Int. J. Electrochem. Sci., 4 **(2009)** 1497 - 1510

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Electroanalytical Determination of Catechin Flavonoid in Ethyl Acetate Extracts of Medicinal Plants

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Received: 30 September 2009 / *Accepted:* 11 November 2009 / *Published:* 1 December 2009

South African Carpobrotus species have been found to contain hydrolyzable 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. In this paper we described the identification and quantification of catechin in C. mellei and C. *quadrifidus* using cyclic and square wave voltammetry. The oxidation potential of catechin ranges from +171.0 mV to +631.6 mV. The oxidation mechanism proceeds in sequential steps, related to the catechol moiety and 3-hydroxyl group. The oxidation of the catechol electron donating group occurs first at low potential and is a reversible reaction. The 3-hydroxyl group oxidises after and undergoes an irreversible reaction. The limit of detection (LOD) of catechin is 0.84 ppm and limit of quantification (LOQ) is 2.79 ppm with R.S.D. equal to 2.5%. The oxidation potentials of the ethyl acetate extracts of *C. mellei* and *C. quadrifidus* correspond with that of catechin. The content of catechin in *C. mellei* and *C. quadrifidus* was 5.0 ppm and 4.7 ppm, respectively. Other analytical techniques such as HPLC, UV-VIS, NMR, and FTIR were also used to validate the presence of catechin in the plant extracts.

Keywords: *Carpobrotus mellei, Carpobrotus quadrifidus*, flavonoid; catechin; cyclic voltammetry; square wave voltammetry

1. INTRODUCTION

Traditional medicine has undergone a major revival and the World Health Organization (WHO) has described traditional medicine as one of the surest means to achieve total health care coverage of the world's population [1]. It is estimated that about 25% of almost all modern medicines are directly or indirectly derived from traditional medicinal plants. Currently, the major pharmaceutical

companies have shown renewed interest in the developments of standardized phytotherapeutic agents with proven efficacy, safety and quality.

The genus C*arpobrotus* belongs to the family *Aizoaceae*, which is well known in South Africa by the name "Mesembryanthemum". Regionally these plants are found in the Western Cape and all the way up to KwaZulu-Natal [2, 3]. *C. edulis* and *C. acinaciformis* are commonly used as traditional medicinal plants. A preparation of leaf juice is gargled to treat infections of the mouth and throat, and is also effective against toothache, earache, and oral thrush. The juice from the leaf also applied externally to treat eczema, wounds and burns [3, 4].

Citric and malic acids, and their calcium salts have been reported to be found in the *Carpobrotus* species, as has the alkaloid mesembrine, which occurs in several genera of *Aizoaceae* [3]. Other South African *Carpobrotus* species were found to contain hydrolyzable tannins, various flavonoids, e.g. rutin and hyperoside, and phytosterols and aromatic acids [5-8]. Flavonoids are increasingly becoming the subject of medical research. Catechin, a monomeric flavanol, has been reported to be present in a variety of foods such as red wine, green tea, fruits and chocolate [9]. Catechin is a hepatoprotective agent, known for its antioxidizing capabilities and membrane-stabilizing properties [10]. Catechin had been used as natural antioxidant in oil fats, supplement for animal feeds, an antimicrobial agent in food and a health functional ingredient in various foods and dietary supplements [11].

Several techniques such as high-performance liquid chromatography (HPLC) have been used with electrochemical detection [12-14], chemiluminescence detection [15] and fluorescence detection [16] to determine and quantify catechin. A detailed electrochemical study of flavonoids including quercetin, quercitrin, rutin, catechin and luteolin, with identification of their oxidation products at a glassy carbon electrode has been reported [17-21]. Fourteen flavonoids aglycones and flavonoid glycoside were evaluated by cyclic voltammetry [22]. Catechin has two different pharmacophores, the catechol moiety in ring B and resorcinol group in the ring A, also the OH group at position 3 in ring C (Figure 1).

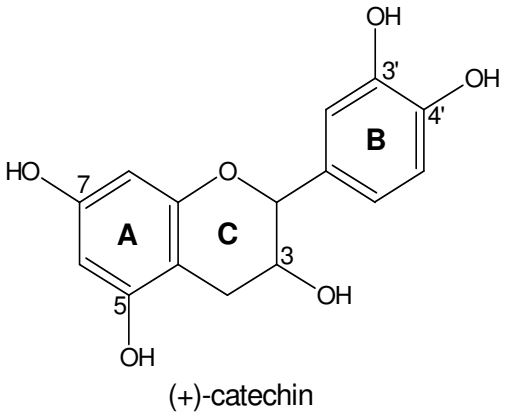


Figure 1. Catechin molecular structure

The aim of the present work is to identify catechin in *C. mellei* and *C. quadrifidus*. The electrochemical mechanism of oxidation of catechin was investigated using cyclic and square wave voltammetry in phosphate buffer pH 7.5 as the supporting electrolyte. The electrochemical oxidation of ethyl acetate extracts of *C. mellei* and *C. quadrifidus* were also investigated using the same methods and then their oxidation potentials were compared with that of catechin. This compound has been shown for the first time to exist in the South African *C. mellei* and *C. quadrifidus*.

