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On the antioxidant properties of three synthetic flavonols

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The antioxidant properties of a series of synthetic and natural flavonoids towards the oxygenated species superoxide radical anion ($O_2^{\cdot-}$) enzimatically generated, were evaluated. 7-Hydroxyflavonol, 7,3′-dihydroxyflavonol and 3′-hydroxyflavonol were synthesised, with a systematic variation of the OH substitution on positions C3, C7, C3′and C4′, and their respective antioxidative abilities compared to those of the already characterised natural flavonoids quercetin, kaempferol and rutin. The efficiency of $O_2^{\cdot-}$ deactivation by the flavonoids does not correlate with their respective determined oxidation potentials, suggesting that the pure one-electron-transfer-mechanism of $O_2^{\cdot-}$ quenching could not be the main scavenging process involved. Experimental evidence demonstrated that the possible inhibition of the $O_2^{\cdot-}$-generator enzymatic system by the flavonoids must be disregarded as a possible indirect cause for the inhibition of the oxidative species. One possible mechanism for the inhibition of $O_2^{\cdot-}$, highly dependent on the substitution pattern of the flavonoid, may be the generation of hydroperoxides or dioxetanes as oxidation products, as already postulated for other biologically relevant compounds. The simultaneous OH-substitution on positions C3 and C7 of the flavonoid skeleton plays a definitive role in the enhancement of the $O_2^{\cdot-}$ inhibitory effect. The replacement of OH by a O-rutinosyl group on position C7 suppresses at all that effect, whereas the absence of an OH group in position 7 significantly reduces the antioxidative power. Finally, the presence of OH groups on positions 3′and 4′ does not produce any determinant effect in the antioxidative behaviour of the flavonoids.

1. Introduction

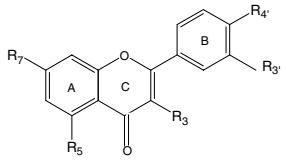
Flavonoids are compounds present in fruits, green plants and certain beverages that have diverse beneficial biochemical and antioxidant effects (Janssen et al. 1998; Csokay et al. 1997; Blasiak et al. 2002). Their dietary intake in mammalians is quite high as compared to other natural antioxidants like vitamins C and E (Kim et al. 2002).

The main antioxidant effect of flavonoids is the protection of cells against the damaging effects of reactive oxygen species, such as singlet molecular oxygen ($O_2(^1\Delta_g)$), peroxyl radicals, hydroxyl radicals, peroxynitrite and superoxide radical anion ($O_2^{\cdot-}$) (Duarte et al. 2002; Tournaire et al. 1993). The last species is a key intermediate in oxygen redox chemistry. It is formed in many living systems and it or subsequent reaction products can have many deleterious effects (for a review see Bielski et al. 1985).

A generalized idea exists in the sense that the chemical structure and the distribution of the hydroxy-groups in the flavonoid skeleton is the determining factor in terms of ability to act as antioxidative protectors (Tournaire et al. 1993; Knekt et al. 2002; Choi et al. 2002; Shen et al. 2002; El-Sukkary and Speier 1981; Criado et al. 1996; Cotelle et al. 1996; Das and Pereira 1990). In particular the 3-OH substituted flavones, generically named flavonols, are considered one of the most powerful antioxidants of the family (Tournaire et al. 1993; El-Sukkary and Speier 1981; Cotelle et al. 1996; Das and Pereira 1990). Nevertheless, we demonstrated that the better ability of mono, di- and tri-hydroxyflavones to act as protectors against ($O_2{}^1\Delta_g$)-iniciated photooxidations of fats is exerted by 7-OH substituted flavones (Criado et al. 1996). These compounds not only effectively deactivate the oxidative species but also do not chemically react with ($O_2{}^1\Delta_g$), whereas 3-OH flavone, a less efficient quencher, is effectively photodegraded. We also studied the activity of 3- and 7-OH flavones as scavengers of $O_2^{\cdot-}$ generated from photoexcited riboflavin (Montaña et al. 2003). In this case only 3-OH flavone constitutes an inhibitor of the oxidative species, being also degraded in the processs of quenching.

