# Antioxidant Activity Coefficient, Mechanism, and Kinetics of Different Derivatives of Flavones and Flavanones Towards Superoxide Radical

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**Abstract**

Ahmed S., Shakeel F. (2012). **Antioxidant activity coefficient, mechanism, and kinetics of different derivatives of flavones and flavanones towards superoxide radical**. Czech J. Food Sci., **30**: 153–163.

A systematic investigation of the electrochemically generated superoxide radical ($O_2^{\bullet -}$) has been conducted in the presence of some flavonoids. Cyclic voltammetry was used to generate $O_2^{\bullet -}$ by reducing the molecular oxygen in DMSO at room temperature. The scavenging of the radical was monitored by the decrease in anodic or/and cathodic currents while linearly increasing the concentration of the flavonoid. The strength of interaction was quantified in terms of the binding constant ($K_b$) values ranging from $1 \times 10^2$ and $5 \times 10^3 M^{-1}$. The antioxidant activity coefficient ($K_{ao}$) was calculated from the linear part of the plot of 'current decrease vs. flavonoid concentration'. Radical scavenging mechanism of the antioxidant was proposed and discussed in terms of the structure activity relationship. Nicholson-Shain method was employed to estimate the bimolecular homogeneous kinetics and on this basis control use of antioxidant is pointed out.

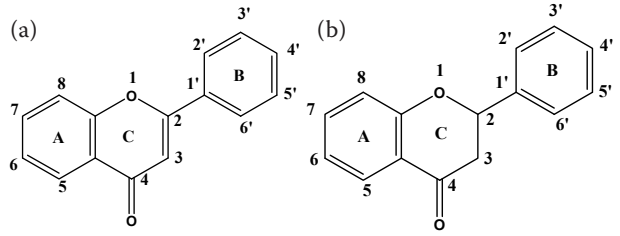
**Keywords**: antioxidant activity coefficient; flavonoids; binding constant; homogeneous kinetics; cyclic voltammetry

Free radicals in biological systems are ubiquitous and they can come from a variety of external sources. Free radicals are either formed by cells through various metabolic processes or produced due to the exposure to different types of radiations, chemicals, environmental stress, and the sun (Harold *et al*. 2000). Imbalanced diet is another cause of the free radicals formation. Most of the free radicals are very unstable and thus highly reactive in nature. Oxygen is an essential ingredient of the cell metabolism and is capable of producing reactive oxygen species (ROS) most of which are free radicals; such as super oxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), hydrogen peroxide ($H_2O_2$), alkyl ($R^{\bullet}$), alkoxyl ($RO^{\bullet}$), and peroxyl ($ROO^{\bullet}$) radicals. The reaction of these species with lipid molecules creates peroxyl radicals and their interactions with nucleic acids and proteins cause certain alterations and change their functions (Edenharder & Grünhage 2003). ROS are also responsible for several degenerative processes and diseases such as aging, emphysema, inflammation, certain cancers, atherosclerosis, liver injury, and many others (Gao *et al*. 1999; Evans 2004).

Superoxide is an anionic free radical with the chemical formula $O_2^{\bullet -}$. It is formed by one-electron reduction of dioxygen ($O_2$) (Ortiz *et al*. 2002), which is present abundantly in nature. Super oxide anion is the most dangerous radical among all oxygen radicals because it has a longer half life and thus can move to a longer distance (Suna *et al*. 2004), and it also acts as an initiator for some other radicals, i.e. hydroxyl radicals. Super oxide radical is toxic in nature and has the ability to react with different organic compounds due to its redox and paramagnetic nature (Mohammad *et al*. 2001).

The compounds that protect cells against the damaging effects of ROS are known as antioxidants. The antioxidant behaviour of a compound is the result of its capacity to inhibit the initiation of free radical or chain breaking in the process propagating oxidation (Madsen *et al*. 1996). Among the known scavengers prevail the low molecular weight antioxidants (LMWA) able to reach the stressed place in the body more efficiently than the high molecular weight antioxidants (HMWA) e.g. enzymes (Kohen *et al*. 2000).

All flavonoids contain 15-carbon atoms in their parent nucleus, which is formed by two benzene rings (A and B) linked through an oxygenated heterocyclic pyrane ring C, i.e. diphenyl propane derivative as shown below for two subclasses used in the present work:




Basic structures of (a) flavone and (b) flavanone

Different classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring while the compounds within a class differ in the substitution pattern in ring A and B. From the antioxidant activity (AOA) point of view, flavones, flavanones, flavanols, biflavones, and biflavanones are the major classes of the flavonoids. A considerable amount of work has been done to determine the AOA of different compounds using different target radicals (Collins *et al*. 2000; Heim *et al*. 2002) and the reports from the near past (Koyama *et al*. 2008; Temraz & Eltantawy 2008; Weber 2009) indicate a growing interest in this field. This is because of two major reasons: firstly, the isolation and synthesis of new compounds, and secondly, the multidimensional utilisation of antioxidants, such as for cosmetics, sunscreens, skin lotions, pharmaceuticals, plastics, and food preservatives (Moure *et al*. 2001; Heim *et al*. 2002; Trombino *et al*. 2004).