2. EXPERIMENTAL PART

2.1. Apparatus and Reagents

Catechin, rutin, quercetin, luteolin and apigenin were purchased from Sigma-Aldrich (Cape Town, South Africa). All aqueous solutions were made up with ultra pure water from a Millipore Milli-Q system (resistivity greater than or equal to 18 MΩ cm) and organic solvents were of analytical grade, acetonitrile was of HPLC grade and purchase from Chemitrix (Cape Town, South Africa). 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). Thin layer chromatography (TLC) was performed on aluminium plates coated with Merck Kieselgel 60 F_{254}. The materials were weighed using Mettler Toledo AB104 analytical scale. A BAS 100*B* electrochemical analyser (Bioanalytical Systems, West Lafayette, Indiana) was used for cyclic and square wave voltammetry measurements.

2.2. Preparation of plant materials

The fresh leaves of *C. quadrifidus* were collected on the 30th November 2004 from Saldanha (Western Cape Province, South Africa), while those of *C. mellei* were collected from Montague (Western Cape Province, South Africa). The leaves were washed with distilled water, air dried for 10 minutes and then weighed. The leaves (300 g) were homogenized in 300 mL of distilled methanol (MeOH) using a domestic blender. An additional 500 mL of methanol 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 liquid was removed and then evaporated to dryness under vacuum at 45 ^{o}C. The crude methanol extract as suspension (dissolved in 100 mL of water and methanol 3:1, v/v) was partitioned with chloroform ($CHCl_3$), ethyl acetate (EtOAc) and butanol (BuOH).

2.3. Electrochemical measurements

For electrochemical measurements, a three electrodes cell consisting of a 3 mm diameter glassy carbon working electrode, a platinum wire auxiliary electrode, and an Ag/AgCl (3 M NaCl) reference electrode were used. Phosphate buffer with pH 7.5 was used as the supporting electrolyte. Before each experiment the glassy carbon electrode was polished successively with 0.05 μm and 0.3 μm alumina

on a microcloth pad and then rinsed with high-purity water. Catechin standard and extracts of *C. mellei* and *C. quadrifidus* were accurately weighed and dissolved in methanol to make a concentration of 0.2 mg mL^{-1}. Cyclic voltammetry measurements were run from –1500 mV to +1500 mV at a scan rate of 200 mV s^{-1}, while square wave measurements were run at the same potential window with frequency of 10 Hz. The solutions were degassed with pure argon.

2.4. HPLC Analysis

The analysis was performed using 1200 series liquid chromatography equipped with capillary pump and UV detector. The instrument was from Agilent Technologies.

2.5. UV-Vis and FT-IR spectroscopy

The UV-Vis spectrum was recorded at room temperature on a GBS UV/VIS 920 which was supplied by Wirsam Scientific (Cape Town, South Africa) and equipped with 1.0 cm quartz cell. FT-IR spectra were recorded on a Spectrum 100 FT-IR spectrometer supplied by PerkinElmer (Cape Town, South Africa) using KBr pellets.

3. RESULTS AND DISCUSSION

3.1. Phytochemical response

Phytochemical screening of chloroform, ethyl acetate and butanol extracts of *C. mellei* and *C. quadrifidus* were run on silica gel thin layer chromatography (TLC) plates with EtOAc/MeOH/H_2O (10:2:1, v/v/v) as solvent system. The dried TLC plates were sprayed with vanillin/H_2SO_4 spray reagent [23]. The TLC results showed ethyl acetate extracts of *C. mellei* and *C. quadrifidus* gave pink colour spot when sprayed with vanillin/H_2SO_4. Catechin have pink colour with R_f value 0.69 on TLC plate after spraying with vanillin/H_2SO_4 (25:1 v/v) and this corresponds with spot of ethyl acetate extracts for *C. mellei* and *C. quadrifidus* (Figure 2).

3.2. Cyclic and Square Wave Voltammetry of Catechin

Cyclic voltammetry (CV) of catechin shows two oxidation peaks (Figure 3A), 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 formed in peak 1. Square wave voltammetry (SWV), which has the shorter analysis time, decrease in problems associated with fouling of the electrode surface and increased sensitivity when compared to CV, was also used [18]. The square wave voltammogram (Figure 3B) also shows two oxidation peaks for catechin and the reduction peak 1'. Furthermore, reversibility of the catechin oxidation reaction is

clearly enhanced in the square wave voltammogram with the peak potentials tabulated in Table 1 and 2.