On this basis we choose two structurally related flavonoid sets in order to investigate the scavenging of superoxide anion radical ($O_2^{\cdot-}$), generated through hypoxanthine-xanthine oxidase system. One of them includes three flavonols, synthesised with a systematic variation of the OH substitution in C3, C7, C3′ and C4′, named 7-hydroxyflavonol (**1**), 7,3′-dihydroxyflavonol (**2**), 3′-hydroxyflavonol (**4**), all of them with an OH group in C3. The derivative **1** has an OH group in position 7 meanwhile flavonoid **4** in

C3′; **2** has OH in C7 and C3′. The second set comprises three natural flavonoids -for which the antioxidative properties have been described in the literature (Carlsson et al. 1976; Tournaire et al, 1993; Cotelle et al. 1996) named quercetin (**3**), kaempferol (**5**) and rutine (**6**). These flavonoid have OH as substituents in C5, C7 and C4′. Compounds **3** and **5** have an additional OH in C3 but quercetin has another OH in C3′. Rutine presents an OH in position C3′ and an O-rutinosyl in C3.



(1)	$R_5 = R_{3'} = R_4 = H$	$R_3 = R_7 = OH$	**7-Hydroxyflavonol**
(2)	$R_5 = R_{4'} = H$	$R_3 = R_7 = R_{3'} = OH$	**7,3′-Dihydroxyflavonol**
(3)	$R_3 = R_5 = R_7 = R_{3'} = R_{4'} = OH$		**Quercetin**
(4)	$R_5 = R_7 = R_4 = H$	$R_{3'} = R_3 = OH$	**3′-Hydroxyflavonol**
(5)	$R_3 = H$	$R_3 = R_5 = R_7 = R_{4'} = OH$	**Kaempferol**
(6)	$R_5 = R_7 = R_{3'} = R_{4'} = OH$	R_3 = O-rutinosyl	**Rutine**

The aim of the present work was to establish any kind of dependence of the antioxidant properties of synthesised flavonols on their chemical structure and redox properties. Results could be of interest for the development of potential drug components for treating pathologies related to biological oxidation and could contribute to a better understanding of the mechanism of the antioxidative effect involving reactive molecular oxygenated species.

2. Investigations, results and discussion

Superoxide radical levels were measured by the nitrite method recording absorbance at 530 nm at different concentrations of flavonoids. A plot of absorbance vs. concentration of flavonoid allows determining the concentration necessary to scavenging 25% of the superoxide radical anion generated ($IC_{25}S$). Typical profiles of absorbance decrease of nitrite at 530 nm vs. flavonoid concentration are displayed in Fig. 1.

Fig. 2 is a graphical representation of the $IC_{25}S$ values obtained. It can be seen that the hydroxyflavonol **1** was the compound most effective as $O_2^{\cdot-}$ scavenger

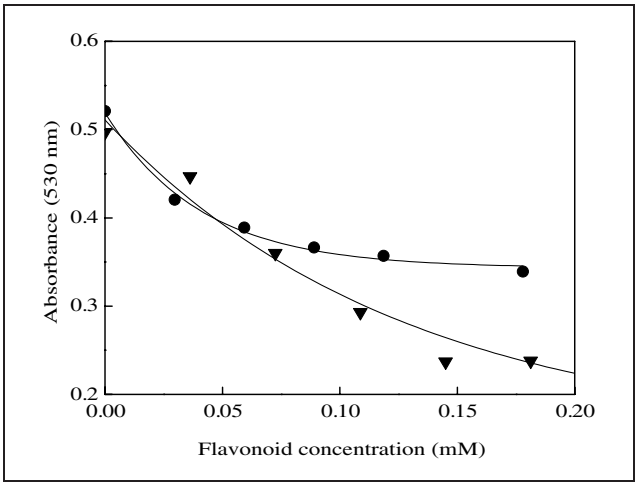


Fig. 1: Profile of inhibition of superoxide radical anion by flavonoids: nitrite absorbance at 530 nm (see Experimental) as a function of quercetin (▼) and kaempferol (●) concentration

