ELECTROCHEMICAL AND ANTIOXIDANT PROPERTIES OF RUTIN

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The mechanism of electrochemical oxidation of rutin on a glassy carbon electrode was studied at different pH by using several electrochemical techniques (cyclic, linear sweep, differential pulse and square-wave voltammetry) in order to give deeper insight into the mechanism of electrochemical oxidation of rutin and adsorption of its oxidation products on a glassy carbon electrode. It was determined that the rutin oxidation process on a glassy carbon electrode is reversible, pH dependent and includes the transfer of 2 e^- and 2 H^+. The products of electrochemical oxidation strongly adsorb on the electrode surface. Maximum surface coverage, Γ_{max}, decreased with increasing scan rate from 3.4×10^{-9} mol cm^{-2} at scan rate 20 mV s^{-1} to 1.5×10^{-9} mol cm^{-2} at scan rate 100 mV s^{-1} and adsorption equilibrium constant was log $K = 4.57 \pm 0.05$. Antioxidant properties of rutin were investigated by a Trolox equivalent antioxidant capacity (TEAC) assay. It was found that the TEAC values of rutin depend on concentration and the EC_{50} value of rutin amounted 0.23.

Keywords: Polyphenols; Antioxidants; Rutin; Electrochemistry; Oxidation; Adsorption; Antioxidant properties; TEAC assay; Differential pulse; Square-wave voltammetry.

Phenolic compounds or polyphenols are naturally occurring group of comp onds which are responsible for brightly coloured pigments present in a variety of fruits and vegetables and protect plants from diseases and ultraviolet light. They are products of the secondary metabolism of plants and arise from two main primary synthetic pathways: the shikimate pathway and the acetate pathway. Depending on their basic structure, polyphenols can be divided into at least 10 different classes. Flavonoids constitute one of the most important groups and their common structure consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (C_6-C_3-C_6). Based on structural differences, flavonoids can be subdivided into several families: flavonols (e.g. quercetin,

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rutin and kaempferol), flavones (e.g. luteolin, apigenin and chrysin), flavanols (e.g. catechin and related compounds) isoflavones (e.g. genistein), flavanones (e.g. hesperidin, naringenin), flavanononones (e.g. astilbine, engeletine), flavanes (e.g. 3,4-*trans*-3′,4′-dimethoxy-6-methyl-2,3-*cis*-flavan), chalkones (e.g. buteine, mareine, floretine), dihydrochalkones (e.g. crotaramine, crotine, acumitine), flavane-3,4-diols (e.g. leukopelargonidine, leukocyanidine) and anthocyanidins (e.g. cyanidin, delphinidin, malvidin, etc. and their related glycosides)[1]. Most of the beneficial health effects of flavonoids (anti-inflammatory, anti-cancer, cardio protective) are attributed to their antioxidant and chelating abilities[2,3]. Since the chemical activities of flavonoids in terms of their reducing properties as hydrogen or electron-donating agents could predict their potential to act as antioxidants (lower oxidation potential points to higher antioxidant activity)[4], studying of electrochemical and antioxidant properties could help in better understanding of the mentioned group of compounds.

In the last two decades flavonoids were studied by different techniques. Electrochemical properties of flavonoids were studied by voltammetric techniques (cyclic voltammetry[5–16,20], differential pulse voltammetry[5,7,10,15,17,18,21], square-wave voltammetry[5,16,19], linear sweep voltammetry[7], chronocoulometry[6]) and UV spectroelectrochemistry[12]. Antioxidant capacity of flavonoids was studied by cyclic voltammetry[19,22–25], differential pulse voltammetry[26], flow injection analysis (FIA)[27,28], chronoamperometry[29], biosensors[21,30], UV/Vis spectroscopy (with different reagents e.g. Folin–Ciocalteau[19,30], FRAP [22], DPPH [19,29], ABTS [19,30], etc.), photochemiluminescence[19], spectrofluorimetry (ORAC method)[30] and electron spin resonance (ESR) spectroscopy[31]. Possible oxidation mechanisms of rutin and its detection limits are given in Table I.

The flavonoid rutin (quercetin-3-*O*-rutinose), also known as vitamin P, is one of the most bioactive flavonoids usually found in plants (especially buckwheat), black tea and apple peels. In humans, it attaches to the iron ion (Fe^{2+}), preventing it from binding to hydrogen peroxide and creating a highly reactive free radical that may damage cells. Rutin may have antioxidant, anti-inflammatory, anti-tumor, anti-thrombotic, cardio protective and antibacterial activity[32]. It increases the strength of the walls of blood capillaries and regulates their permeability. Therefore, rutin can reduce the symptoms of many other capillary diseases. The chemical structure of rutin is given in Fig. 1.