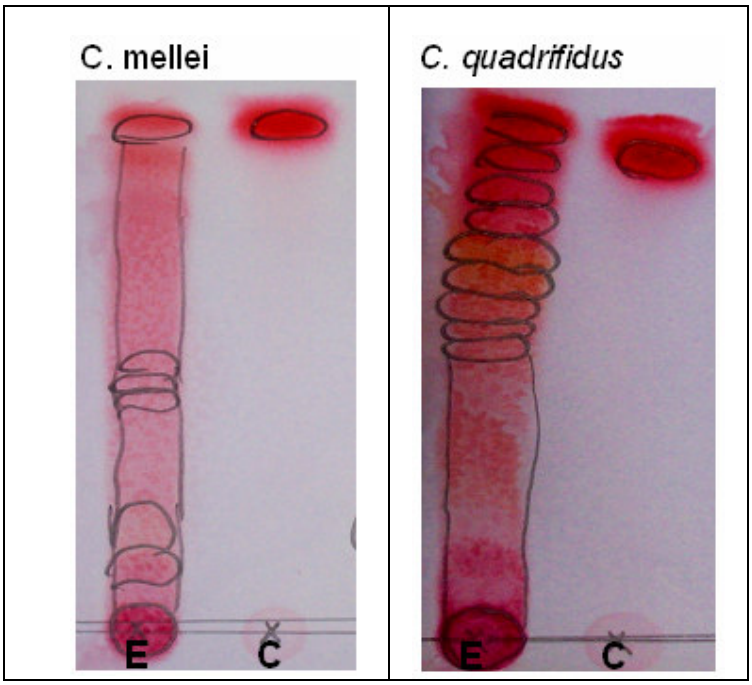


Figure 2. TLC of ethyl acetate extract (**E**) and catechin (**C**).

Catechin has hydroxyl groups attached to the ring structures which may be electrochemically oxidised. Electrochemical studies reveal trends in the electron donating abilities of flavonoids. It showed that the catechol moiety is more easily oxidizable than the resorcinol group [24]. 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 and square wave voltammograms. The oxidation process involves a two electron - two proton reversible reactions and forms *o*-quinone (Scheme 1) [19, 25, 26]. The process has a formal potential (E°′) of 171 mV. The peak corresponding to catechol moiety of catechin has been shown to be dependent on the pH and that at high pH *o*-quinines generated in the reaction are unstable. The reaction at higher pH follows a different pathway than at lower pH and the reaction products are easily oxidised [19]. The 3-hydroxyl group at ring C of catechin is oxidized (peak 2) next and undergoes an irreversible oxidation reaction [26]. Its been reported that peak 2 is dependent on pH ranging between pH 3 and 9; but for pH < 3 or pH > 9 the electron transfer reaction is pH independent. This is in agreement with the studies done on 3-hydroxyflavone and 7-hydroxyflavone. 3-hydroxyflavone which exhibited the oxidation peak at 500 mV, while the oxidation peak of 7-hydroxyflavone occurred at higher potentials, and this was done at pH 7 [27]. Catechin contains the resorcinol group namely, the 5,7-dihydroxyl group at ring A. The oxidation peak for the resorcinol group was not observed using cyclic and square wave voltammetry. But it was reported to occur at very high positive potentials and irreversible, this was observed using differential pulse voltammetry [19].

The calibration curve of catechin was constructed by a plot of oxidative peak current (I_p) versus concentrations (Figure 4). It shows that the I_p of catechin increases with increasing concentration and after I_p achieved its maximum value and tends to level off with further addition of catechin as expected for a process that was limited by adsorption of analyte. The linear plot of I_p versus the concentration of catechin (Figure 4) shows a linear range 6.7 to 16.7 ppm with a correlation coefficient (R^2) of 0.9882. Limit of detection (LOD) of the measurement of catechin was 0.84 ppm and was calculated using the equation LOD = 3 S.D./m. Where S.D. is the standard deviation and m is the slope of the calibration curve. The limit of quantification (LOQ) of catechin was found to be 2.79 ppm. LOQ is considered as the lowest concentration of each single compound providing a signal-to-noise ratio of approximately 10 (R.S.D = 2.5%). These results agree closely with that reported for the detection of catechin using HPLC and electrochemical sensor using β-cyclodextrin incorporated carbon paste modified electrode (Table 3). The results obtained for the quantification limit suggest that the proposed method is sufficiently sensitive for the determination of catechin.