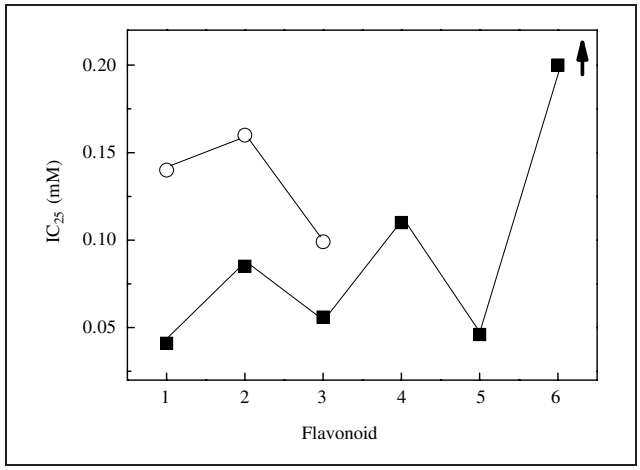


Fig. 2: Profile of 25% of superoxide radical anion inhibitory concentration ($IC_{25}S$) by flavonoids **1**–**6** (●); Profile of 25% of hypoxanthine oxidase activity inhibitory concentration ($IC_{25}X$) by flavonoids **1**–**3** (O). Lines connecting the experimental points were drawn in order to clarify the figure. The symbol ↑ in the figure indicates that no inhibition of superoxide radical anion was observed up to the flavonoid **6** concentration 0.2 mM

($IC_{25}S = 0.037$ mM), whereas **6** was the weakest one, since no inhibition of $O_2^{\cdot-}$ generated by hypoxanthine-xanthine oxidase system was observed up to a concentration of 0.2 mM, for this compound.

Flavonols are considered relatively efficient reducing agents (Jorgensen and Skibsted 1998) and depending on the redox potentials of the individual flavonol and the relative stability of the one-electron oxidised product, different alternative reactions can occur. In many cases it has been demonstrated that the reaction of certain substrates with $O_2^{\cdot-}$ proceeds through an electron transfer reaction (Bielski et al. 1985; Fang et al. 1998). In the present case, since $O_2^{\cdot-}$ has a reduction potential of -0.33 V (Fang et al. 1998), the reaction with the flavonoids studied herein, with oxidation potentials ranging between $+0.88$ and $+1.33$ V is thermodynamically feasible. Nevertheless, a representation of the $IC_{25}S$ values as function of the respective oxidation potentials for flavonols **1**–**6** (Fig. 3), shows that only compounds **3**, **4** and **5** behave in the expected

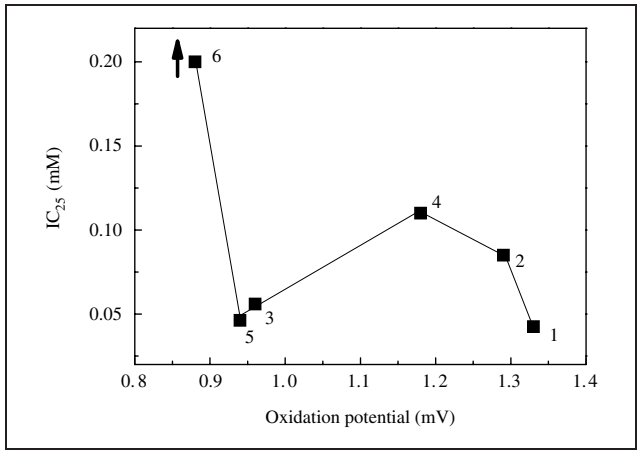


Fig. 3: Superoxide radical anion inhibitory concentration ($IC_{25}S$) by flavonoids **1**–**6** as a function of the respective oxidation potentials. Lines connecting the experimental points were drawn in order to clarify the figure. The symbol ↑ in the figure indicates that no inhibition of superoxide radical anion was observed up to flavonoid **6** concentration 0.2 mM

fashion (i.e. increase of the $IC_{25}S$ value as the oxidation potential increases). Furthermore, compound **6**, which should be in electrochemical terms the more favoured flavonoid for this kind of processes, is the compound that exhibits the highest $IC_{25}S$ value.

The results, clearly show that at least some of the flavonoids **1**–**6** interact with $O_2^{\cdot-}$ in a way different from the simple electron transfer process.