The purpose of this study was to apply electrochemical methods (cyclic, differential pulse and square-wave voltammetry) in systematic investigation of electrochemical properties of rutin and to study rutin adsorption on the

TABLE I
Possible oxidation mechanisms of rutin and its limits of detection (LOD)

Method	LOD, mol dm^{-3}	Possible oxidation mechanism	Ref.
Cyclic voltammetry, differential pulse voltammetry, square-wave voltammetry	n.a.[a]	2 e^-, 2 H^+ reversible oxidation (3′,4-dihydroxy group) followed by irreversible oxidation (5,7-dihydroxy group)	5
Cyclic voltammetry	1×10^{-8}	2 e^-, 2 H^+ reversible oxidation (3′,4-dihydroxy group)	6
Differential pulse voltammetry	2.51×10^{-8}	2 e^-, 2 H^+ reversible oxidation (3′,4-dihydroxy group)	7
Cyclic voltammetry	n.a.[a]	quasi-reversible electrochemical process involving 2 e^-	8
Cyclic voltammetry	n.a.[a]	2 e^-, 2 H^+ reversible oxidation (3′,4-dihydroxy group)	9
Differential pulse voltammetry	1×10^{-8}	quasi-reversible electrochemical process involving 2 e^-	10
Linear sweep voltammetry	1.5×10^{-7}	irreversible process	13
Cyclic voltammetry	n.a.[a]	oxidation (3′,4-dihydroxy group) followed by irreversible oxidation (5,7-dihydroxy group)	15
Square-wave voltammetry	1×10^{-8}	n.a.	16
Differential pulse voltammetry	1×10^{-8}	n.a.	17
Differential pulse voltammetry	4×10^{-8}	2 e^-, 2 H^+ oxidation (3′,4-dihydroxy group) followed by irreversible oxidation (5,7-dihydroxy group)	18
Square-wave voltammetry	1×10^{-9}	n.a.	19
Cyclic voltammetry	3.58×10^{-7}	2 e^-, 2 H^+ reversible oxidation (3′,4-dihydroxy group)	20
Square-wave voltammetry	1.75×10^{-7}	n.a.	21
Cyclic voltammetry, rotating ring-disk voltammetry	n.a.[a]	2 e^-, 2 H^+ quasi-reversible oxidation (3′,4-dihydroxy group) followed by irreversible oxidation (5,7-dihydroxy group)	34

[a] n.a. = not available.

glassy carbon electrode (by linear sweep voltammetry), in order to give deeper insight into the mechanism of electrochemical oxidation of rutin and adsorption of its oxidation products on a glassy carbon electrode. Antioxidant capacity and EC_{50} value of rutin were determined by a Trolox equivalent antioxidant capacity (TEAC) assay. The obtained results can be used to explain the connection between electrochemical properties of rutin and its biological activity.

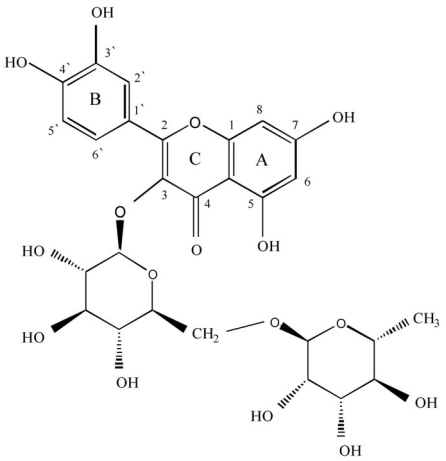

FIG. 1
Chemical structure of rutin (3′,4′,5,7-tetrahydroxyflavone-3β-D-rutinoside)

EXPERIMENTAL

All chemicals were of the highest purity commercially available and were used without further purification. Rutin trihydrate ($C_{27}H_{30}O_{16}\cdot 3H_2O$) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma (St. Louis, MO, USA). (±)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Fluka (Buchs, Switzerland). All other chemicals were obtained from Kemika (Zagreb, Croatia). Stock solution of rutin $c = 1 \times 10^{-2}$ mol dm^{-3}) was prepared in methanol and kept in the refrigerator (solution was stable for at least 1 month). Prior to use, stock solution was diluted to the desired concentration with buffer supporting electrolyte (I_c = 0.1 mol dm^{-3}). Buffer supporting electrolyte solutions were prepared in high-purity water from a Millipore Milli-Q system (resistivity greater than or equal to 18 MΩ cm). A phosphate buffer solution of pH 8.0 and 7.0, an acetate buffer solution of pH 5.5, 4.5 and 3.5, and a hydrochloride buffer solution of pH 2.5 were used.

