracellular fluids (SOD3). SOD1 and SOD3 contain copper and zinc in its reactive centre, while SOD2 contains manganese.

5.2.5.2 Xanthine Oxidase (XOD)

Molybdenum containing hydroxylases catalyze the incorporation of oxygen derived from water in substrates in a manner whereby reducing equivalents are generated rather than consumed (Huber et al., 1996). The molybdenum is associated with a pterin deriviate, called molybdopterin, to form the molybdenum cofactor. Xanthine oxidase is a representative member of this group of enzymes. Xanthine oxidase (XOD) is an enzyme that catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. The active site of XOD is composed of a molybdopterin unit with the molybdenum atom coordinated by terminal oxygen, sulphur atoms and a terminal hydroxide (Hille, 2006). In the reaction with xanthine to form uric acid, an oxygen atom is transferred from molybdenum to xanthine. The reformation of the active molybdenum centre occurs by the addition of water. In humans, XOD is found in the liver and not free in blood and is also used to treat gout.
5.3. Experimental

5.3.1 Apparatus and reagents

A BAS 100B electrochemical analyser (Bioanalytical Systems, West Lafayette, Indiana) equipped with a three electrode system and a 20 mL capacity cell were used for all the voltammetric measurements. A platinum electrode with a surface area of 0.0176 cm² was used as working electrode, a platinum wire as auxiliary electrode and an Ag/AgCl (3 mol L⁻¹ NaCl) as reference electrode. All aqueous solutions were made up with high-purity water from a Millipore Milli-Q system (resistivity greater than or equal to 18 MΩ cm). The pH of the phosphate buffer was measured using a pH meter (Hanna instrument, HI 221 Calibration Check Microprocessor pH meter with accuracy of ± 0.05). Nafion 117 solution (5% in mixture of lower aliphatic alcohols and water) were supplied by Fluka (South Africa), superoxide dismutase (SOD) from bovine erythrocytes 4470 U mg⁻¹, xanthine (2,6-dihydroxy purine) sodium salt and xanthine oxidase (XOD) from buttermilk 0.6 U mg⁻¹ were purchased from Sigma (South Africa). Chemicals such as ascorbic acid, potassium hydroxide (KOH), fluorescein sodium salt, TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), Iron (III) chloride hexahydrate (FeCl₃ 6H₂O), sodium acetate were purchased from Sigma (South Africa), while di-hydrogen orthophosphate-1-hydrate (NaH₂PO₄·H₂O), di-sodium hydrogen orthophosphate dehydrate (Na₂HPO₄·2H₂O) were purchased from Merck (South Africa). Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid, AAPH (2,2'-Azobis (2-methylpropionamidine) dihydrochloride and DPPH’ (2,2-diphenyl-1-picrylhydrazyl) were purchased from Aldrich (South Africa). Organic solvents for chemical analysis were of analytical grade and purchased from Saarchem.

The ultraviolet (UV) measurements were performed using Multiskan spectrum and Fluoroskan Ascent from thermo Electron Corporation.
5.3.2 Determination of antioxidant activity by spectrophotometry

Radical scavenging activity of plant extract of *C. mellei* and *C. quadrifidus* was determined spectrophotometrically using assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH·), oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP). The methanolic extracts of *C. mellei* and *C. quadrifidus* were used for these tests. The extracts were dissolved in methanol to make different concentration. The following concentrations were prepared: 5.39, 5.64 and 6.14 mg mL⁻¹ from the methanol extract of *C. mellei* and 5.27, 5.71 and 7.88 mg mL⁻¹ from *C. quadrifidus*.