From the work done so far, one can see that there are areas which need further attention. Mono derivatives of flavonoids having substituents other than hydroxyl group have not been much investigated so far. There are hardly any reports presenting kinetic measurements along the AOA data. Mechanistic study of the flavonoid-radical interaction has always remained an interesting task. The present article underpins the aforementioned three aspects while using superoxide anion as the target radical. This work will be a good addition to emulate the antioxidant behaviour of the monosubstituted flavonoids and a better way to understand their free radical scavenging activity mechanism. In addition to this, the relevance of kinetic and thermodynamic parameters for antioxidant properties is also pointed out.

## MATERIAL AND METHODS

***Reagents***. Dimethyl sulphoxide (DMSO) purchased from LAB-SCAN/Analytical Sciences was used as solvent without further purification. Tetrabutyl ammonium perchlorate (TBAP) of Fluka, 99%, was used as the supporting electrolyte and its concentration was kept at 0.1M. flavone (F, 99%) was purchased from Fluka, 6-chloro flavone (6ClF, 98%), 6-bromo flavone (6BrF, 97%), 6-methyl flavone (6MF, 99%), 5, 6, 7-trihydroxy flavone (567THF, 98%), 3-methoxy flavone (3MOF, 99%), 6-methoxy flavanone (6MOFv, 98%), and 6-hydroxy flavanone (6HFv, 99%) were obtained from Aldrich.

***Instrumentation***. Cyclic voltammetric measurements were carried out using Eco Chemie Autolab PGSTAT 302 potentiostat/galvanostat (Utrecht, the Netherlands) along with the software GPES 4.9. All the experiments were carried out in a double walled electrochemical cell (Model K-64 PARC and conventional three electrode system was employed. Glassy Carbon (GC) electrode having an area of 0.013 $cm^2$ was used as a working electrode. The electrode surface was polished before each measurement. Polishing was done with commercial talcum powder (Tibet) and was followed by washing with distilled water and the solvent in use. Platinum wire was used as a counter electrode and Silver-silver chloride (Ag/AgCl, 3M KCl) of Metrohm Company with a plastic tip was used as a reference electrode.

***Procedure***. Super oxide anion radical was generated in DMSO containing 0.1M TBAP. The scan rate was kept at 20 mV/s and the potential window was – 1.0–0.0 V. The atmospheric solubility of oxygen in DMSO was 2.1mM (Tsushima *et al*. 1994). The flavonoid was added incrementally to the *in situ* generated radical and the resultant behaviour was

recorded. From the change in the shape of voltammograms, the AOA was assessed and quantified using pertinent mathematical formulations. All measurements were made in triplicate (or even more times) to ensure the reproducibility of the results. The uncertainties in the obtained peak potentials and peak currents were ≤ ±5 mV and ≤ ±0.5%, respectively. To support the experimental results for mechanistic studies, the charge densities for each element in the compound were calculated from HyperChem (Vers. 8.0 release) software after optimising the geometry of the compound.

## RESULTS AND DISCUSSION

### Voltammetric behaviour of super oxide anion radical

The superoxide anion radical was generated by one electron reduction of the atmospheric molecular oxygen ($O_2$) dissolved in DMSO at room temperature (28 ± 1°C). The cyclic voltammogram of super oxide radical showed one electron reversible process (Figure 1), having well developed and well resolved oxidation and reduction peaks with the peak separation ($\Delta E_p$) value of 66 mV, well in agreement with the reported data (Moure *et al*. 2001). It is worth mentioning that the stability of superoxide radical in aqueous medium is quite low because of the high reactivity of the radical with water as discussed elsewhere (Mohammad *et al*. 2001).

It was observed during the experimentation that the reproducibility of the cyclic voltammogram

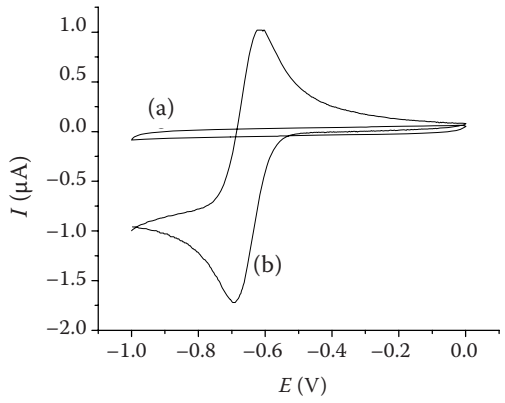



Figure 1. Cyclic voltammograms of (a) medium (DMSO + TBAP) and (b) $O_2^{\bullet-}$ in the medium on GC as working electrode vs. Ag/AgCl as reference at 28°C with scan rate of 20 mV/s