The electrochemical experiments were preformed with an EG&G Princeton Applied Research Model 273A potentiostat controlled by a computer in a three-electrode system, consisting of a glassy carbon working electrode (Bioanalytical System, d = 3 mm), a saturated

calomel reference electrode (SCE) and a platinum counter electrode. The glassy carbon working electrode was polished with α-Al_2O_3 powder (0.05 μm, Buehler, USA) before each measurement. Linear sweep voltammograms were performed at 20, 50 and 100 mV s^{-1}. Cyclic voltammetry scan rate was 50 mV s^{-1}. The differential pulse voltammetry conditions were: scan increment 5 mV, pulse amplitude 50 mV, pulse width 70 ms and scan rate 5 mV s^{-1}. Square-wave voltammetry conditions used were: frequency 50 Hz, pulse amplitude 50 mV and scan increment 2 mV (effective scan rate 100 mV s^{-1}).

Antioxidant properties of rutin were investigated by a TEAC decolourisation assay on a J. P. Selecta UV/Vis spectrophotometer Model UV 2005. A stock solution of ABTS radical cation was prepared by mixing 0.2 ml ammonium peroxodisulfate solution (c = 65 mmol dm^{-3}) with 50 ml of ABTS solution (c = 1 mmol dm^{-3}) in phosphate buffer (I_c = 0.1 mol dm^{-3} NaH_2PO_4, pH 7.40). The mixture was left to stand overnight. A 0.5 ml of ABTS radical cation stock solution was put into the 1 cm glass cuvette, diluted with 2 ml of phosphate buffer and the absorbance of the ABTS radical cation at 734 nm was read. Subsequently, 0.1 ml of sample solution was added into the cuvette, the solution was quickly mixed and the absorbance was monitored at 734 nm for 60 s. The decrease of absorbance after 60 s (ΔA_{sample}) was compared with the decrease of absorbance caused by the addition of 0.1 ml of Trolox solution (c = 400 μmol dm^{-3}) (ΔA_{Trolox}) and the TEAC value was calculated according to the formula[27]

$$\mathrm{TEAC} = \Delta A_{\mathrm{sample}}\, c_{\mathrm{Trolox}}\, (\Delta A_{\mathrm{Trolox}}\, c_{\mathrm{sample}})^{-1} \,. \qquad (1)$$

The pH measurements were carried out at room temperature with a Metrel MA 5736 pH-meter.

RESULTS AND DISCUSSION

Cyclic Voltammetry

In order to obtain general information regarding electrochemical properties and possible surface activity of rutin the cyclic voltammetry measurements were performed. Cyclic voltammograms of rutin (c = 1 × 10^{-5} mol dm^{-3}) were recorded in buffer solutions (I_c = 0.1 mol dm^{-3}) in pH range 2.5–8.0. An example of cyclic voltammogram of rutin recorded at pH 4.5 from 0 to 0.6 V is shown in Fig. 2B. It can be seen that there is one reversible oxidation peak at the potential, $E_{p,a}$ = 0.401 V, which corresponds to the oxidation of the 3′,4′-dihydroxy substituent on the B-ring of rutin. The reduction peak of the 3′,4′-diquinone formed during rutin oxidation process, appeared at $E_{p,c}$ = 0.364 V. The peak separation is $\Delta E_p = E_{p,a} - E_{p,c}$ = 37 mV, which points to reversible electrode reaction involving two electrons on the glassy carbon electrode[5,7–9]. The reversibility of the rutin oxidation process can be seen from Fig. 2C as well, since the decrease of anodic potential limit has shown that the reduction peak appears even if the anodic poten-

tial is inverted immediately after the oxidation peak of rutin. It can also be seen from the Fig. 3A since linear dependence was found between anodic peak current and the square root of scan rate, which also shows that the rutin oxidation is controlled by diffusion[11]. Adsorption process on the electrode surface was confirmed by increase of reduction peak current with increasing scan rate, $I_{p,c}(\nu)^{-1/2}$ vs log ν plot (Fig. 3B)[33]. Even dough, there can be found a second oxidation peak of rutin above 0.6 V in the relevant literature[5,9], extension of measurements to 1.2 V has not revealed a second oxidation peak of rutin in our cyclic voltammetry measurements (Fig. 2A).