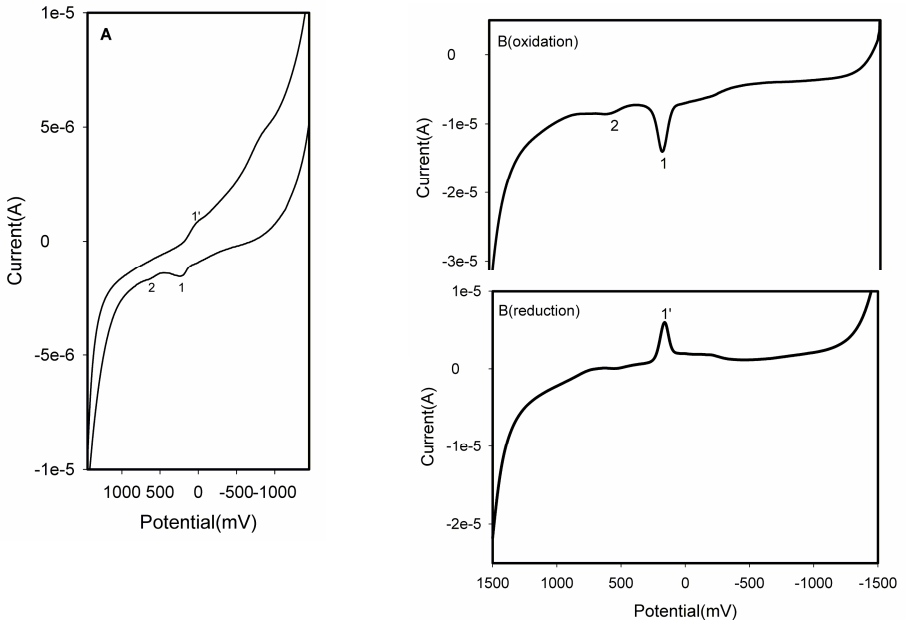


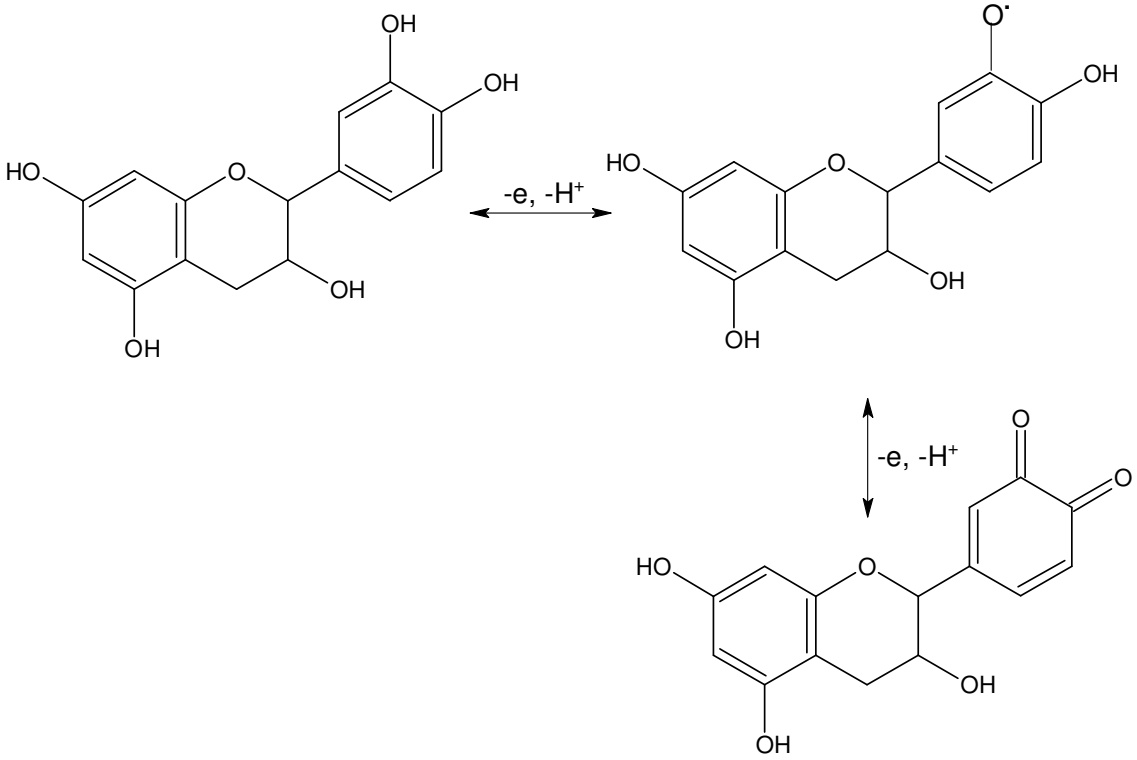
Figure 3. Cyclic (**A**) and square wave (**B**) voltammograms of 16.67 µg mL^{-1} catechin in 0.05 mol L^{-1} phosphate buffer pH 7.5, scan rate 200 mV s^{-1} and frequency 10 Hz.

Table 1. Peak Potentials from Cyclic Voltammetry

Samples	**Peak 1 (mV)**	**Peak 2 (mV)**	**Peak 1' (mV)**
Catechin	+197.0	+612.7	+70.7
C. mellei	+201.5	+500.0	+37.3
C.quadrifidus	+182.2	+507.0	+70.1

Table 2. Peak Potentials from Square Wave Voltammetry

Samples	Peak 1 (mV)	Peak 2 (mV)	Peak 3 (mV)	Peak 4 (mV)	Peak 1' (mV)
Catechin	+171.0	+631.6			+171.0
C. mellei	+158.1	+602.6	+1072.6		+150.6
C.quadrifidus	+166.7	+464.9	+657.9	+1043.9	+164.5



