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Evaluation of Antioxidants in Herbal Tea with a Laccase Biosensor

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In this study, a glassy carbon electrode (GCE) modified with laccase was used as the working electrode for analysis of phenolic compounds. The electrochemical behaviour of rutin and ascorbic acid were used to assess the antioxidant capacities (trolox reagents) for the estimation of total phenolic (TP) content in two herbal tea samples common in South Africa. The result showed a positive linear correlation between the trolox equivalent antioxidant capacities (TEAC) and TP content ($R^2 = 0.9812 \pm 0.012$), which indicated that phenolic compounds could be one of the main components responsible for the antioxidant activities in the tea samples investigated. The experimental results obtained using a Differential Pulse Voltammetry (DPV) suggested that indeed laccase is a suitable biosensor showing good reducing properties. The scavenging ability of 2,2'-Azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), a diammonium salt assessed using UV-Visible spectrophotometry in the sample extract yielded half maximal effective concentration (EC_{50}) values of 10.80 µg/ml and 11.62 µg/ml for ascorbic acid and rutin respectively.

Keywords: Laccase, Antioxidants, Biosensor, Voltammetry, UV-Visible spectrophotometry

1. INTRODUCTION

Plant phenolics are commonly found in both edible and non-edible plants, and have been reported to have multiple biological effects, including antioxidant activity. Their potential for the maintenance of health and protection from coronary heart disease and cancer has raised interest among scientists and food industrialist because antioxidants have an ability to retard oxidative degradation of lipids and thereby improve the quality and nutritional value of foods [1-3]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in

absorbing and neutralizing free radicals, quenching singlet and triplet. Many naturally occurring products have been reported to contain large amounts of antioxidants other than vitamin C, E and carotenoids. The antioxidant activity of polyphenol has been extensively evaluated by several researchers as can be seen in the review by Lotito and co-workers [3]. Apparently, there appears to be a general correlation between antioxidant properties and the total polyphenolic content [4]. Slight differences in the range of antioxidant activities might be attributed to variations in the amounts of different phenolic substances and the additional presence of other antioxidant components.

Several methods that have been employed for the evaluation of antioxidant compounds in pharmaceutical formulations, fruit juices, teas, wines, urine, plasma etc.; includes the following: high-performance liquid chromatography electrochemical detector (HPLC-ECD) [5], HPLC-UV [6-8], Folin–Ciocalteu (FC), [6], spectrophotometer [6, 9-11], potentiometric [12], electron paramagnetic resonance spectroscopy (EPRS) [13], capillary electrophoresis (CE) and Voltammetry [6, 10, 14, 15]. Electrochemical techniques are used to perform redox mechanism studies utilizing modified electrodes which are thought to be more sensitive than bare electrodes.

The laccase (benzenediol:oxygenoxidoreductase, E.C. 1.10.3.2) molecule is a dimericortetrameric glycoprotein with a molecular mass ranging from about 50 to 100 kDa. They are characterized by four copper atoms per monomer distributed in three redox sites produced by higher plants, microorganisms, mainly fungal species [16-18] including insects and bacteria [18]. An important feature of the structure of laccase is the covalently-linked carbohydrate moiety which may contribute to the high stability of the enzyme [19]. It displays a broad specificity for the reducing substrates including phenols, polyphenols, aminophenols, aromatic diamines and even certain inorganic compounds by a one-electron transfer mechanism [20]. Thus wider range of applications include effluent decolouration, detoxification, pulp bleaching, removal of phenolics from wines, organic synthesis, biosensors, synthesis of complex medical compounds and dye transfer blocking functions in detergents and washing powders, many of which have been patented [21, 22]. Laccases from various sources have successfully been immobilized on various supports, but covalent immobilization of laccase directly on an electrode surface is not commonly employed in biosensor applications [23].

Literature studies revealed that the assessment of laccase activity in fungal cultures is achieved by using a good mediator. Most commonly antioxidant capacity is measured using ABTS [2, 2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], a synthetic mediator which exhibits fast electron transfer kinetics between the electrode surface and laccase to possess a formal potential resembling a type 1 copper ion [22]. It is well known that ABTS undergoes two consecutive one-electron oxidations [13] with the stabilities of $ABTS^{+}$ and $ABTS^{2+}$ dependent on solution composition, pH, electrode material, etc.