5.3.2.1 2,2-diphenyl-1-picrylhydrazyl (DPPH·) Assay

When DPPH· reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour from deep violet to light yellow were measured at 517 nm with UV spectrophotometer. Radical scavenging activity of methanol extracts was measured by slightly modified method of Brand-Williams *et al.* (1995). Ascorbic acid was used as standard and at the following concentrations in methanol: 0, 20, 50, 100, 250 and 500 (μmol L⁻¹). The solution of DPPH· in methanol (100 μmol L⁻¹) was prepared freshly before UV measurements. An aliquot of ascorbic acid solutions (10 μL) were mixed with DPPH· solution (300 μL), and were allowed to stand at room temperature for 15 minutes and then the absorbance at 517 nm was measured. The methanolic plant extracts were treated similarly and the absorbance was measured. All determinations were performed in triplicate.
5.3.2.2 Oxygen Radical Absorbance Capacity (ORAC) Assay

In the presence of free radicals or oxidant species the protein β-phycoerythrin (β-PE) loses over 90% of its fluorescence within 30 minutes (Cao et al., 1995). The addition of antioxidant species, which react with the free radicals, inhibits the diminution of the fluorescence of this protein. The inhibition caused by the action of the free radicals is correlated with the sample’s antioxidant activity. The 2,2′-Azobis (2-methylpropionamidine (AAPH) is used to generate peroxide radicals. ORAC assay was carried out on Fluoroskan Ascent Spectro Fluorophotometer at an excitation wavelength of 480 nm and an emission wavelength of 530 nm at temperature 37 °C. Phosphate buffer (75 mmol L⁻¹) pH 7.4 was prepared by mixing NaH₂PO₄·H₂O and Na₂HPO₄·2H₂O. Trolox was used as a standard and at the following concentrations were prepared in buffer solution: 0, 83, 167, 250, 333 and 417 μmol L⁻¹ were prepared. Fluorescein sodium salt (0.45 mg mL⁻¹) and AAPH (25 mg mL⁻¹) were dissolved in phosphate buffer. Trolox (12 μL), fluorescein (138 μL) and AAPH (50 μL) solutions were mixed together and the absorbance was measured immediately. The methanolic plant extracts were treated similarly and the absorbance was measured. All determinations were performed in triplicate.
5.3.2.3 Ferric Reducing antioxidant Power (FRAP) Assay

This method is based on the ability of the antioxidants to reduce Fe\(^{3+}\) to Fe\(^{2+}\). FRAP assay measures directly the reducing activity of a sample, which is an important parameter for a compound to be a good antioxidant (Benzie and Strain, 1996). Ascorbic acid was used as a standard and at the following concentrations was prepared in methanol: 0, 50, 100, 200, 500 and 1000 μmol L\(^{-1}\). A mixture of FeCl\(_3\) (9.3 mg mL\(^{-1}\)) in water, TPTZ (18 mg mL\(^{-1}\)) in 0.1 M HCl and acetate buffer pH 3.4 (by mixing sodium acetate and acetic acid) was prepared. The mixture gave a straw colour. Ascorbic acid solution (10 μL) and mixture solution (300 μL) were mixed together and gave a purple-blue colour. The mixture was allowed to stand for 30 min. at room temperature. The absorbance was measured at 593 nm using a Multiskan Spectrum. The methanolic plant extracts were treated similarly and the absorbance was measured. All determinations were performed in triplicate.

5.3.3 Superoxide Dismutase (SOD) Biosensor

5.3.3.1 Preparation of SOD Biosensor

Before each experiment the platinum electrode was polished with 0.05 μm, 0.3 μm and 1.0 μm alumina on a microcloth pad and then rinsed with ultra pure water, Ag/AgCl electrode was washed with ultra pure water and platinum wire was heated using Bunsen burner. 1% Nafion solution was prepared in ethanol and the pH was adjusted to neutral pH by adding few drops of 6% ammonium solution. The superoxide dismutase (SOD) enzyme was physically entrapped in 1% Nafion. 2 μL of SOD/Nafion layer solution was dropped on the clean platinum electrode, rinsed with ultra pure water to remove excess of SOD/Nafion and then air dried at room temperature for 10 minutes. The biosensor was put at 4 °C when not in use.
5.3.3.2 Principle of SOD Biosensor