Scheme 1. Oxidation of catechol moiety of catechin to *o*-quinone

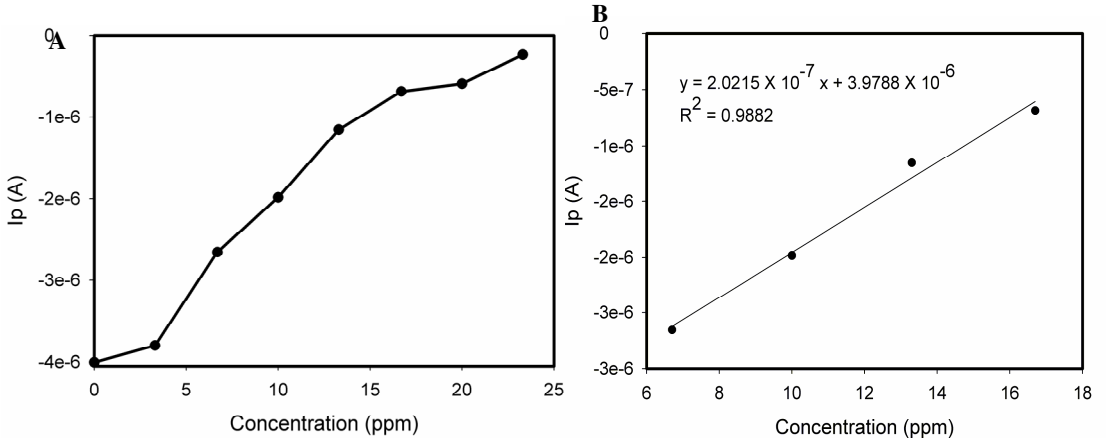


Figure 4. Plot of anodic peak current versus concentration of catechin. Plot **B** is the extracted linear region from **A**

Table 3. Analytical characteristics of the calibration graph of different techniques

Technique	R^2	LOD (ppm)	LOQ (ppm)	R.S.D (%)	Reference
Electrochem. analysis	0.9882	0.84	2.79	2.5	This paper
HPLC	0.9999	0.37	1.23		[28]
Electrochem. sensor		1.35	3.45	1.95	[29]

3.3. Cyclic and Square Wave Voltammetry of ethyl acetate extract

The cyclic voltammetry 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 corresponds to reduction of the oxidation products formed 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 5 and Table 1). Square wave voltammetry 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 6 and Table 2).

The first oxidation peak (peak 1) in the cyclic and square wave voltammograms of ethyl acetate extracts of *C. mellei* and *C. quadrifidus* closely corresponds to the catechol moiety - 3',4'-dihydroxyl group in ring B of catechin and therefore is a two electron two proton reversible reaction (Table 1 and 2). Oxidation potentials of *C. mellei* (peak 2) and *C. quadrifidus* (peak 3) corresponds to that of catechin (peak 2) which involves the oxidation of 3-hydroxyl group in the C ring (Table 2). Oxidation potentials of quercetin, rutin, luteolin and apigenin were determined by cyclic voltammetry and square wave voltammetry by the addition of the relevant standard solution to the phosphate buffer (Table 4.1. and 4.2). A standard addition method was applied for the determination of catechin content in ethyl acetate extracts of *C. mellei* and *C. quadrifidus*. The SWV voltammograms when 6.67 $\mu g\ mL^{-1}$ of catechin was added to *C. mellei* and *C. quadrifidus* extracts showed a marked increase in current for the peak assigned to catechin (Figure 7). This confirmed the presence of catechin in extracts, as compared to other flavonoids investigated, based on their oxidation potentials established using standard solutions. The total catechin content in *C. mellei* was found to be 5.0 ppm and that of *C. quadrifidus* is 4.7 ppm.

In subsequent HPLC analysis (Zorbex C18, water/acetonitrile gradient analysis) further attempts to distinguish between catechin and epicatechin (the closest neighbour in terms of structure, that catechin could be confused with) were made. The acetonitrile content of the mobile phase was increased from 10-100% at a flow rate of 0.8 mL/min. The retention time for the catechin standard under mobile phase gradient control between 40-60% acetonitrile was observed at t_R = 12.06 and epicatechin standard was observed at t_R = 12.81 (Figure 8). The peak for catechin in the ethyl acetate

extract of C. *mellei* and C. *quadrifidus* was observed at retention time (t_R) = 12.06 min using the same mobile phase gradient and instrumental parameters as for standards. In this way HPLC was able to corroborate the conclusions drawn from CV and SW analysis that the flavonoid present in the extracts (at potentials less than 200 mV) was indeed catechin and not epicatechin.

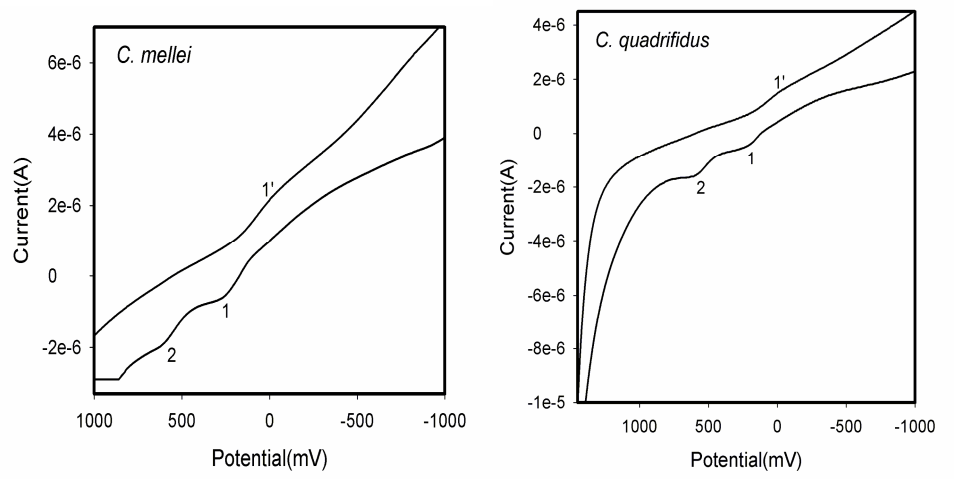


Figure 5. Cyclic voltammograms of the ethyl acetate extracts with concentration 16.67 μg mL^{-1} 0.05 mol L^{-1} phosphate buffer pH 7.5, scan rate 200 mV s^{-1}