In the present work, the antioxidant properties of herbal tea samples have been investigated electrochemically with an in-house laccase modified biosensor. Since most of the herbs are believed to be associated with antioxidant activities and have many beneficial effects. For this purpose two herbal tea samples were evaluated as sources of antioxidants using ascorbic acid and rutin as trolox compounds. In addition, the spectrophotometric test measuring the antiradical capacity by reduction of the stable free radical ABTS was also performed in this study.

2. EXPERIMENTAL

2.1. Apparatus

All electroanalytical measurements were performed with a 797 VA Computrace from Metrohm (Herisau, Switzerland). It is a three electrode system of a 3mm diameter rotating disc electrode (RDE), reference electrode made of Ag/AgCl (saturated AgCl, 3 M KCl), and the auxiliary electrode that is made of platinum wire. Scans were evaluated with the Metrohm 797 VA potentiostat. A 781 pH/Ion meter coupled with 801 stirrer supplied by Metrohm (Herisau, Switzerland) was used to adjust the pH of the buffer solutions. All working solutions including the buffer were prepared with deionized water using the Aqua MaxTM Basic 360 Series water purification system supplied by Trilab (Durban, SA). The electrochemical buffers together with the samples were refrigerated at 4 °C. A Varian 50 UV/Visible spectrophotometer with 1.0 cm path length cells was used to perform spectroscopic measurements. The software provided with the spectrophotometer enabled automatic peak evaluation for current signal, absorbance and estimation of the concentration in a standard addition mode. All analytical measurements were performed at room temperature.

2.2. Reagents and Chemicals

All reagents used in this study were analytical grade. Ascorbic acid, potassium persulphate ($K_2S_2O_8$) and sodium hydroxide (NaOH) were obtained from *Saarchem* (Gauteng, SA). Rutin trihydrate (78095) and 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma (Durban, South Africa SA), Methanol with a purity ≥ 99.9 % was obtained from Merck (Durban, SA), Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) was obtained from Capital Lab Supplies (Durban, SA). Nujol mineral oil and Graphite powder (282863) < 20 μm were purchased from Aldrich (SA). Nitrogen of 99.99 % purity was supplied by AFROX (Durban, SA). Alumina powder ≤ 3μm was supplied by Metrohm (Durban, SA). Laccase derived from *Trametesversicolor* (51639) and supplied by Sigma (Durban, SA) was used for the preparation of the biosensor surface (paste) and the two herbal tea samples Green tea and Rooibos tea, were purchased at a local supermarket in Durban, SA.

2.3. Preparation of standards

An adjusted phosphate buffer pH 7.4 of 0.1 M was prepared and used to make standards. The stock solutions of 1 mM ascorbic acid and rutin were prepared by dissolving equivalent mass in the methanol: buffer (50:50 v/v). Diluted working standard solutions were prepared from the stock solution with the buffer. The standard addition curve was prepared by using 10.0 $mg.l^{-1}$ solutions of ascorbic acid and rutin. Total Antioxidant values are expressed in terms of rutin and ascorbic acid equivalent per mass, as they were used as reference compounds in this work.

2.4. Preparation of biosensor

Enzymes may be immobilized by a variety of methods (adsorption, entrapment, crosslinking and covalent bonding) mainly based on chemical and/or physical mechanisms. In this study, adsorption of the enzyme on the polished surface was performed by immobilization of approximately 0.5 g paste with the composition of laccase, nujol and graphite on the electrode. The glassy carbon electrode was cleaned manually with Alumina Al_2O_3 and electrochemically within a -1.0 to +1.0V. Thereafter 2 ml of 40 mM laccase standard was used for film deposition on the paste for duration of 10 min. This was to ensure maximum electrical contact between the biosensor electrode and the measuring solution.