The biosensor used to determine the superoxide radical ($O_2^{\cdot-}$) was obtained by coupling an amperometric electrode for hydrogen peroxide with the superoxide dismutase (SOD) enzyme immobilised on 1% Nafion. The superoxide radical is produced directly in the aqueous solution by oxidation of xanthine to uric acid in the presence of xanthine oxidase (Campanella et al., 2004b; Bonanni et al., 2007):

$$\text{Xanthine} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{XOD}} \text{Uric acid} + 2\text{H}^+ + \text{O}_2^-$$

Superoxide dismutase (SOD) on the platinum electrode catalyzes the dismutation reaction of the superoxide radical with release of oxygen and hydrogen peroxide (Campanella et al., 2000; Campanella et al., 2005):

$$2\text{H}^+ + 2\text{O}_2^{\cdot-} \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2$$

The $\text{H}_2\text{O}_2$ can be detected at the electrode surface. The current generated by oxidation of hydrogen peroxide at the working electrode is proportional to the concentration of superoxide radical in solution. The scheme of the SOD biosensor is shown in scheme 5.2.
Scheme 5.2 Superoxide Dismutase (SOD) biosensor (SOD/Nafion/Pt)
5.3.3.3 Measurement of procedure

All measurements were performed in a standard three electrode cell containing 0.1 mol L\(^{-1}\) phosphate buffer (5 mL) pH 7.0 at 25 °C. SOD/Nafion/Pt was placed in the cell and allowed to stabilize with a constant stirring rate of 300 rpm. 0.12 mg mL\(^{-1}\) of xanthine oxidase (XOD) enzyme (500 µL) was added and mixed thoroughly, and then different concentrations of 0.01 mol L\(^{-1}\) xanthine were added to the solution. The calibration plot was current as a function of increasing xanthine concentration. The addition of a sample characterised by antioxidant properties results in a decrease of signal strength as the antioxidant species react with the superoxide radical, thus reducing its concentration in solution. All experiments were done in the presence of air. The value of the relative antioxidant capacity is expressed by the algorithm:

\[
\text{Relative antioxidant capacity (RAC)} = 1 - \frac{m_b}{m_a}
\]

where \(m_a\): the slope of the straight line obtained by successive xanthine additions

\(m_b\): the slope of the straight line obtained by successive additions of xanthine in presence of sample with antioxidant properties
5.4. Results and Discussion

5.4.1 DPPH’, ORAC and FRAP Assays

The DPPH’ and FRAP values of the methanolic extracts of *C. mellei* and *C. quadrifidus* were evaluated by correlation of the absorbance of the sample from the calibration curve constructed from the standard ascorbic acid solutions in the concentration range 20 – 1000 μmol L\(^{-1}\) and expressed as μmol of ascorbic acid g\(^{-1}\) extract, while the ORAC values using fluorescence values are expressed as μmol of Trolox g\(^{-1}\) extract. The results of DPPH’, ORAC and FRAP are shown in Table 5.1. *C. mellei* and *C. quadrifidus* showed to have high values for DPPH’, ORAC and FRAP assays, suggesting both *C. mellei* and *C. quadrifidus* have antioxidant compounds.

Table 5.1 Antioxidant activities of methanolic extracts determined by DPPH’, ORAC and FRAP assays

<table>
<thead>
<tr>
<th>Species</th>
<th>DPPHa (μmol g(^{-1}) extract)</th>
<th>ORACa (μmol g(^{-1}) extract)</th>
<th>FRAPa value (μmol g(^{-1}) extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. mellei</em></td>
<td>759.6±33.77</td>
<td>1613.79±115</td>
<td>2148±18.52</td>
</tr>
<tr>
<td><em>C. quadrifidus</em></td>
<td>647.9±19.87</td>
<td>1591.52±60</td>
<td>1879±154.3</td>
</tr>
</tbody>
</table>

a: Expressed as a mean value ± standard deviation
5.4.2 Superoxide dismutase (SOD) Biosensor