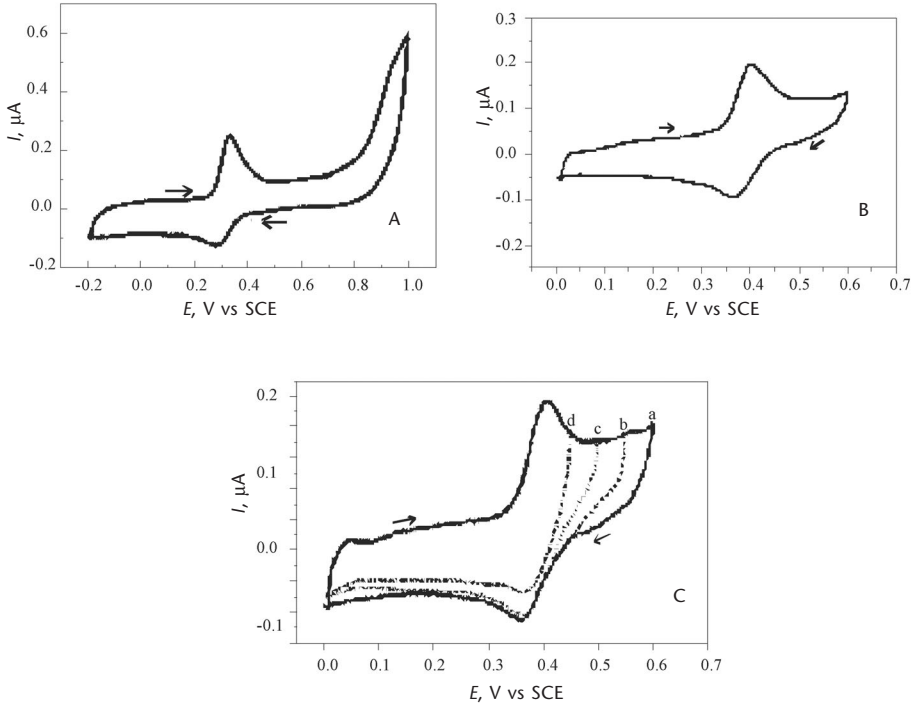

FIG. 2
Cyclic voltammograms of rutin ($c = 1 \times 10^{-5}$ mol dm^{-3}) in acetate buffer ($I_c = 0.1$ mol dm^{-3}, pH 5.5 (A); pH 4.5 (B)) at scan rate 50 mV s^{-1}. (C) Cyclic voltammogram of rutin ($c = 1 \times 10^{-5}$ mol dm^{-3}) in acetate buffer ($I_c = 0.1$ mol dm^{-3}, pH 4.5) at scan rate 50 mV s^{-1}. First scan (between 0 and 0.60 V) (a), second scan (between 0 and 0.55 V) (b), third scan (between 0 and 0.50 V) (c), forth scan (between 0 and 0.45 V) (d)

Linear Sweep Voltammetry

According to data found in literature, the products of electrochemical oxidation of rutin strongly adsorb on the glassy carbon electrode surface[5]. In order to study this phenomena linear sweep voltammetry was used. The relationship between peak current (I_p) and rutin concentration (from 8×10^{-6} to 1×10^{-4} mol dm^{-3}) in acetate buffer (I_c = 0.1 mol dm^{-3}, pH 4.5) was studied at scan rates 20, 50 and 100 mV s^{-1}. The results have shown that anodic peak current increased proportionally with scan rate and rutin concentration. Linear sweep voltammetry data obtained at different scan rates were