2.5. Preparation of samples (Extraction of phenolic content)

The extracts of herbal tea samples were obtained by brewing/heating approximately 2.0 g of tea sample in a vacuum flask with 100 ml of hot water at 65 ^{0}C for duration of 30 min while placed in rotatory hot plate ensuring maximum yield and minimal loss through evaporation since the analytes are volatile. After extraction, the obtained aqueous herbal tea extracts were subjected for the analysis of their total phenolic contents and radical scavenging capacity with voltammetry and *ABTS* radical scavenging methods.

2.6. Bio-Electrochemical measurements

Cyclic voltammetric measurements were run from -1.00 to +1.00 V at the laccase immobilized glassy carbon electrode with a scan rate of 100 mV s^{-1}, while stirring the measuring solution. A volume of 15 mL of the supporting electrolyte was placed in the electrochemical cell and the 1 ml of the standard solution was added into the cell by micro-pipette. The solution was purged with pure nitrogen prior to electrochemical measurements. The same procedure was followed for sample analysis. The standard addition method was applied adding successive aliquots of 10 μL as 10 μM ascorbic and rutin standard solutions to the electrochemical cell. Differential pulse voltammetry (DPV) was recorded at a scan rate of 5 mV s^{-1} and a pulse amplitude of 50 mV with the potential ranging from -200 mV to +600 mV. After the stirrer was stopped, the potential was scanned towards a positive potential. All measurements were carried out at room temperature (23 ± 3 ^{o}C).

2.7. Oxidation of phenols by ABTS

The free radical scavenging activity was measured in terms of hydrogen donating ability or free radical scavenging ability by using the stable ABTS. A series of rutin and ascorbic acid standards were separately mixed with 2.0 ml of 25 mM ABTS enzyme that was incubated at 25°C for duration of 30 min to allow for the reaction to reach equilibrium. The λ_{max} of each compound was determined prior to the absorbance measurements for the reaction mixtures at respective wavelengths with a double beam UV/Visible spectrophotometer. The calibration curve was plotted for absorbance of Trolox (rutin and

ascorbic acid) versus the concentrations taking into consideration the absorbance of the blank. Different volumes of the tea extracts (0.1 to 1 ml) were mixed with 2 ml of ABTS and buffer in a test tube to a total volume of 3.5 ml. The reaction mixtures were allowed to stand in the dark for 5 min before the absorbance at 734 nm were recorded. All the measurements were performed in triplicate. The concentration of sample, required to scavenge 50% of ABTS free radicals was determined from the calibration plot as EC_{50} value.

2.8. Data Evaluation

The statistical significance between antioxidant activity AA, Total Phenolic TP content and TEAC values of the extracts were evaluated by analysis of variance (ANOVA) incorporated with Grubb's test for outlier identification using STATGRAPHICS Plus version 5.1 and Microsoft excel® 2010. Peak evaluation in voltammetry was performed with the Nova Software 1.3 ® 2008, accompanying the VA 797 Computrace.

3. RESULTS AND DISCUSSION

3.1. Optimization of experimental procedure

One of the most important aspects for the performance of laccase biosensors lies in their immobilization of the enzyme on the electrode surface. Accordingly one of the goals of this study was to propose an alternative approach with a view to achieving better sensitivity and reproducibility. The enzyme denaturation is one way in which the biochemical properties of laccase can be affected resulting in poor electronic coupling between the enzyme and the electrode [24].