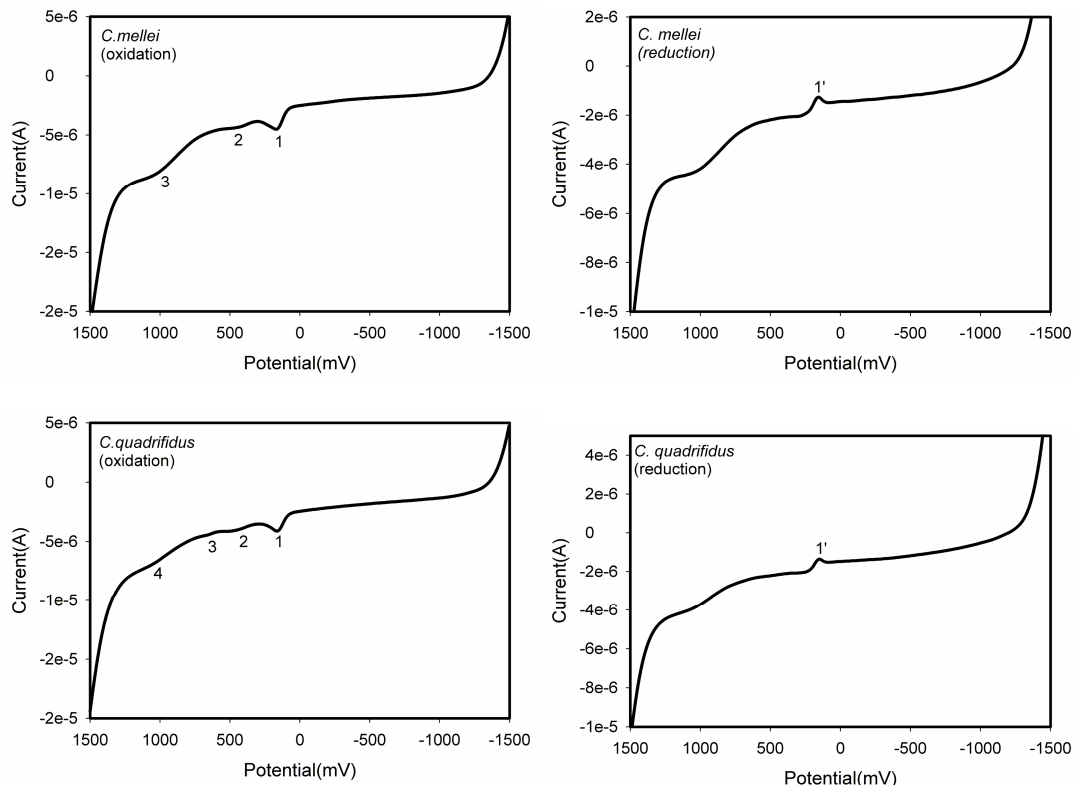


Figure 6. Square wave voltammograms of ethyl acetate extracts with concentration 16.67 μg mL^{-1} in 0.05 mol L^{-1} phosphate buffer pH 7.5, frequency 10 Hz.

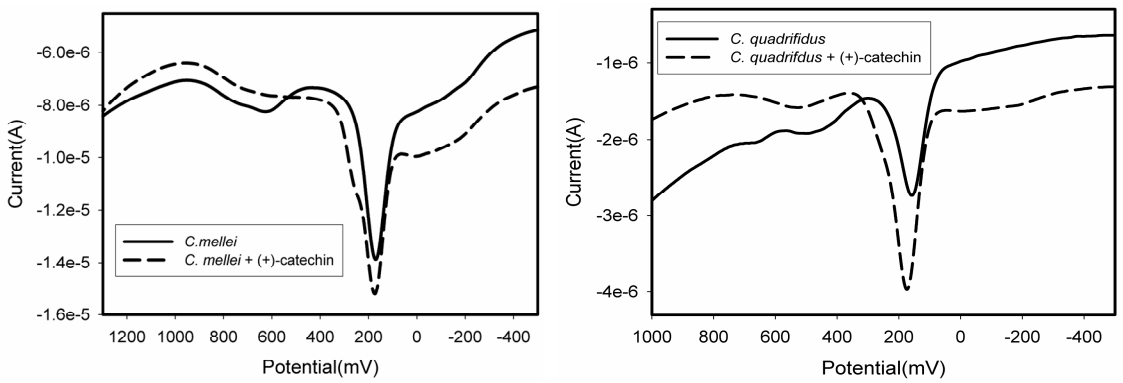


Figure 7. Square wave voltammograms of 16.67 μg mL^{-1} *C. mellei* and *C. quadrifidus* with addition of 6.67 μg mL^{-1} catechin in 0.05 mol L^{-1} phosphate buffer pH 7.5, frequency 10 Hz.