Electrochemical behaviour of the SOD biosensor (SOD/Nafion/Pt) in the presence of superoxide radical was observed. Superoxide radicals were generated based on the xanthine-xanthine oxidase system. A square wave voltammogram of Nafion/Pt and SOD/Nafion/Pt in phosphate buffer pH 7.0 at frequency 10 Hz is shown in Figure 5.1. Catalytic current was observed at +815.4 mV for SOD/Nafion/Pt electrode, but no response was observed at the Nafion/Pt electrode in the presence of $O_2^{-\cdot}$. These results demonstrate that the observed current response is ascribable to the SOD/Nafion modified electrode. The direct electron transfer of the enzyme at the bare platinum electrode is slow, and therefore no oxidation peak was observed for SOD/Pt (Hong et al., 2006; Tian et al., 2004b). Nafion improves the stability of the enzyme and offers a biocompatible microenvironment on the electrode surface (Andrieux et al., 1990b; Furbee Jr. et al., 1993; Liu and Deng, 1995).
The activity of the SOD/Nafion-modified electrode as a superoxide radical sensor was examined in 0.1 mol mL\(^{-1}\) phosphate buffer pH 7.0 and the result is shown in Figure 5.2. It was observed that the anodic peak current increased when the concentration of xanthine increased. The observed current is attributed to the catalytic response of superoxide radicals (O\(_2^•\)\(^−\)). Figure 5.3 shows the plot of \(I_p\) versus xanthine concentration and Table 5.2 shows the main analytical data obtained for the calibration curve of xanthine.
Figure 5.2 Square wave voltammogram showing responses of 2 μL SOD/Nafion/Pt to superoxide radical after addition of 1 mmol L⁻¹ xanthine.

Figure 5.3 Plot of Ip versus xanthine concentration in phosphate buffer.
5.4.3 Amperometric response of SOD biosensor

Amperometric response of the SOD/Nafion electrode was obtained in a cell containing phosphate buffer pH 7.0 (5 mL) with 0.12 mg mL\(^{-1}\) xanthine oxidase (XOD) (50μL). A constant potential of +650 mV was applied and the current was measured as a function of time. The solution was kept under gentle stirring and then application of a potential to the electrode, the background current was allowed to decay to steady state. A single injection of 0.01 mol L\(^{-1}\) of xanthine (50 μL) was pipetted into the phosphate buffer solution with XOD to generate superoxide radical (O\(_2^{•-}\)) and the response was recorded. For the linearity of the electrode response, successive additions of the required amount of xanthine and the current-time response was continuously recorded. Figure 5.4 shows the current-time response of the SOD electrode to successive addition of xanthine. The response of the enzyme electrode is rapid (2 - 3 s). This strongly indicates that O\(_2^{•-}\) generated by the XOD/xanthine system is involved in the current response measured at SOD/Nafion/Pt electrode.
5.4.3.1 Linearity and detection limit of SOD biosensor

By using amperometric responses obtained in Figure 5.4, a calibration curve was obtained as indicated in Figure 5.5. From this curve, we can say that the SOD biosensor obeyed the normal Michaelis-Menten kinetics. A linear relationship exists for the xanthine concentration range from 0.2727 to 0.7273 μmol L\(^{-1}\) with regression equation \( y = -9.510 \times 10^{-9} x - 2.313 \times 10^{-9} \) and correlation coefficient (\( r^2 \)) of 0.9974 (Figure 5.5, inset). Detection limit, which was calculated based on a signal-to-noise ratio of 3, is 0.03918 μmol L\(^{-1}\) and sensitivity of 1.44 μA (μmol L\(^{-1}\))\(^{-1}\).
5.4.4 Plant material analysis