It is well known that hypoxanthine is oxidised successively to xanthine and then to uric acid by xanthine oxidase (Grossman and Moldave 1967). Molecular oxygen is the electron acceptor in this reaction. One of the products is the carcinogenic species $O_2^{\cdot-}$. It is well known that polyphenols, and particularly flavonols, act as $O_2^{\cdot-}$ scavengers (Hu et al. 1995). However, some authors suggest that the flavonoid-mediated-decrease of $O_2^{\cdot-}$ activity, when the oxidative species is generated through the xanthine oxidase system, could be a secondary effect, that would occur as a consequence of the inhibition of xanthine oxidase activity by flavonoids (Ruch et al. 1989). This information led us to investigate the possible effects of the synthesized flavonols on the enzimatic activity of xanthine oxidase, since the method for $O_2^{\cdot-}$ generation employed in this work for the determination of $IC_{25}S$ values involves that enzymatic reaction. The enzimatic inhibition assay was performed for compounds **1**–**3**, chosen as representative samples, since these compounds exhibit excellent relative levels of $IC_{25}S$. The data, obtained under the same experimental conditions employed in the $O_2^{\cdot-}$-inhibition assay, was plotted as absorbance of nitrite (proportional to the XO activity) vs. flavonoid concentration.

Relative values were fitted by regression and the 25% inhibition concentration ($IC_{25}X$) was graphically evaluated. Fig. 2 shows the $IC_{25}X$ values, being the natural flavonoid **3** ($IC_{25}X = 0.099$ mM), employed for comparative purposes, the most efficient inhibitor of the xanthine oxidase enzymatic activity. The $IC_{25}X$ values are much higher that the respective $IC_{25}S$ ones, clearly indicating that the scavenging of the species $O_2^{\cdot-}$ is not an apparent or secondary effect, governed by the inhibition of the xanthine oxidase activity.

As said, a certain connection between chemical structure of a given flavonoid and its protective antioxidant properties is currently accepted (Tournaire et al. 1993; Shen et al. 2002; El-Sukkary and Speier 1981; Criado et al. 1996; Cotelle et al. 1996).

Das and Pereira (1990) tested the antioxidative action against thermal autoxidation of refined palm oil – a process occurring through free radicals (Carlsson et al. 1976) – by several flavonoids, including compounds **3**, **5** and **6** (Cotelle et al. 1996). Structure-reactivity analysis reveals that the flavonoid molecule with polyhydroxylated substitutions on A and B rings and a free 3-OH group confers to the compound potent antiperoxidative properties. The antioxidative power decreased in the order **5** > **3** > **6**. in agreement with our results. In coincidence, Cotelle et al. (1996) provided evidence in the sense that 2′,3′,4′-OH-substituted flavones exhibit interesting antioxidant properties to scavenge free radicals, whereas 7-OH flavones are effective to competitively inhibit xanthine oxidase.

Matsuura et al. (1970) studied the proclivity of substituted flavonols to photosensitised oxygenation, which is in some way a measure of their potential antioxidant protective effect. These compounds, when irradiated in the presence of a dye-sensitizer, gave the respective depsides as main reaction products. The authors demonstrate that the corresponding O−Me – substituted derivatives at the flavonol position are almost unreactive upon sensitised photoirradiation.

The antioxidant activity of flavonoids as ($O_2{}^1\Delta_g$)-quenchers was studied in 1993 by Tournaire et al. The main results indicate that the efficiency of physical (unreactive) quenching of ($O_2(^1\Delta_g)$) is mainly controlled by the presence of the cathecol structure on ring B, whereas the structure of ring C, particularly the presence of OH group activating the double bond on position 3, is the main factor determining the efficiency of effective oxidation. The photooxidation quantum yields behave in the order **5** ≈ **3** ≫ **6**, in excellent coincidence with our trend for the inhibitory effect towards $O_2^{\cdot-}$.

El-Sukkary and Speier (1981) demonstrated that a series of 3-hydroxyflavones are effectively oxidised by chemically generated $O_2^{\cdot-}$ in THF. The overall process results in the oxidative cleavage of the heterocyclic ring. A radical intermediate is assumed, formed by hydrogen abstraction by $O_2^{\cdot-}$, which further reacts with $O_2^{\cdot-}$ to give the final products via hydroperoxide and dioxetane intermediates. Flavonols containing H; OH or OMe as a substituent on position 7 were all oxidisable with relative yields of the main oxidation product of 1; 0.6 and 0.8 respectively.