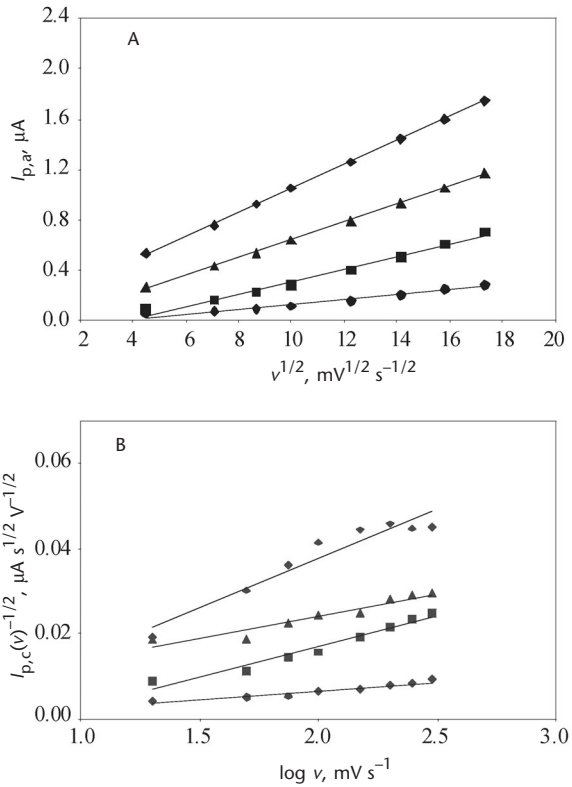

FIG. 3
(A) Anodic peak current of rutin solutions (I_c = 0.1 mol dm^{-3}, pH 4.5) as a function of the square root of scan rate. c(rutin) = 1 (●), 10 (■), 70 (▲), 200 (◆) μmol dm^{-3}. (B) Dependence of $I_p(v)^{-1/2}$ for cathodic peak current of rutin solutions (I_c = 0.1 mol dm^{-3}, pH 4.5) as a function of the logarithm of scan rate. c(rutin) = 1 (●), 10 (■), 70 (▲), 200 (◆) μmol dm^{-3}

used to calculate surface coverage (Γ) of the glassy carbon electrode according to the formula[7]

$$\Gamma = q(nFA)^{-1} \tag{2}$$

where q is the electric charge, n is the number of electrons exchanged during the oxidation process ($n = 2$), F is the Faraday constant and A is the area of the glassy carbon electrode ($A = 0.071$ cm^2). The linear sweep voltammetry results were fitted according to Langmuir adsorption isotherm (Fig. 4) which is usually expressed as

$$(\Gamma)^{-1} = (\Gamma_{max})^{-1} + (Kc_{eq}\Gamma_{max})^{-1} \tag{3}$$

where Γ_{max} stands for the maximum surface coverage, K is the adsorption equilibrium constant and c_{eq} is the equilibrium concentration of rutin. There are two linear regions in Fig. 4. At lower concentrations of rutin (till c(rutin) ~ 2.5 × 10^{-5} mol dm^{-3}) anodic peak current is a linear function of the rutin concentration (the adsorption of rutin oxidation products on the electrode surface occurs). At higher concentrations of rutin (above c(rutin) ~

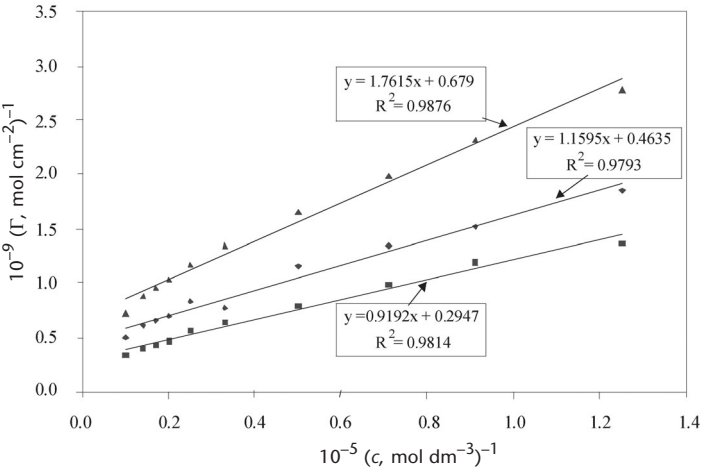

FIG. 4
Langmuir adsorption isotherms of rutin in acetate buffer (I_c = 0.1 mol dm^{-3}, pH 4.5) on the glassy carbon electrode at room temperature (t = 25 °C) and at different scan rates. Scan rate: 20 (■), 50 (●), 100 (▲) mV s^{-1}

2.5×10^{-5} mol dm^{-3}), the increase of peak current slows down, which could be explained by increase of interactions between molecules adsorbed on the electrode surface and diffusion current[6]. The obtained values of maximum surface coverage decreased with increasing scan rate from $\Gamma_{max} = 3.4 \times 10^{-9}$ mol cm^{-2} at scan rate 20 mV s^{-1} to $\Gamma_{max} = 1.5 \times 10^{-9}$ mol cm^{-2} at scan rate 100 mV s^{-1}. Calculated adsorption equilibrium constant for rutin adsorption on a glassy carbon electrode surface was log $K = 4.57 \pm 0.05$.