The same measurement was then repeated, but this time adding also methanolic extract of *C. mellei* and *C. quadrifidus* to the cell containing 0.1 mol L\(^{-1}\) phosphate buffer and then proceeding as described above. Figures 5.6 and 5.7 show the square wave voltammograms of both species added to xanthine. If the methanolic extracts display antioxidant activity, the observed peak currents will be lower than in the preceding case, in proportion to the decrease in concentration of the superoxide radicals in solution. Plot Ip versus xanthine concentration of *C. mellei* and *C. quadrifidus* are shown in Figures 5.8 and 5.9 and present a lower slope value than in the preceding case as shown in Table 5.1. This shows that *C. mellei* and *C. quadrifidus* have antioxidant activity. Table 5.2 shows the relative antioxidant capacity (RAC) of both species, *C. quadrifidus* display an antioxidant activity is generally higher than that of *C. mellei*. Table 5.4 shows comparison of other SOD
biosensor on different electrode and immobilization is different. The results show that the SOD biosensor developed can be used to determine the antioxidant activity.

Figure 5.6 Square wave voltammogram showing responses of 2 μL SOD/Nafion/Pt to superoxide radical after addition of 1 mmol L⁻¹ xanthine in presence of 0.5 μmol L⁻¹ of methanolic extract of *C. mellei*
Figure 5.7 Square wave voltammogram showing responses of 2 μL SOD/Nafion/Pt to superoxide radical after addition of 1 mmol L⁻¹ xanthine in presence of 0.5 μmol L⁻¹ of methanolic extract of *C. quadrifidus*.
Figure 5.8 Plot of Ip versus xanthine concentration in presence of 0.5 \( \mu \text{mol L}^{-1} \) methanolic extract of \( C. \text{mellei} \)

Figure 5.9 Plot of Ip versus xanthine concentration in presence of 0.5 \( \mu \text{mol L}^{-1} \) methanolic extract of \( C. \text{quadridius} \)
Table 5.2 Analytical data obtained for the calibration curves of xanthine, *C mellei* and *C. quadrifidus*

<table>
<thead>
<tr>
<th>Species</th>
<th>Equation of calibration curve</th>
<th>Correlation coefficient (R²)</th>
<th>Linear range (mmol L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of antioxidant species</td>
<td>$y = 1.5 \times 10^{-6} x + 2.104 \times 10^{-6}$</td>
<td>0.9773</td>
<td>0.2 – 1.0</td>
</tr>
<tr>
<td>Presence of <em>C. mellei</em></td>
<td>$y = 1.145 \times 10^{-6} x + 2.025 \times 10^{-6}$</td>
<td>0.9637</td>
<td>0.2 – 0.4</td>
</tr>
<tr>
<td>Presence of <em>C. quadrifidus</em></td>
<td>$y = 8.7 \times 10^{-7} x + 2.14 \times 10^{-6}$</td>
<td>0.9685</td>
<td>0.2 – 0.4</td>
</tr>
</tbody>
</table>

Table 5.3 Relative antioxidant capacity (RAC) of *C. mellei* and *C. quadrifidus*

<table>
<thead>
<tr>
<th>Species</th>
<th>RAC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. mellei</em></td>
<td>24</td>
</tr>
<tr>
<td><em>C. quadrifidus</em></td>
<td>42</td>
</tr>
</tbody>
</table>
Table 5.4 Comparison of the analytical parameters of SOD biosensor

<table>
<thead>
<tr>
<th>SOD biosensor</th>
<th>LOD (μmol L⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized by nafion on platinum electrode</td>
<td>0.03</td>
<td>This work</td>
</tr>
<tr>
<td>Immobilized by silica-PVA sol-gel on gold electrode</td>
<td>0.1</td>
<td>(Di et al., 2004)</td>
</tr>
<tr>
<td>Oriented on gold electrode via self assembled reaction of cysteine</td>
<td>0.006</td>
<td>(Tian et al., 2002a,b; Ohsaka et al., 2002)</td>
</tr>
<tr>
<td>Polymerization of pyrrole on platinum electrode</td>
<td>0.015</td>
<td>(Mesáros et al., 1998b)</td>
</tr>
</tbody>
</table>