Table 4.1 Peak Potentials from Cyclic Voltammetry

Samples	Peak 1 (mV)	Peak 2 (mV)	Peak 3 (mV)	Peak 1' (mV)
Quercetin	+97.0	+392.8	+891.3	+88.2
Rutin	+233.8	+874.9		+225.7
Luteolin	+281.8	+968.4		+227.3
Apigenin	+709.0	+932.8		

Table 4.2 Peak Potentials from Square Wave Voltammetry

Samples	Peak 1 (mV)	Peak 2 (mV)	Peak 3 (mV)	Peak 4 (mV)	Peak 1' (mV)
Quercetin	+125.0	+712.2	+896.7		+125.0
Rutin	+217.0	+842.1			+217
Luteolin	+250.4	+948.7			+250.4
Apigenin	+634.5	+861.3			

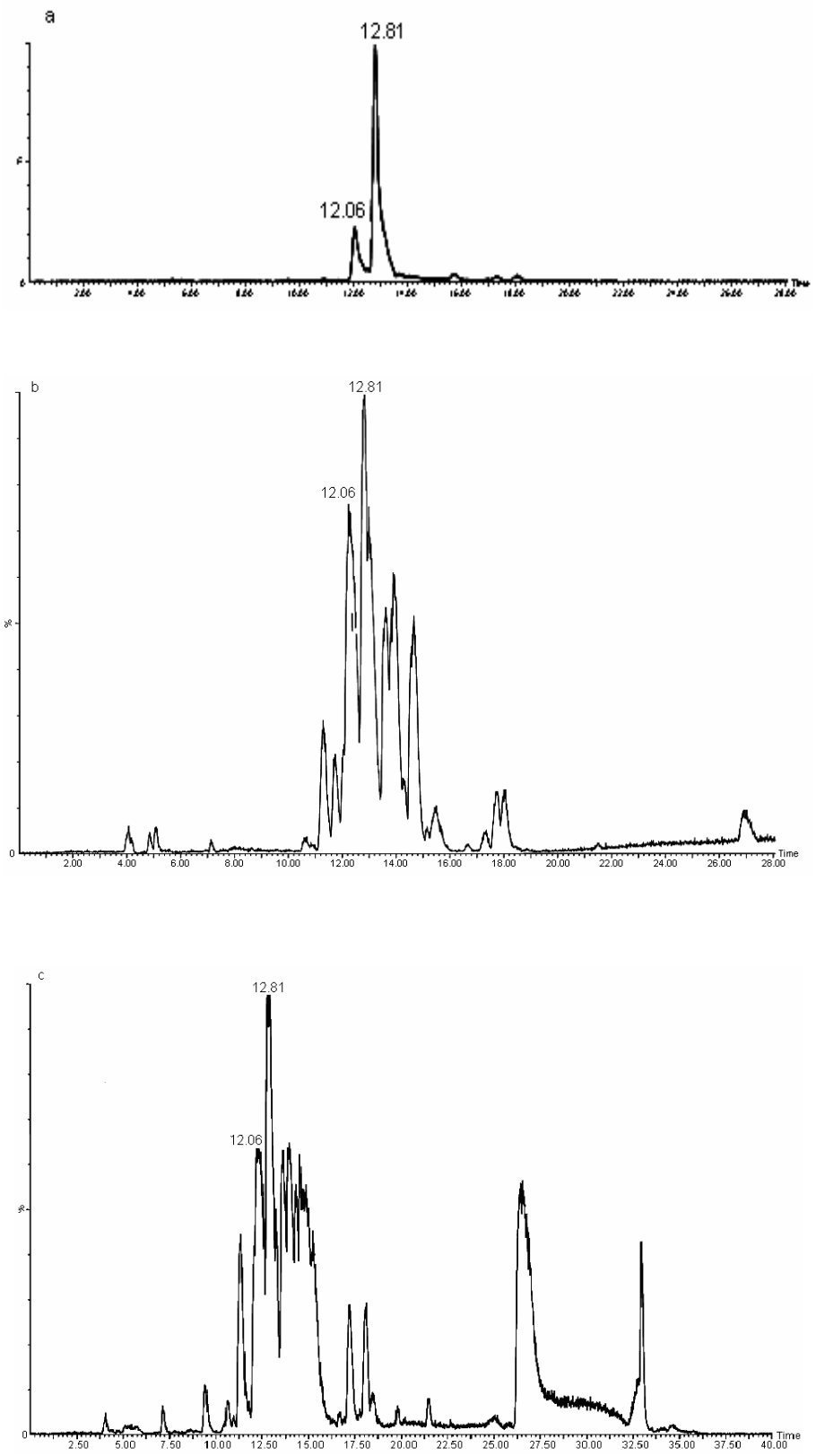


Figure 8. HPLC chromatograms showing (**a**) catechin t_R = 12.06 min and epicatechin t_R = 12.81 min for standards (**b**) *C. mellei* extract and (**c**) *C. quadrifidus* extract.

3.4 Ultraviolet-visible (UV-vis) and Infrared (IR) spectroscopy of ethyl acetate extract

Infrared (IR) and ultraviolet-visible (UV-Vis) spectroscopy were used to confirm the presence of catechin in ethyl acetate extracts of *C. mellei* and *C. quadrifidus* as reported using electrochemical analysis. The FT-IR absorption spectra of catechin and ethyl acetate extracts overlap confirming the presences of catechin in the plant extracts (Figure 9). 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* overlap with that of catechin, which confirms the presences of the catechin. The UV-vis absorption spectra of *C. mellei* and *C. quadrifidus* show two absorption bands, a strong one at 218 nm (Band II) and a weak one at 282 nm (Band I) (Figure 10). 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 [30].