was affected both in terms of the peak shift and peak height. To probe the cause, the experiment was conducted several times while closely observing each variable (RE, WE, solubility of $O_2$ etc). It was observed that cathodic and anodic peaks appeared in the potential ranges of –0.700 V to –0.688 V and –0.624 V to –0.612 V, respectively, vs. Ag/AgCl as reference electrode. The fluctuation of about ±12 mV could not be assigned to the experimental uncertainty (which is ≤ ±5 mV). This fluctuation is rationalised in terms of the difference between the solvent in the cell (DMSO in this case) and in the reference electrode filling solution (water + KCl). Due to the different solvents at the electrode-solution interface, $O_2$ showed uncertain electrochemical behaviour at the interface. Note that the solubility of $O_2$ significantly differs in DMSO (2.1mM) and in $H_2O$ (1mM) (Vasudevan &Wendt 1995). Moreover, the pre equilibrium time of the redox process is considerably affected due to the highly sensitive nature of atmospheric molecular oxygen. Similarly, the cathodic and anodic peak heights (current values) varied between –0.912 μA to –1.330 μA and 1.182 μA to 1.692 μA, respectively. The average change was about ±0.50 μA and was attributed to the variation in the solubility (concentration) of oxygen. A reference electrode with the same filling solvent as that used in the cell could resolve this problem. Unfortunately it was not available in the laboratory in the time frame of this work. Despite this problem the task of AOA measurement was not affected as it was the relative effect of flavonoid on the voltammogram of the $O_2^{\bullet-}$ radical. As far as the stability of the $O_2$/ $O_2^{\bullet-}$ in an unperturbed solution is concerned, $O_2^{\bullet-}$ is stabilised by the ion pair formation and solvation process with tetrabutylammonium per chlorate cation. Here, the oxygen radical can act as a strong Bronsted base, a nucleophile and as a one electron donor.

### Voltammetric behaviour of flavonoids

Before investigating the effects of flavonoids on the superoxide radical, their voltammetry was conducted. It was found that all the compounds were CV inactive in the potential window of –2.0 V to +2.0 V except 5, 6, 7-trihydroxy flavone and 6-hydroxy flavanone (figures not shown). It has been reported that such compounds show redox behaviour due to the presence of hydroxyl groups

in their structure (Collins *et al*. 2000). All compounds including these exceptions were inactive in the potential window of super oxide radical (–1.0 V to 0.0 V), therefore they are not further discussed here.

### Voltammetric behaviour of super oxide radical in the presence of flavonoids

The effect of increasing flavonoid concentration on the peak current and peak potential of the super oxide anion radical was investigated. Scavenging behaviour of all the compounds was checked from minimum concentration (0.1mM) up to maximum concentration (20.0mM) by stepwise addition of flavonoid to the solution having super oxide anion radical. For baicalein, the maximum effect was observed at 10.0mM which remained constant afterwards. For such a case, the increment was further reduced.

It is well known that the addition of a flavonoid to the solution containing super oxide radical causes a decrease in the current (at least in one; cathodic or anodic) and a positive shift in the peak potential value, which is due to the scavenging activity of the added flavonoid (Kohen *et al*. 2000). The decrease in the current is directly proportional to the concentration of the radical and is a direct measure of the antioxidant activity (Feroci & Fini 2007). However, the scavenging mode and magnitude differ, i.e. it is a structure-activity relationship. In the current studies, as far as the change in CV response is concerned, three different types of behaviour were observed and are discussed separately.

### Decrease in anodic current: hydrogen atom transfer mechanism

The cyclic voltammograms of the super oxide anion radical in the presence of antioxidants (F, 6ClF, 6BrF, 6MF, and 6MOFv) showed a decrease in anodic current only for some initial increments while at higher flavonoid concentrations the cathodic current also started to decrease slightly. The representative CV behaviour of all such cases is shown below in Figure 2. The percentage decrease in the current values corresponding to 8mM concentration for all the flavonoids is presented in Table 1. As the super oxide radical formation occurs on reducing molecular oxygen and its existence and stability is indicated from the oxidation peak current in the forward scan, the decrease in anodic current corresponds to the interaction with the additive, hence its scavenging magnitude. From Figure 2 is it obvious that the depletion in anodic current remains linear for the initial concentrations. Afterwards, this decrease in the current gets gradually smaller and becomes constant at some concentration value (e.g. 8mM for 6ClF, 4.5mM for 6MOFv) and beyond. On the other hand, a small decrease in cathodic current shows a lower availability of dissolved $O_2$ (as it has been consumed in $O_2^{\bullet -}$ form) for the time interval. Thus, the substrate (additive) reacts irreversibly with $O_2^{\bullet -}$. Sometimes a very small irregularity in the current may be possible when the cell is open to atmospheric oxygen.