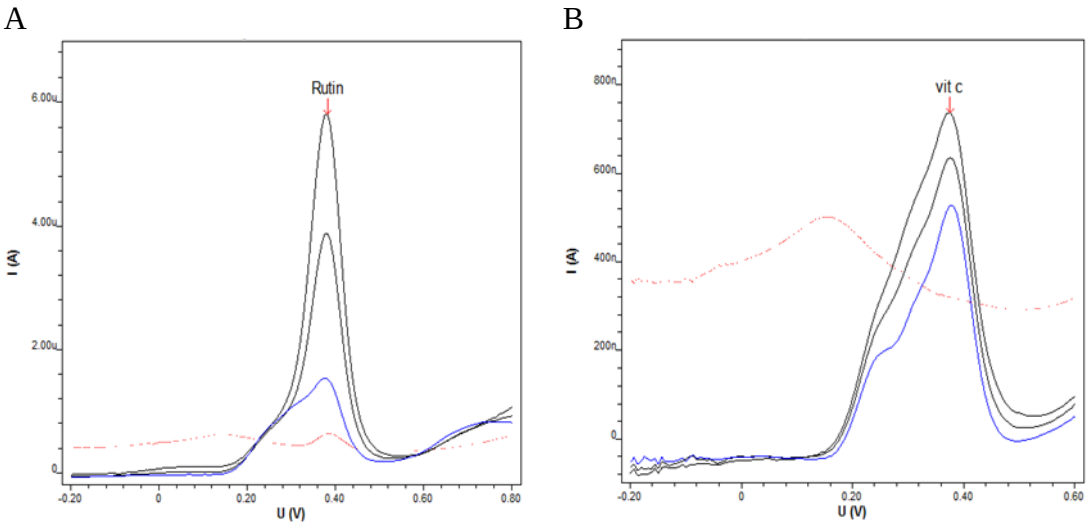


Figure 1. shows DP voltammogram of recorded at scan rate 5 $mV.s^{-1}$, pulse amplitude 50 mV, initial potential -200 mV and final potential +600 mV. **A**: rutin and **B**: ascorbic acid equivalent Green tea. ---blank, ——sample, — standard addition 1 and 2.

Immobilization is therefore achieved by fixing the enzymes onto or within a solid support resulting in an immobilized enzyme system by mimicking the natural mode of occurrence in living cells where enzymes are attached to the cellular membranes, the system stabilizes the structure of enzymes; hence their activities are utilized accordingly. Figure 1 depicts that the peak potentials and voltammetric responses (I_p) are strongly influenced when the concentration of buffer is not sufficient enough to maintain the pH at the surface of working electrode, even if a new peak is produced [14].

Moreover, values of the electrode potential are dependent upon the redox behaviour of the compounds thus reflecting their ability to lose electrons. Figure 1 shows that the herbal tea samples increase their reducing abilities when the concentration/volume of the extracts are correspondingly increased, resulting in superimposed voltammograms. Despite the fact that the experimental measurements were performed in triplicate, the current signal (I_p) of the sample does not disappear, thus confirming that our conditions were well optimized. The optimized current response signal was crucial due to the relatively large molecular size of laccase enzyme which prevents the direct immobilization of the multilayer on the surface from an aqueous medium. One of the goals of this work was to propose an alternative approach with a view to achieving better reproducibility and sensitivity. The sensitivity of the laccase immobilized electrode was approximated around 1.5 uA/5 µM. The proportionality of the current to the concentration of the analyte allowed for the quantification total phenolic contents (see Table 1) of the analyte using differential pulse voltammetry (DPV). However, the methodology required continuous renewal of the electrode surface between runs.

Table 1. Total Phenolic (TP) Contents of tea samples obtained with Differential Pulse Voltammetry (DPV) results. Number of replicates (n) = 3

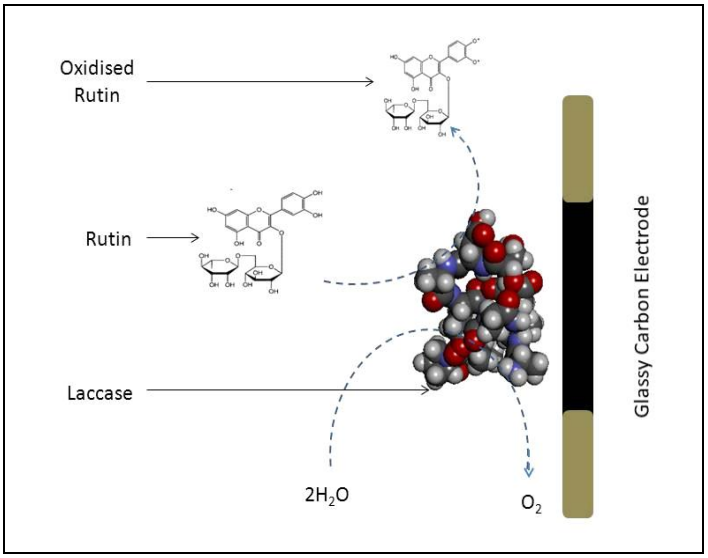
Sample	Analytes	$TP_{biosensor}$ (mg/g)	Corr. R^2
Green Tea	Rutin	7.282± 0.18	0.9976
	Ascorbic Acid	5.35± 0.09	0.9993
Rooibos	Rutin	5.282± 0.23	0.9976
	Ascorbic Acid	2.253± 0.34	0.9952

Table 1 shows that rutin which represent polyphenolic content is available in greater quantity in both samples in contrast to ascorbic acid, a phenolic equivalent content. These results suggest that there is a greater quantity of polyphenolics than phenols in the evaluated tea samples.