From a combined analysis of our results and the compiled literature data, several conclusions arise:

a) A significant point is the absence of a correspondence between the oxidation potential values and the antioxidant capacity of the studied flavonoids. Similar overall results are well documented from some radicals such as tyrosyl and tryptophyl derivatives. In these cases, even when the one-electron oxidation step is a thermodynamicaly viable process, the substrates mainly react with $O_2^{\cdot-}$ by addition, generating hydroperoxydes that yield stable products, whereas the electron transfer component is, at most, a minor component (Oldham 1986). This behaviour, in our case, could explain why the electron transfer step, which is a crucial step in reactions occurring through a $O_2^{\cdot-}$-mediated mechanism (El-Sukkary and Speier 1981; García 1994) is not the determining step when the oxidative species is enzymatically generated. According to our present results, the chemical structure of the flavonoid plays the dominant role in the scavenging of the species $O_2^{\cdot-}$, suggesting that the oxidative reaction could also occur via hydroperoxides.

b) It is evident for the $O_2^{\cdot-}$ inhibition process, the dominant active flavone structure is given by the simultaneous presence of OH groups in positions C3 and C7. In compound **6**, with a rutinosyl group in position C3 and OH group in C7, the antioxidant capacity is practically suppressed at all. In parallel, compound **3**, with an H-substituted C7 and an OH in C3, also exhibits the weakest antioxidative potential within the set of synthetic flavonoids.

c) The simplest synthetic dihydroxyflavonoid **1**, with OH substitution only on C3 and C7 was the most efficient $O_2^{\cdot-}$ suppressor. Its antioxidative behaviour is even superior to recognised antioxidant natural flavonoids such as quercetin and kaempferol.

d) The scarce difference in antioxidant activity exerted by compounds **1** and **5** indicate the irrelevance of the OH substitution in C4′.

e) The $IC_{25}S$ values for several 3,7-dihydroxyflavonoids, assayed as a representative sampling, indicate that these compounds do not inhibit the enzymatic generation of the species $O_2^{\cdot-}$ at concentration levels competitive with the respective quenching of the oxidative species.

3. Experimental

3.1. Chemicals

7-Hydroxyflavonol, 7,3′-dihydroxyflavonol and 3′-hydroxyflavonol were synthetized, purified and identified as described below (Tanaka and Sujino, 2001). Quercetin, Kaempferol and Rutine were naturally obtained from Organic Chemistry Area, San Luis National University. Hypoxanthine and xanthine oxidase were purchased from Sigma Chem. Co.

3.2. Synthesis of the flavonoids

3.2.1. Synthesis of 7-hydroxyflavonol (**1**)

Crystals of 2′,4′-dihydoxychalcone (0.250 g 100% pure), an aq. NaOH solution (2.5 mL 8 M) and a 30% hydrogen peroxide solution (2.15 mL) were stirred at room temperature for 2 h. The reagent mixture was neutralized with HCl (6 M) in an ice bath. The precipitate was suction filtered, water washed, dried and recrystallized from a methanol-water mixture to give a beige crystalline solid yielding 95% of the pure product. The structure of **1** was determined by the chromatographic and spectroscopic data: Rf (TLC): 0.22; UV λ_{max} (MeOH) nm: 330 (18162.96); 253 (16672.64); 226 (20416.64). EI-MS m/z (rel. int.) 270 [M^+] (100); 137 (11.56).

3.2.2. Synthesis of 7,3′-dihydroxyflavonol (**2**)*:*

Crystals of 2′,4′,3-trihydroxychalcone (0.500 g 100% pure), an aq. NaOH solution (5 mL 8 M) and a 30% hydrogen peroxide solution (5 mL) was stirred at room temperature for 2 h. The reagent mixture was neutralized with HCl (6 M) in an ice bath. The precipitate was suction filtered, water washed, dried and recrystallized from methanol-water mixture. The product was purified by chromatoghaphy using a Sephadex LH 20 column and methanol as eluent. The structure of **2** was determined by the chromatographic and spectroscopic data: Rf (TLC): 0.14; UV λ_{max} (MeOH) nm: 318.6 (16052.30); 244.8 (15447.56). EI-MS m/z (rel.int.) 270 [M^+] (100); 137 (52.01); 121 (30.89); 120 (15.08).