Differential Pulse Voltammetry

Differential pulse voltammetry was used for investigation of rutin oxidation peak current (I_p) as a function of pH (from pH 2.5 to 8.0). As is shown in Figs 5A and 6, the highest peak current was around pH 5.5 (the same result

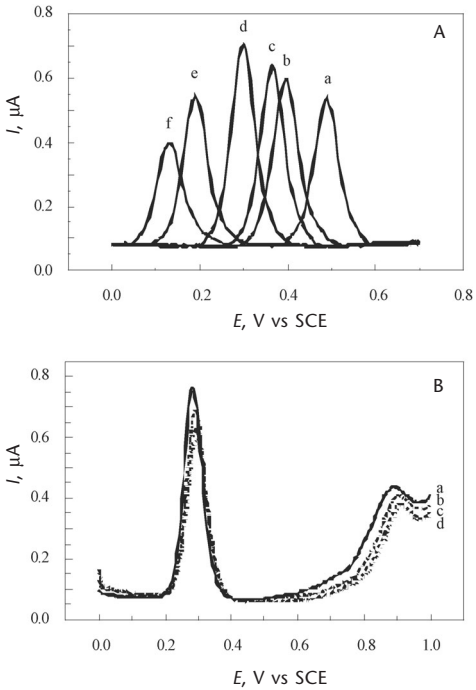

FIG. 5
(A) Differential pulse voltammograms of rutin ($c = 1 \times 10^{-5}$ mol dm^{-3}) as a function of pH: 2.5 (a), 3.5 (b), 4.5 (c), 5.5 (d), 7.0 (e), 8.0 (f) at scan rate 5 mV s^{-1}. (B) Differential pulse voltammograms of rutin ($c = 1 \times 10^{-5}$ mol dm^{-3}) in acetate buffer ($I_c = 0.1$ mol dm^{-3}, pH 5.5) at scan rate 5 mV s^{-1}. First scan (a), second scan (b), third scan (c), forth scan (d)

was obtained by cyclic voltammetry) and it decreased in more acidic and alkaline media. The plot of peak potential (E_p) vs pH showed linearity (Fig. 6) with the slope 60.6 mV, which corresponds to the mechanism involving the same number of protons and electrons and agrees with data found in literature[5,7,9]. It can be concluded that the oxidation of the 3′,4′-dihydroxy substituent on the B-ring of rutin is a 2 e^- – 2 H^+ reversible process on the glassy carbon electrode, which depends on pH.

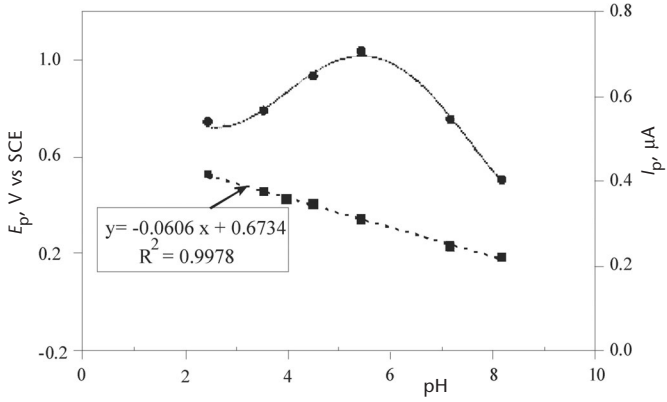

FIG. 6
Rutin oxidation peak current (I_p) and peak potential (E_p) as a function of pH

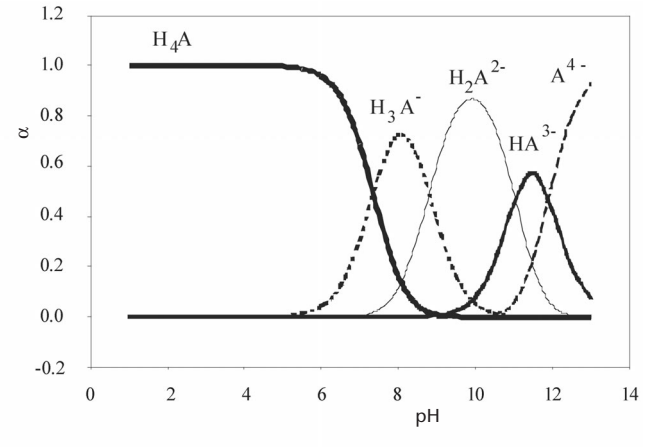

FIG. 7
Dissociation diagram of rutin: undissociated form of rutin (H_4A), deprotonated forms of rutin (H_3A^-, H_2A^{2-}, HA^{3-} and A^{4-})