3.2. Redox reactions at a biosensor electrode

Laccase enzyme exhibits good electron transfer kinetics. Accordingly, the determination of the phenolic content in tea samples was performed with an immobilized laccase biosensor electrode. In general terms, substrate oxidation by laccase involves a single-electron reaction that generates a typically unstable free radical, which undergoes a second enzyme-catalysed oxidation or otherwise a non-enzymatic reaction such as hydration, disproportionation or polymerization. The mechanism of the

oxidation of rutin resulting in the formation of the conjugate base is pictorially depicted in Scheme 1, and the corresponding cyclic voltammogram is shown in Figure 2B.



Scheme 1. Shows the mechanism of rutin oxidation by a laccase immobilized on the surface of the Glassy Carbon Electrode (GCE).

It can be seen that a deprotonation of the antioxidant molecules had taken place at ±0.45 V resulting in the formation of the conjugate base as can be seen in Scheme 1.

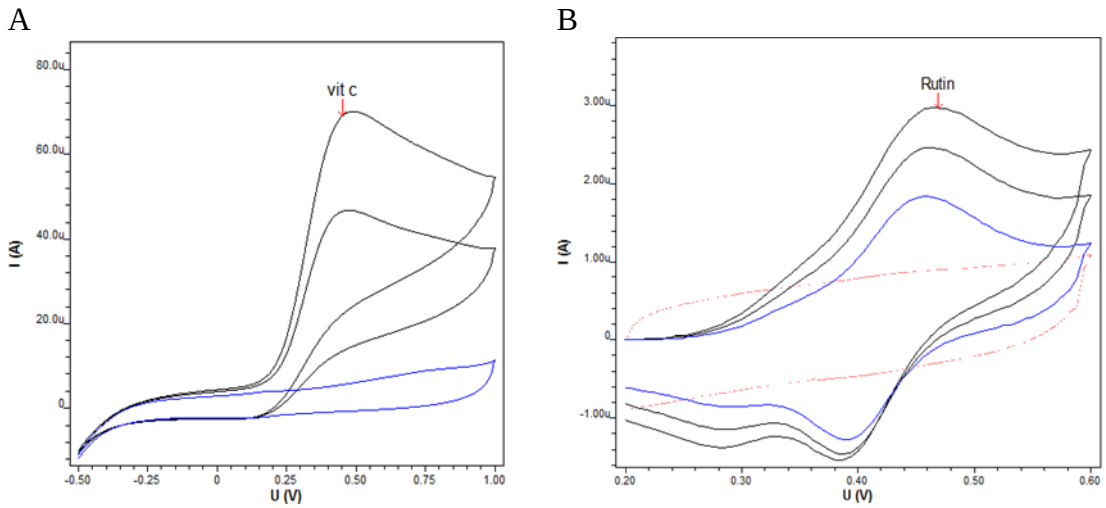


Figure 2. CVs recorded at a laccase modified electrode in 0.1 M PBS pH 5.5; Scan rate 100 m.Vs^{-1} in the potential range of -0.2 to 1.0 V. **A**: Vit C and **B**: Rutin obtained from a Green tea sample.

Laccase oxidizes its substrate molecule by taking four electrons from the compound while the four Cu^{2+} of its active center are reduced to Cu^{+}. The reduced laccase returns to a rest status by transferring the electrons to produce water. It is this mediated electron transfer that forms the basis of

their application as enzymes for phenol determination and the prediction of redox mechanisms. The oxidation of the reducing substrates involves the free radical formation upon the transfer of a one electron laccase.