3.2.3. Synthesis of 3′-hydroxyflavonol (**4**)*:*

Crystals of 2′,3-dihydroxychalcone (0.500 g 100% pure), an aq. NaOH solution (5 mL 8 M) and a 30% hydrogen peroxide solution (4.15 mL) was stirred at room temperature for 2 h. The reagent mixture was neutralized with HCl (6 M) in an ice bath. The precipitate was suction filtered, water washed, dried and recrystallized from methanol-water mixture. A beige crystalline solid was obtained yielded 95% of the pure product. The structure of **3** was determined by the chromatographic and spectroscopic data: Rf (TLC): 0.32; UV λ_{max} (MeOH) nm: 344.4 (20665.47); 304.4 (12807.99); 243.8 (24782.82). EI-MS m/z (rel. int.) 254 [M^+] (100); 121 (53.83).

3.3. Generation and inhibition of superoxide radical anion

Superoxide radical anion was generated enzymatically by a hypoxanthine-xanthine oxidase system and was detected by the nitrite method (Hu et al. 1995). The test solutions were prepared by mixing 2 mL of stock solution (1.2×10^{-3} M hypoxanthine, 1.2×10^{-3} M hydroxylamine and 6×10^{-4} M EDTA), 1.4 mL buffer (0.0624 M KH_2PO_4 and 0.0498 M $Na_2B_4O_7$) with flavonoids (or without flavonoids as controls). Flavonoids were prepared in concentrated methanolic solutions and 25 to 200 μL were added (completing to 200 μL with MeOH) in the test tubes. The reaction was started by adding 10 μL xanthine oxidase (final concentration: 2×10^{-3} U/mL). After incubation (30 min, 37 °C), 0.3 mL HCl (1 M) and 0.1 mL dye reagent (sulfanilic acid 0.019 M and N-1-naphtylethylenediamine 2.60×10^{-3} M) were added. The mixtures were allowed to stand for 45 min at room temperature and absorbances at 530 nm were determined in a spectrophotometer Shimadzu UV-160A. The scavenging activities were determined with different concentrations of flavonoids.

3.4. Inhibition assay of xanthine oxidase by flavonols

Xanthine oxidase (XO) enzyme activity was determined as described by Kuppusamy et al. (1990). A XO solution (final concentration 2×10^{-3} U/mL) was preincubated with 0.2 mL of methanolic flavonoid solution and 1.4 mL pH 7 buffer for 15 min at 37 °C. The volume was made up to 3.2 mL with distilled water. Control tubes contained the enzyme, methanol, buffer and water in the same proportion as assay tubes were prepared. After preincubation, assay was started by adding 0.4 mL of a stock solution containing hypoxanthine (6×10^{-3} M), hydroxilamine (6×10^{-3} M) and EDTA (3×10^{-3} M) to each tube and incubated for 30 min at 37 °C. Reactions were stopped by addition of 0.3 mL HCl (1 M) and the coloration reaction was carried out in the same way as described above.
On the basis of data obtained in the enzymatic inhibition assay, absorbance of nitrite (proportional to the XO activity) was plotted vs. flavonoid concentration. Values were fitted by regression and the 25% inhibition concentration ($IC_{25}X$) was graphically evaluated.

3.5. Determination of the half-wave potentials

Electrochemical measurements were performed in a two-compartment Pyrex cell. The working electrode was a Pt wire. Before each measurement the Pt wire electrode was cleaned by heating it up to the red on a burner flame. The counter electrode was a platinum foil of large area (approximately 2 cm^2). The reference electrode was an aqueous saturated calomel electrode (SCE). Solutions were deaerated by bubbling pure nitrogen. All measurements were performed at 25 ± 0.5 °C, employing H_2O/NaOH 10 mM as solvent.
The convolution of faradaic current was calculated by applying the algorithm proposed by Oldham (1986) to the background corrected voltammograms. The measuring system for linear scan voltammetry (LSV) was constructed from a EG & G PARC Model 273 potentiostat run with model PAR270 electrochemical analysis software.

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