The scavenging results are in agreement and the effect can be attributed to the hydrogen atom transfer (HAT) from the antioxidant to the radical, and to the formation of phenoxyl radical and a conjugate base of hydrogen peroxide. This behaviour of a hydrogen atom transfer is analogous to that reported in literature (Bourvellec *et al*. 2008). The charge density values calculated from HyperChem (Shakeel 2010) further support the nucleophilic attack of the super oxide radical on the hydrogen atom in C-3 position. Keeping in mind the basic structure of flavone, as discussed above, a comparison was made between these five flavonoids. It is obvious from Table 1 that 6ClF has the highest depletion in anodic current followed by 6MOFv while the remaining three offered comparable magnitude. The chloro group is electron withdrawing by inductive effect and

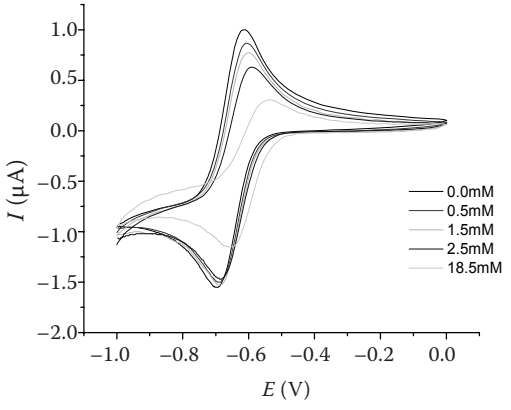



Figure 2. Cyclic voltammograms of $O_2^{\bullet -}$ in presence of 6MOFv at different concentrations in DMSO + 0.1M TBAP on GC as working electrode vs. Ag/AgCl as reference at 28°C with scan rate of 20 mV/s

Table 1. Decrease % in current values, binding constant ($K_b$), change in free energy of reaction ($\Delta G$) and antioxidant activity constant ($K_{ao}$) for the flavonoids

| Antioxidant | % decrease | | $K_b \times 10^{-2}$ ($M^{-1}$) | $-\Delta G^\circ$ (kJ/mol) | $K_{ao} \times 10^{-2}$ ($M^{-1}$) |
|---|---|---|---|---|---|
| | $I_{pc}$ | $I_{pa}$ | | | |
| 567THF | 33 | 100 | 50.41 | 21.34 | 12.3 |
| 6HFv | – | 100 | 8.12 | 16.77 | 4.6 |
| 3MOF | 58 | 62 | 6.58 | 16.24 | 0.7 |
| 6ClF | 26 | 66 | 4.86 | 15.48 | 1.2 |
| 6MOFv | 32 | 61 | 1.91 | 13.15 | 0.8 |
| F | 18 | 52 | 1.28 | 12.14 | 0.4 |
| 6BrF | 26 | 50 | 1.09 | 11.75 | 0.5 |
| 6MF | 36 | 48 | 0.95 | 11.39 | 0.9 |

% decrease was calculated for a concentration of 8mM for all compounds

electron donating by resonance, however, the former effect is dominant. As mentioned above, ring C is more susceptible to the interaction with the additive, therefore a substituent in ring A or B is less influential than that in ring C. The same has been proved from the charge density calculations showing that H in C-3 is more acidic, thus giving the highest probability of the reaction at this position (Shakeel 2010). Methoxy substituent behaving as an electron withdrawing group reduced the anodic peak current linearly up to 4.5mM, afterwards the linearity was lost, depicting the scavenging of the radical (Figure 2). Further, when the anodic peak was scavenged completely, further additions shifted the peak towards more positive potential values while making no change in the anodic and cathodic currents. This shifting could be attributed to the H-bonding between host-guest structures as discussed elsewhere for other systems (Ahmed *et al*. 2007).

The bromo group has a smaller electron withdrawing effect because of lower electronegativity in comparison to the chloro group. Thus again HAT mechanism operates to scavenge the free radical. Similarly, 6MF followed the pattern. In all these cases, the hydrogen of the C-3 in ring C revealed maximum charge density value being more acidic in nature and the nucleophilic attack of superoxide radical is thought to occur at this hydrogen atom. In overall conclusion, it is an $E_rC_i$ mechanism i.e. reversible electron transfer followed by an irreversible chemical reaction (i.e. hydrogen atom transfer).

In the case discussed above, this change in the cathodic current is attributed to the mechanism of interaction between the free radical and the scavenger. When there is no significant modification in the cathodic current, then the HAT mechanism (concerted H-atom abstraction) operates between the free radical and the scavenger while a decrease in the cathodic current shows the electron transfer mechanism followed by proton transfer.