5.5 Conclusion

The antioxidant activity of *C. mellei* and *C. quadrifidus* was determined using superoxide dismutase (SOD) biosensor. Superoxide dismutase enzyme was physically immobilized by nafion on platinum electrode surface. The preparation of the SOD biosensor was simple. The direct electron transfer of SOD in the nafion was realized at the platinum electrode without any mediators or promoters. Therefore, a third-generation amperometric biosensor for the measurement of antioxidants was developed.
6.1 Conclusions

It was shown by polyphenolic content that the methanolic extracts of *C. mellei* and *C. quadrifidus* are rich in polyphenolic compounds, among which flavonols and flavanols are present. The content of flavanols for both *C. mellei* was found to be 0.15 mg g$^{-1}$ plant extract, while *C. quadrifidus* is 0.16 mg g$^{-1}$ plant extract. Using ESI-HPLC-MS, 8 flavonoids were identified and the identified constituents were flavanols: catechin, coumarylquinic acid, epicatechin-epicatechin and epicatechin; the flavonol: isorhamnetin; and flavonol glycosides: rutin, quercetin-hexose (hyperoside) and myricetin-deoxyhexose. These constituents may play an important role in the antimicrobial activity of *C. mellei* and *C. quadrifidus*.

Flavonoid constituents in *C. mellei* and *C. quadrifidus* were also determined by using cyclic and square wave voltammetry. All the experiments were run in 0.05 mol L$^{-1}$ phosphate buffer pH 7.5 at the scan rate of 200 mV s$^{-1}$. The results for quercetin, rutin, catechin, luteolin and apigenin showed that the oxidation reaction is strongly related to their structure. Quercetin, rutin, catechin and luteolin have the catechol moiety (3',4'-dihydroxyl group) in the B ring. The oxidation of the catechol moiety of all mentioned flavonoids occurs first, and at low positive potential. The oxidation process involves a two electron-two proton reversible reaction and forms an o-quinone. Apigenin does not have a catechol moiety, consequently the results showed the oxidation of the 4’ hydroxyl group to occur at high potential and the oxidation reaction is irreversible. The hydroxyl group in the C ring and resorcinol group (5,7-dihydroxyl group) in the A ring of quercetin, rutin, catechin, luteolin and apigenin
oxidise thereafter and undergo an irreversible reaction. The oxidation peak potentials of the flavonoids were affected by the composition and pH of the electrolyte solution. The results for the ethyl acetate and butanol extracts of *C. mellei* and *C. quadrifidus* showed that the extracts are electrochemically oxidizable. The oxidation potentials of the ethyl acetate and butanol extracts range from +150.6 mV to +1072.6 mV and +165.8 mV to +908.7 mV, respectively. Many oxidation peaks were observed and this may be due to the flavonoids identified by HPLC. The oxidation potentials of the ethyl acetate extracts of *C. mellei* and *C. quadrifidus* closely resembled that of catechin. UV-vis and FTIR spectroscopy results confirmed the presence of catechin in the ethyl acetate extract. During the electro-oxidation, the absorbance bands of catechin and ethyl acetate extracts at 214 and 278 nm showed changes in wavelength, suggesting the formation of o-quinonic structure at the catechol moiety.