Differential pulse voltammetry revealed a second oxidation peak of rutin around 0.9 V. Both peaks decreased with successive scans (Fig. 5B) for all pH studied which confirmed adsorption of oxidation products of rutin on the glassy carbon electrode surface. The products of oxidation of rutin can also undergo homogenous chemical reactions with water following its oxidation at a glassy carbon electrode[5,34].

Dissociation diagram of rutin (Fig. 7) shows that its spontaneous deprotonation does not occur below pH 5 which means that the neutral rutin molecule participate in electrochemical oxidation reaction and adsorption processes up to pH 5. At higher pH values (pH 5–8), spontaneous deprotonation of rutin occurs, different dissociated species of rutin are formed and the possibility of occurrence of much more complex electrochemical and adsorption processes exists. Dissociation constants (pK_{a7} = 7.35; $pK_{a4'}$ =

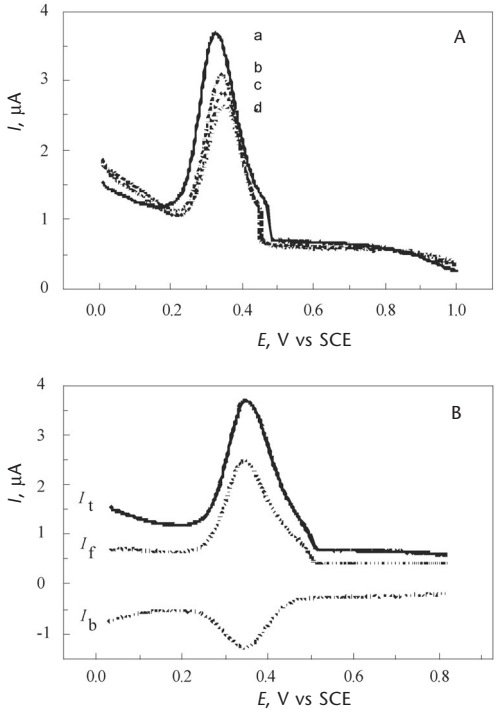

FIG. 8
Square-wave voltammograms of rutin (c = 1 × 10^{-5} mol dm^{-3}) in acetate buffer (I_c = 0.1 mol dm^{-3}, pH 5.5) at scan rate 100 mV s^{-1}. (A): first scan (a), second scan (b), third scan (c), forth scan (d). (B): total current (I_t), forward current (I_f), backward current (I_b)

8.80, $pK_{a3'}$ = 11.04 and pK_{a5} = 11.90) were obtained by spectrophotometry and potentiometry[35].

Square-Wave Voltammetry

Square-wave voltammograms were recorded over the same pH interval investigated by differential-pulse voltammetry. The results have confirmed adsorption of oxidation products of rutin on the electrode surface since the oxidation peak currents decreased with increasing scan number (Fig. 8A). The advantage of this method compared to differential pulse and cyclic voltammetry lies in greater speed of analysis, lower consumption of electro active species and reduced problems with blocking of the electrode surface. The current is sampled in positive and negative-going pulses, so the oxidation and reduction peaks of electro active species can be obtained at the same time. The reversibility of the first oxidation peak of rutin was also confirmed since both oxidation (forward current) and reduction (backward current) peaks appeared at the same potential, E_p = 0.33 V (Fig. 8B). A possible oxidation mechanism of rutin, which corresponds to the oxidation of 3′,4′-dihydroxy substituent on the B-ring of rutin and includes transfer of two electrons and protons is given in Fig. 9. The mechanism involves ion-