The electrochemically formed superoxide anion radical reacts with the flavonoid (antioxidant) and forms an electro inactive species which is a radical of flavonoid (phenoxy radical) and a conjugate base of the hydrogen peroxide. The redistribution of electrons takes place and phenoxy radical is stabilised by resonance. But on the other hand, the conjugate base of hydrogen peroxide is not stable and reacts with flavonoid, taking a proton to form a relatively stable product $H_2O_2$. The proposed pathway of the interaction between flavonoid and the superoxide anion radical is (HAT) and this mechanism can be described as follows (see Eqs 1–3). The same has been reported elsewhere for similar compounds (Nanni *et al*. 1980).

$$O_2 + e^- \leftrightarrow O_2^{\bullet -} \quad (1)$$

$$O_2^{\bullet -} + FlH \rightarrow [Fl\text{-}H\text{-}O_2^{\bullet -}]^{\pm} \rightarrow HO_2^- + Fl^{\bullet} \quad (2)$$

$$HO_2^- + FlH \rightarrow H_2O_2 + Fl^- \quad (3)$$

### Decrease in both the anodic and cathodic currents: Electron transfer mechanism

The cyclic voltammograms of super oxide anion radical in the presence of 3-methoxy flavone is presented in Figure 3a. The addition of 3-methoxy flavone decreases the anodic and cathodic

currents by approximately same magnitude of 62% and 58%, respectively (Table 1), and shifts both peak positions towards more positive values. Maximum effect was observed up to 8mM concentration while further additions of flavonoid had little effect on the peak potentials and peak currents. The decrease in the anodic current is attributed to the decrease of the radical concentration upon scavenging while the simultaneous decrease in the cathodic current indicates electron transfer (ET) mechanism from the antioxidant to the super oxide radical immediately followed by a proton transfer.

The charge density values informed that ring B of the compound is involved in the reaction between flavonoid and the radical. Methoxyl group possesses coplanar Ar-O-bond in which π orbitals of the aromatic ring tend to overlap with the lone pair electron orbitals of the methoxy oxygen, leading to delocalisation of the non-bounding oxygen electrons and strengthening the Ar-O-bond. This also results in an increased electron density in the aryl ring carbons *ortho* and *para* of the methoxy group. Therefore, the presence of the C-2 and C-3 double bond and 3-position substitution in C ring with '4-one' will stabilise the radical by resonance within the molecule. Thus, conjugation is important for the antioxidant activity. It is accepted that the torsion angle of the B-ring with respect to the rest of the molecule influences the free radical scavenging ability (Heim *et al*. 2002).

All the compounds tested showed immediate and frequent interactions with the superoxide radical upon their addition to the solution but 3-methoxy flavone started to interact after about two hours passing. Moreover, during the measurements it was noticed that the stock solution of the compound recrystallises after a lapse of three hours. The slow interactions could be attributed to the high stability of the compound or conformational changes before the interaction.

As far as the mechanism of interaction is concerned, the observed behaviour demonstrated ET process. It is well established that whenever there is an ET, it will be accompanied by a proton transfer from the antioxidant to the free radical to end by a stable product. In the case of protic solvents, this proton may be obtained from the solvent if this is more acidic than the flavonoid used. One possibility is direct ET and the situation will be as given in Equation (2) while the other is indirect ET as shown in Eqs (4) and (5).

$$O_2^{\bullet -} + FlH \rightarrow HO_2^{\bullet} + Fl^- \quad (4)$$

$$HO_2^{\bullet} + O_2^{\bullet -} \rightarrow HO_2^- + O_2 \quad (5)$$

**Decrease in anodic current and increase in cathodic current: Proton transfer mechanism**

The anodic current decreased very rapidly and the peak was completely scavenged when the flavonoids 6HFv and 567THF (having hydroxyl group(s) in their structure) were added into the solution of super oxide, while the cathodic current increased gradually by increasing the concentration of flavonoid (Figure 3b). The maximum effect of the added concentration of baicalein was observed from 0.1mM up to 0.6mM, anodic current decreased very rapidly up to this concentration and at last the radical was scavenged completely, while the cathodic peak showed a smaller decrease in the current value. Further additions caused a small

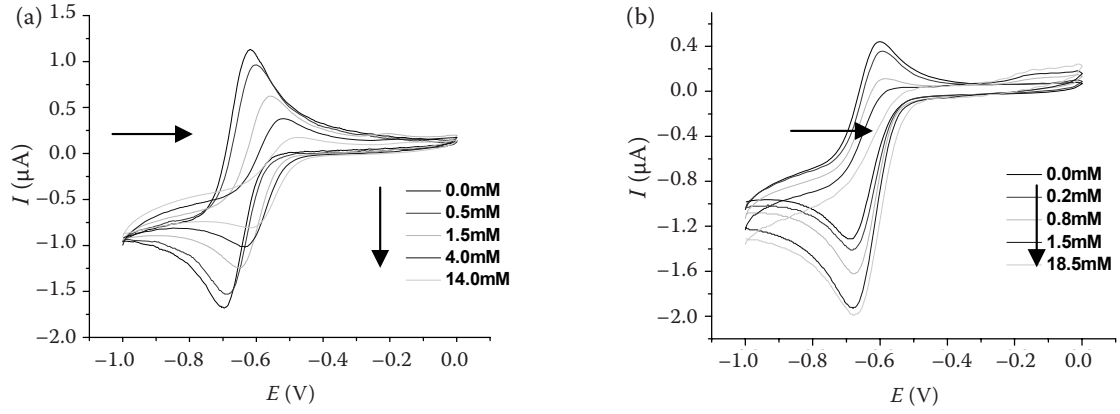



Figure 3. Cyclic voltammograms of $O_2^{\bullet -}$ in presence of (a) 3MOF (b) 6HFv at different concentrations in DMSO + 0.1M TBAP on GC as working electrode vs. Ag/AgCl as reference at 28°C with scan rate of 20 mV/s