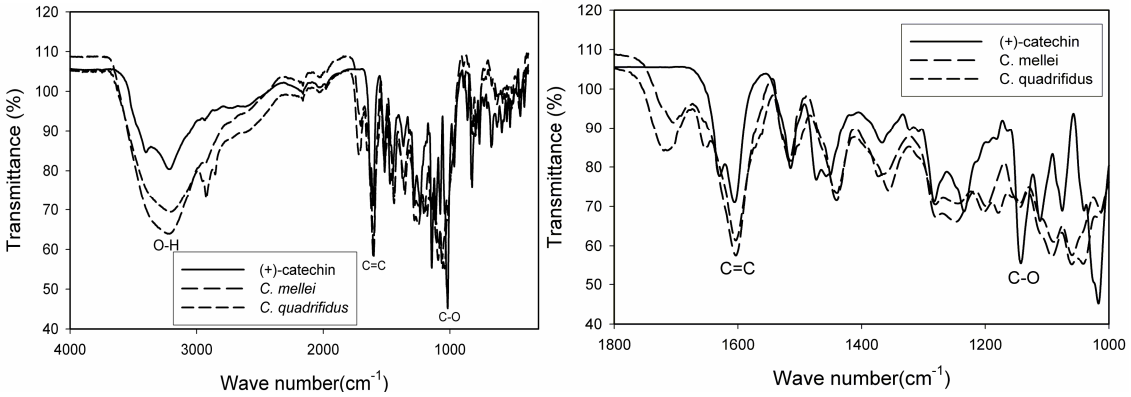


Figure 9. FT- IR spectra for catechin, *C. mellei* and *C. quadrifidus*

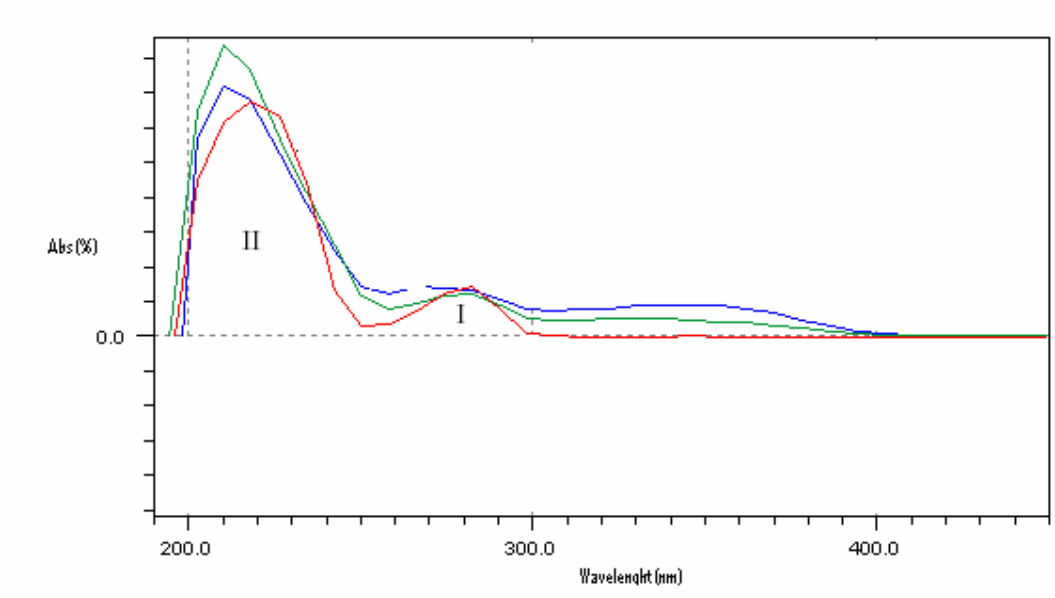


Figure 10. UV-vis spectra of catechin (red), *C. mellei* (blue) and *C. quadrifidus* (green)

4. CONCLUSIONS

This study revealed for the first time the identification of catechin in ethyl acetate extracts of *C. mellei* and *C. quadrifidus* using cyclic and square wave voltammetry. The presence of catechin in the ethyl acetate extracts of *C. mellei* and *C. quadrifidus* was further confirmed by HPLC, UV-VIS and FT-IR spectroscopy. The LOD of 0.84 ppm and LOQ of 2.79 ppm (R.S.D. = 2.5%), obtained from voltammetry compares well with that of the well known sensitive HPLC technique. Oxidation potentials for both *Carpobrotus* species is similar to that of pristine catechin with the other constituents in the crude extracts causing the little differences observed. The content of catechin in ethyl acetate extracts of *C. mellei* and *C. quadrifidus* is 5.0 ppm and 4.7 ppm, respectively. The identification of catechin in these plants will help explain some reasons behind their medicinal properties. This work also adds to the library of plants that may be tapped into for modern drug or pharmaceutical application in the nearest future.

ACKNOWLEDGEMENTS
We would like to thank National Research Foundation of South Africa for the financial support of this project.

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