Figure 2 shows the cyclic voltammograms of ascorbic acid and rutin at a surface of the biosensor electrode at the same pH 5.5. The oxidation of ascorbic acid involves two electrons and two protons to produce dehydroascorbic acid, followed by an irreversible reaction at pH lower than 5.5 [14]. The compounds containing the catechol group are known to be oxidised in the presence of polyphenol oxidase forming 1.2-benzoquinone however, the reaction can be reversible [8]. In this study it was found that ascorbic acid is irreversible hence it shows a reduction peak at $E_{1/2} = E_{pa} = 0.45$ V, while rutin showed E_{pa} and E_{pc} at 0.46 and 0.39 V respectively. The $E_{1/2}$ of rutin was calculated to be 0.43 V. The low value of $E_{1/2}$ for rutin is related to the free radical scavenging capacity which is primarily attributed to the high reactivities of the hydroxyl substituents that participates in protonation reactions [2].

3.3. Trolox Equivalent Antioxidant Capacities (TEAC) and EC_{50}.

The ABTS radicals produced in the presence of potassium persulphate $K_2S_2O_8$ are responsible for the absorbance at 734 nm and also for a visible deep purple color that is stable at room temperature. Initially, the existence of phenol in samples was tested by mixing ABTS radical solution and a sample extract, resulting in a positive test of extensive colour change observed after 1 min of mixing in a dark environment. The activities are shown in Figure 3.1.

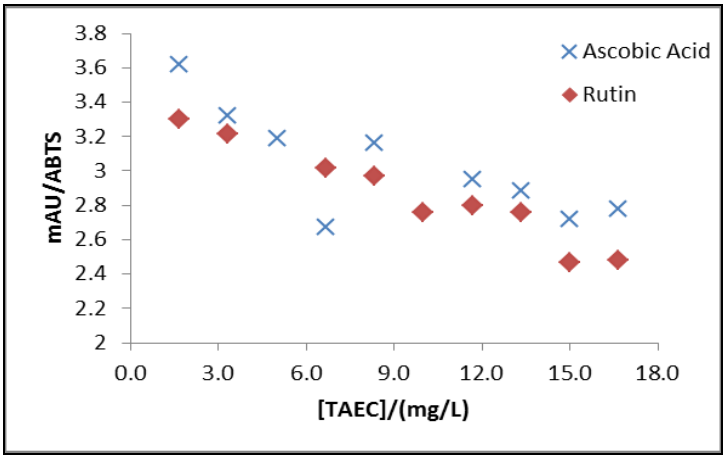


Figure 3.1. Total Antioxidant TEAC obtained with ABTS method

This activity showed a reasonable correlation of the phenolic content as the concentration correlation sample extract was increased by changing the volume. ABTS is a relatively stable free radical but colourises in the presence of antioxidants. The conjugate base formed during the protonation possesses an additional double bond, which increases the number of π electrons relative to

the cation, causing an increased delocalization of the electrons, resulting in significant shifts of the absorption wavelength of the phenolic compound. In the reaction mixture, ABTS accepts an electron donated by an antioxidant compound present in the sample, and loses colour (see Figure 3.1). The linear model describing the relationship between TEAC and Total Phenolic content obtained with a laccase biosensor were fitted and shown in Figure 3.2 and corresponding analysis in Table 2.

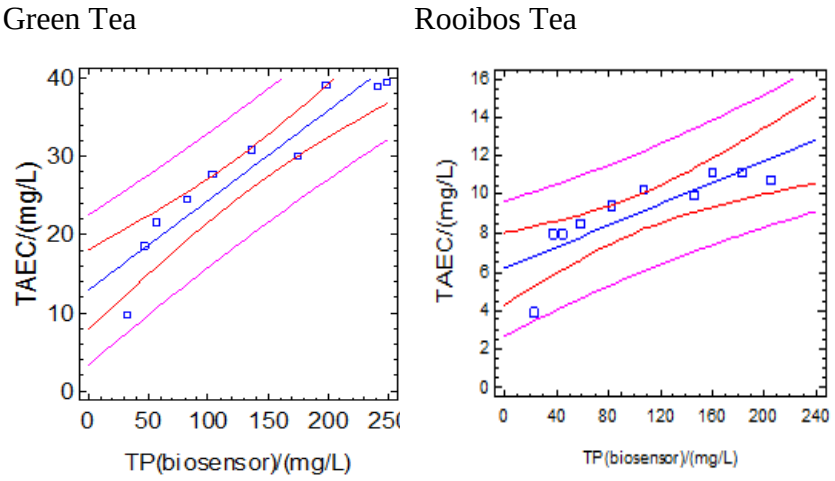


Figure 3.2. comparison between TEAC against Total Phenolic content obtained with a laccase biosensor electrode