increase in cathodic peak current and resulted in a large positive shift in the peak potential. The depletion of anodic current and a positive shift in the cathodic peak could be due to the protonation reaction. HyperChem data validated the reactivity of the *ortho* position in ring A of the molecule. The hydrogen of the *ortho* position was found to have a more positive charge density value than the others. While the *para* and *meta* positions have equal chances for the super oxide radical attack, the *para* position being more acidic is more susceptible for nucleophilic attack. In the case of 6HFv, the anodic current was decreased very rapidly and the peak was completely scavenged at 1.5mM while the cathodic current increased gradually by increasing the concentration of flavonoid.

It is well known that the super oxide radical abstracts proton when it reacts with a week acid (Collins *et al*. 2000). By nature, antioxidants are weak acids and the OH group containing antioxidants are capable of donating proton(s). The increase in the reduction current of super oxide radical by the addition of hydroxyl group(s) containing flavonoids shows that electron is accepted or proton is donated by the flavonoid (Ziyatdinova *et al*. 2005; Arshad *et al*. 2009). To conclude which of the two processes occurred, the charge density values were analysed. The charge density data (Shakeel 2010) favoured the proton transfer from OH group of the flavonoid radical. In the presence of these two flavonoids (6HFv and 567THF), an additional small anodic peak appeared prior to the oxidation wave of superoxide (Figure 3b) which is due to the oxidation of phenoxide ($Fl^-$). This is a strong indicative of protonation reaction (Macias-Ruvalcaba *et al*. 2004), thus supporting the proposed protonation pathway. Either disproportionation will take place (H-atom transfer between two $HO_2^\bullet$) or it will undergo reduction to form $HO_2^-$ following the protonation as shown in Eq. (4). The $HO_2^\bullet$ will be reduced by taking an electron; there are two possibilities for this reduction: it will be reduced either on the electrode surface or in the solution (Eq. 5). The lifetime of $HO_2^\bullet$ is longer than 1 ms, so it will form at a larger distance from the electrode surface, hence it will be impossible for it to diffuse back to the electrode surface and to get reduced. Therefore, it is expected that the second electron transfer will take place in the solution, and that it will be the ECE mechanism. This $HO_2^-$ will be protonated by the flavonoid and converted to $H_2O_2$ (as shown previously in Eq. 3).

As expected, out of all tested compounds, the powerful scavenging behaviour is shown by the compound(s) having more hydroxyl groups in their structure because the hydrogen of OH is more acidic. It is important to mention that the decrease in the cathodic current does not occur because of the interaction of the neutral molecular oxygen. It is rather an 'EC' type mechanism in which the electron transfer occured to its full satisfaction followed by chemical reaction. Here, it is the interaction of flavonoid with the superoxide anion radical, i.e. the reduced molecular oxygen. Moreover a positive shift (less negative potential) has been observed in most of the cases as predicted by Nernst equation while showing the consumption of $O_2^{\bullet-}$ during the reaction course. The absence of a pre peak or/and negative shift in the potential excludes the possibility of interaction of molecular oxygen with flavonoid.

## Thermodynamic parameters

To quantify the results, the strength of interaction between super oxide radical and antioxidant was estimated in terms of the binding constant $K_b$. Based on the decrease in the peak current, the binding constant ($K_b$) of super oxide radical with the additive was calculated using the following equation (Feng *et al*. 1997):

$$\log\left(\frac{1}{[AO]}\right) = \log K_b + \log\left(\frac{I_p}{I_{po} - I_p}\right) \quad (6)$$

where:

$I_{po}$, $I_p$ – peak currents of super oxide anion radical in the absence or presence of additives, respectively

[AO] – concentration of antioxidant

Thermodynamic parameter ($\Delta G^\circ$) was calculated using the $K_b$ value obtained from the above equation. The representative plots are given in Figure 4.