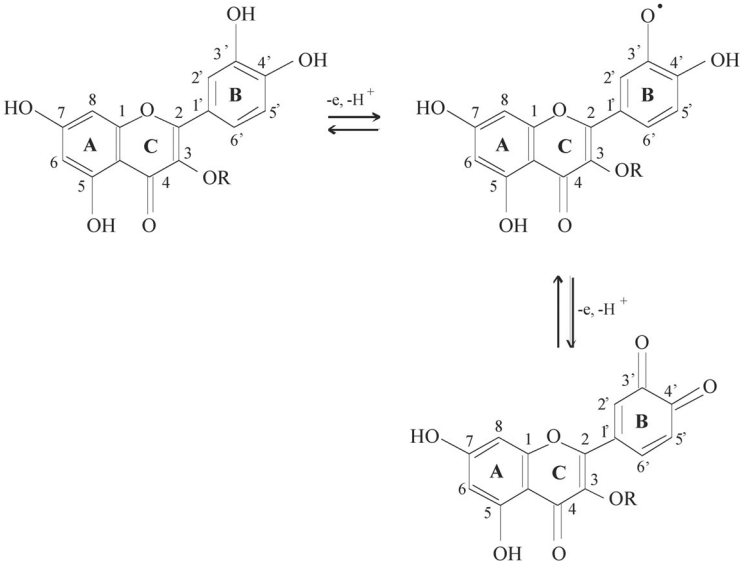

FIG. 9
A possible mechanism of electrochemical oxidation of rutin. R = rutinose ($C_{12}O_9H_{21}$)

ization of rutin, 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 dehydrorutin. The latter species is rapidly protonated and then dehydrated to yield the final product of 3′,4′-diquinone[5,7]. It was found that rutin and its oxidation products are both adsorbed on the electrode surface[12]. The oxidation mechanism is pH dependent and the optimal pH value for the rutin oxidation process was found to be around pH 5.5 which agrees with data found in ref.[17]. The second oxidation peak of rutin found in differential pulse voltammogram, which corresponds to the oxidation of 5,7-dihydroxy group on the A-ring of rutin, is an irreversible oxidation process[15].

TEAC Assay

The TEAC assay is an electron transfer based assay. It includes two components in the reaction mixture, antioxidants and oxidant (also the probe) and is based on the following electron-transfer reaction[36].

probe (oxidant) + e (from antioxidant) → reduced probe + oxidized antioxidant (*4*)

The probe itself is an oxidant (ABTS) that abstracts an electron from the antioxidant. Antioxidant is a substance which scavenges reactive oxygen/nitrogen species to stop radical chain reaction or it can inhibit the reactive oxidants from being formed in the first place, causing colour changes of the probe (from dark blue to colourless). In this study, the antioxidant capacities of rutin solutions of different concentrations, expressed as Trolox equivalents, were determined by the classic TEAC decolourisation assay (the procedure was taken from ref.[27]). It was found that TEAC values of rutin are concentration dependant (Fig. 10), which agrees with data found in literature[37]. Percent inhibition (% inhibition) of rutin was plotted as a function of rutin concentration (data not shown) in order to determine the EC_{50} value of rutin. The EC_{50} value is the ratio of the antioxidant concentration necessary for decreasing the initial ABTS concentration by 50% to the initial ABTS concentration. The concentration of rutin, c (in μmol dm^{-3}), necessary for ABTS initial concentration decrease to 50% of the initial ABTS concentration, was calculated from linear regression equation:

$$\% \text{ inhibition} = 0.2136\, c(\text{rutin}) + 8.6746\ . \qquad (5)$$

The determined concentration of rutin was divided with ABTS initial concentration (c = 1 mmol dm^{-3}) and the obtained EC_{50} value of rutin was 0.23, which is similar to data found in ref.[24]. The results obtained in this study could be used to explain the connection between electrochemical properties of rutin and its biological activity.

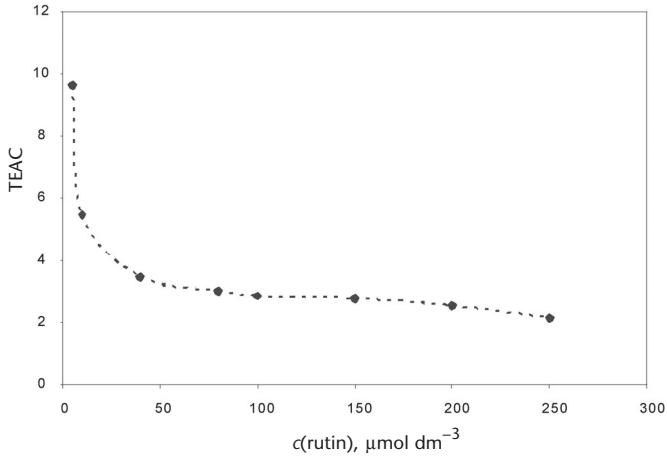

FIG. 10
The TEAC values (n = 3, SD = ±0.05) of rutin measured after 1 min in different concentrations by TEAC assay

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