A closer inspection of Table 2 depicts that the P-values from ANOVA for both samples evaluated are less than 0.01, hence there is a statistically significant relationship between TEAC and Total Phenolic content at the 99 % confidence level.

Table 2. Analysis of Variance (ANOVA) in the model of Total Phenolic (TP) content and Trolox Equivalent Antioxidant Content (TEAC)

Sample	Source	Sum of Squares	Df	F-Ratio	P-Value	Correlation Coefficient	R-squared (%)	Standard Error of Est
Green tea	Model	765.16	1	61.87	0.0004	0.9410	88.55	3.517
	Residual	98.94	8					
	Total (Corr.)	864.15	9					
Rooibos	Model	30.01	1	17.98	0.0028	0.8319	69.21	1.292
	Residual	13.35	8					
	Total (Corr.)	43.36	9					

The R-Squared statistic indicates that the model as fitted explains 88.55 % and 69.21 % of the variability for both Green tea and Rooibos respectively. The correlation coefficients 0.9410 and

0.8319, indicated that a relatively stronger relationship between the variables is attained in Green tea. Seemingly, the model for Green tea showed a higher Standard Error of Estimate of 3.52 higher than that of Rooibos 1.29.

The data used for the evaluation of this relationship (in Figure 3.2) was subjected to the Grubbs test for outlier identification. One point in each series was identified as outlier and eliminated, hence nine points were retained. Furthermore the effective concentration of antioxidants (see Table 3) able to scavenge 50 % of the ABTS radical (EC_{50}) for the Green tea and Rooibos tea extracts was calculated to be 11.62 µg/ml and 10.80 µg/ml, as opposed to that of 15.23 ug/ml and 13.31 ug/ml for ascorbic acid and rutin respectively as they are well-known antioxidant.

Table 3. Antioxidant Activities (AA) and EC_{50} determined using ABTS free radical scavenging assay

Sample	AA_{ABTS} (mmol/L)	EC_{50} (µg/ml)
Rooibos	41.65	10.80
Green tea	35.43	11.62

The two compounds studied showed difference in free radical scavenging activity rutin> ascorbic acid. This different behaviour is attributed to the higher ability of diphenols in the B ring of Rutin to quench free radicals in solution by easier donation of hydrogen of hydroxyl groups and to stabilize the respective radicular forms due to electron delocalization forming double bonds (See Scheme 1). Javanmardi and co-workers concluded that antioxidant activity of plant extracts is not limited to phenolics [4]. From the spectroscopic analysis (see Table 3), it shows that both samples had been oxidized in the presence of ABTS. Initially, the absorbance of tea samples was the lower than that of Green tea sample. After 5 min of storage in the dark environment, both samples exhibited good effect in inhibiting ABTS oxidation.

5. CONCLUSION

In the present study an in-house laccase modified biosensor was used to assess the antioxidant properties of locally available herbal tea samples by electrochemical and spectrophotochemical methods. The results showed a positive linear correlation between the TAEC and TP content (R^2 = 0.9812 ± 0.012), suggesting that phenolic compounds could be one of the main components responsible for the antioxidant properties of tea samples. The proposed methodology for the modification of the GCE using laccase was found to be sensitive and able to provide good reproducibility for the detection of TP. Consequently, it is rational to conclude that the influence of a biosensor depends on the method of evaluation, the type of radical used, and whether the oxidizable substrate contains a catechol ring which may contribute to the total antioxidant activity. Moreover, in

this work it was observed that a good response factor obtained in both voltammetry and UV methods suggested the presence of high antioxidant content in herbal tea samples.

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