The compound which shows a high value of the binding constant reveals a strong interaction with the radical and shows a large decrease in the anodic current value. From Table 1 is it apparent that the % decrease of the anodic current of super oxide anion radical and binding constant ($K_b$) values follow the following order:

567THF > 6HFv > 3MOF > 6ClF > 6MOFv > F > 6BrF > 6MF

Baicalein shows the highest value of the binding constant ($K_b$) and a large % decrease in the anodic

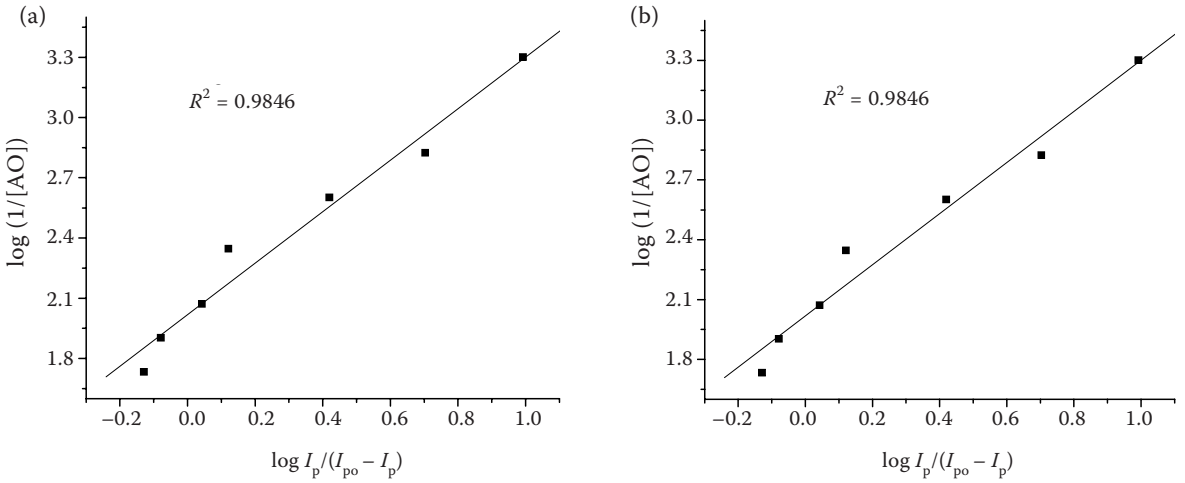



Figure 4. Plots to determine binding constant ($K_b$) using equation log (1/[AO]) vs log [$I_p/(I_{po} - I_p)$] for (a) 6MF and (b) 6MOFv

current values while 6HFv comes next to it, which ensures a strong interaction between the antioxidant and super oxide radical. The value of the change in free energy ($\Delta G^\circ$), calculated using the obtained $K_b$ value, shows spontaneity of the reaction between super oxide radical and additives.

### Antioxidant activity coefficient ($K_{ao}$)

The relative capacity of flavonoids to scavenge the target radical was determined as antioxidant activity coefficient ($K_{ao}$) from the degree of the interaction of the compound/substrate with the super oxide anion radical. The constant $K_{ao}$ is defined as the ratio of the current density values, with and without the addition of the substrate to the free radical. To quantify this effect the following equation (Korotkova *et al*. 2003) was employed:

$$K_{ao} = \frac{\Delta j}{(j_o - j_{res})\,\Delta C} \quad (7)$$

where:

$\Delta j$ – change in the oxygen anodic current density with the addition of the substrate

$j_o$ – limiting current density of oxygen without the substrate in the solution

$j_{res}$ – residual current density of oxygen

$\Delta C$ – change in the concentration of the substrate (mol/l)

The equation was employed only for the region in which a linear change occurred in the value, i.e. at low concentration of additives. From Table 1 is it apparent that the tested compounds follow the following order for $K_{ao}$:

567THF > 6HFv > 6ClF > 6MF > 6MOFv > 3MOF > 6BrF > F

Among the flavones tested, the most powerful antioxidant behaviour is shown by the baicalein with (3 hydroxyl groups), while flavanone having one hydroxyl group is next to it. This order shows that the $K_{ao}$ depends upon hydroxyl groups (hydrogen bond accepter or donor) and hydrophobic aryl rings which is in agreement with the earlier discussion. Keto group is electron withdrawing and it changes the properties of the rings but flavone is planar while flavanone is non planar, so it will be easier for flavones to be oxidised than flavanones. The phenoxyl radical formed after the reaction with super oxide is more stable in the case of trihydroxy flavone than 6HFv because the saturation of the flavanone ring breaks up the resonance within all aromatic rings. Trihydroxy flavone has aromaticity and a highly hydrophobic character which is absent in flavanones, thus it shows a high $K_{ao}$. Similary, it is reported that unsaturated molecules show a high stability of phenoxyl radical and a strong antioxidant activity in comparison with the saturated molecules (Harborne 1994).

In most cases, antioxidant activity constant ($K_{ao}$) values are in accordance with the % decrease in the current values and $K_b$ data (given in Table 1) as expected and logical. However, in some cases (e.g. 3MOF and 6MF) this is not true because of the fact that the equation used to estimate the $K_{ao}$ entails only linear portion of the plot, i.e. the concentration range of the antioxidant which systematically scavenges the free radical (Figure 4).