The study supports the use of cyclic and square wave voltammetry for simple and rapid identification of flavonoids in *Carpobrotus* species. No sample pre-treatment is required in the proposed CV and SWV methods in contrast to the HPLC method which always requires prior filtration or sample clean-up.
The identification of flavonoids in *C. mellei* and *C. quadrifidus* by HPLC and cyclic and square wave voltammetry confirm the importance of *Carpobrotus* species as excellent sources of plant antioxidants. Superoxide Dismutase (SOD) biosensor was used to determine antioxidant activity. The superoxide dismutase enzyme was physically entrapped in 1% Nafion and dropped coated on the platinum electrode. The superoxide radical was directly produced in solution by oxidation of xanthine to uric acid in the presence of xanthine oxidase enzyme. Superoxide dismutase on the platinum electrode catalyzes the dismutation reaction of the superoxide radical with release of oxygen and hydrogen peroxide. The hydrogen peroxide can be detected at the electrode surface. SOD biosensor showed to have a detection limit of 0.03918 μmol L⁻¹ and sensitivity of 1.44 μA (μmol L⁻¹)⁻¹. The results have shown that the methanolic extracts of *C. mellei* and *C. quadrifidus* contain antioxidants. The relative antioxidant capacity (RAC) of *C. mellei* and *C. quadrifidus* were found to be 24% and 42%, respectively. Spectrophotometric techniques such as DPPH˙, oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) were used to corroborate the results found using the SOD biosensor. SOD biosensor and spectrophotometry results suggested that *C. mellei* and *C. quadrifidus* have high antioxidant activity.
6.2 Future Work and Recommendations

Following aspects of determining the antioxidant properties of natural products from *C. mellei* and *C. quadrifidus* presented in this work warrant further investigation:

1. More experimental work needs to be done on isolating all the identify flavonoids from the plant extracts into pure compounds using different chromatographic techniques. These pure compounds can then be tested for antimicrobial activity, especially the antibacterial activity.

2. More work must be done on complete isolation, characterization and structural elucidation of all compounds following the electrochemical oxidation of the ethyl acetate and butanol extracts. Preparative separation and verification of the oxidized products can be achieved by a combination of column chromatography, GC-MS and LC-MS-MS.

3. The antioxidant activity of *C. mellei* and *C. quadrifidus* should be further investigated using a superoxide dismutase biosensor, where the SOD enzyme is immobilised on nano-particles.
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APPENDIX

Appendix A

Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula:

\[ C = \frac{c \times V}{m} \]

Where \( C \): total content of phenolic compounds, mg g\(^{-1}\) plant extract in GAE

\( c \): the concentration of gallic acid from the calibration curve, mg mL\(^{-1}\)

\( V \): the volume of extract, mL

\( m \): the weight of pure plant methanolic extract, g

The content of flavonols, in quercetin equivalents (QE) was calculated by the following formula:

\[ X = \frac{C \times V}{m} \]

Where \( X \): flavonol content, mg g\(^{-1}\) plant extract in QE

\( C \): the concentration of quercetin solution from calibration curve, mg mL\(^{-1}\)

\( V \): the volume of plant extract, mL

\( m \): the weight of plant extract, g
Polyphenolic content

\[ y = 0.0067x + 0.0228 \]
\[ R^2 = 0.999 \]

Flavanone content

\[ y = 0.0003x + 0.0231 \]
\[ R^2 = 0.970 \]
Flavonol content

Absorbance

Concentration (mg L$^{-1}$)

$y = 0.0020x - 0.0019$

$R^2 = 0.999$

Flavanol content

Absorbance

Concentration (mg L$^{-1}$)

$y = 0.0301x - 0.0009$

$R^2 = 0.999$
Appendix B

*C. mellei* (Ethyl acetate extract)

Compound 5

![Compound 5 Diagram]

Compound 6

![Compound 6 Diagram]
Compound 7

Compound 8

Compound 9
C. quadrifidus (Ethyl acetate extract)

Compound 5

Compound 6
Compound 10

C. mellei (Butanol extract)

Compound 10
C. quadrifidus (Butanol extract)

Compound 5

Compound 7
Compound 2
Appendix C

Quercetin

Rutin
Catechin

Luteolin
Apigenin