This is understandable and, as it was discussed earlier, that the variation in the concentration of the added antioxidant changes the free radical scavenging mode and thus the strength of interaction as well. The substantial change in Gibbs free energy values shows not only the spontaneity of the reaction between the additives and the super oxide anion radical but it also contributes to the relative stability of the resultant products.

## Kinetics

Kinetics of the free radical scavenging was assessed in terms of the second order homogenous rate constant ($K_2$). For this purpose pseudo first order rate constants ($k_f$) for the reaction of additives and super oxide radical were calculated using the Nicholson-Shain equation (Nicholson & Shain 1964).

$$E_p = E_{1/2} - \frac{RT}{nF}[(0.78 - \ln\sqrt{\frac{k_f}{a}})] \quad (8)$$

where:

$a = (RT/nF)\,\nu$

$E_{1/2}$ – reversible half wave potential of super oxide radical

$E_p$ – peak potential after the addition of a higher concentration of the additive

$\nu$ – scan rate (mV/s)

The values of $k_2$ for the compounds were calculated from the following relationship:

$$k_2 = \frac{k_f}{[AO]} \quad (8)$$

[AO] is the concentration of antioxidants, which was present in large excess in order to obtain the pseudo first order condition. From the $k_2$ Gibbs energy of activation ($\Delta G^*$) was calculated using the Arrhenius form of the rate constant relationship.

$$k_2 = \frac{kT}{h}\, e^{-\Delta G^*/RT} \quad (9)$$

where:

$k$ – Boltzman constant

$R$ – gas constant

$h$ – Plank's constant

The data obtained is presented in Table 2 along with the Gibbs activation energies ($\Delta G^*$). For two substrates containing hydroxyl groups, the calculation of $k_2$ was not possible because the anodic peak was scavenged completely and pseudo first order condition was not fulfilled. The obtained second order rate constants follow the following order:

Table 2. Homogeneous rate constant ($k_2$) and energy of activation ($\Delta G^*$) for the flavonoids

| Compounds | $k_2 \times 10^{-5}$ ($M^{-1}s^{-1}$) | $\Delta G^*$(kJ/mol) |
|---|---|---|
| 3MOF | 501 | 29.36 |
| 6MF | 76 | 39.86 |
| 6ClF | 48 | 41.02 |
| F | 17 | 43.53 |
| 6MOFv | 15 | 38.13 |
| 6BrF | 2 | 49.60 |

3MOF > 6MF > 6ClF > F > MOFv > 6BrF

It must be noted that the $k_2$ values are not necessarily proportional to the $K_{ao}$. The former tells how fast is the antioxidant behaviour while the latter is an estimate of the scavenging capacity, i.e. an antioxidant having a very high value of the antioxidant activity coefficient ($K_{ao}$) may have a very small value of $k_2$, showing a very slow reaction to scavenge the radical with a high antioxidant capacity. Significantly large values of the order of $10^5$ to $10^7$ indicate a fast reaction between the radical and the flavonoids used. The obtained $\Delta G^*$ values are positive and in the range of the activation controlled reactions in contrast to the electrochemical production of superoxide anion radical which is purely a diffusion controlled process. Small values of $K_b$ and $K_{ao}$ classify (e.g of 3MOF) the flavonoids as weak antioxidants but, on the other hand, large $k_2$ values suggest a fast reaction. Thus even with a weak antioxidant the effect occurs within a short time interval. It is interesting that a weak antioxidant can be used effectively for certain applications in which the reaction is desired to occur in a short time interval. An *in vivo* study of this type would be a nice idea to pursue especially in the field of pharmaceutics and food preservation.

**Future prospects**: The relevant work underway in the lab includes the study of similar systems and some plant species in organic and aqueous media to mimic the *in vivo* situation. Further, the electrochemical generation of $OH^{\bullet}$ radical and the investigation of its fate are also in progress.

## CONCLUSIONS

All compounds cause a decrease in the anodic current of super oxide radical which shows their scavenging ability, i.e. the antioxidant activity and change in cathodic current are mechanism dependent. All the cases followed $E_rC_i$ mechanism where the following chemical reaction due to the additive was characterised as; (*i*) hydrogen atom transfer (HAT), (*ii*) electron transfer (ET), (*iii*) proton transfer (PT). Flavonoids containing hydroxyl group(s) in their structure were found more powerful antioxidants in comparison to the others. Quantification, in terms of the binding constant ($K_b$) and antioxidant activity ($K_{ao}$), complements the percentage decrease in the anodic current and the scavenging behaviour. The value of homogeneous rate constant was the highest for 3MOF and minimum for 6BrF. The results indirectly support the $E_rC_i$ pathway and point towards the control use of antioxidants on the basis of their effectiveness and rate of free radical scavenging activity.

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Received for publication November 17, 2010
Accepted after corrections March 28, 2011

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