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Antioxidant effect of flavonoids after ascorbate/Fe^{2+}-induced oxidative stress in cultured retinal cells

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Abstract

In this study, we investigated the structure–activity relationship of four flavonoids, i.e. eriodictyol, luteolin, quercetin, and taxifolin, in cultured retinal cells after ascorbate/Fe^{2+}-induced oxidative stress. The relative order of antioxidant efficacy, determined by the thiobarbituric acid method, was the following: eriodictyol > quercetin > luteolin > taxifolin. Upon preincubation, the flavonoids were also effective in reducing the extent of lipid peroxidation. Oxidative stress, determined by the changes in fluorescence of 2′,7′-dichlorodihydrofluorescein, was also decreased in the presence of the flavonoids, showing the following order of antioxidant efficacy: eriodictyol > taxifolin ≈ quercetin > luteolin. Ascorbate/Fe^{2+}-induced oxidative stress or incubation in the presence of the flavonoids did not significantly affect the viability of retinal cells. We also evaluated the degree of membrane partition of the flavonoids. In this system, the results strongly suggest that the higher antioxidant activity of the flavonoids is not correlated with the presence of a double bond at C_2–C_3 and/or a hydroxyl group at C_3 on the C ring, but rather may depend on the capacity to inhibit the production of reactive oxygen species to interact hydrophobically with membranes. Eriodictyol was shown to be the most efficient antioxidant in protecting against oxidative stress induced by ascorbate/Fe^{2+} in the retinal cells. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Antioxidants; Flavonoids; Oxidative stress; Retinal cells

1. Introduction

Although the etiology of many diseases of the CNS is still unclear, several lines of evidence indicate that the generation of ROS plays an important role in many neuropathological conditions, such as cerebral ischemia and Parkinson's or Alzheimer's disease [1,2]. Consequently, there is a major interest in developing efficient antioxidants that can protect against neuronal cell injury and death without showing toxic effects. The flavonoids are a group of polyphenolic compounds ubiquitously found in plants and one of the most important classes of compounds with biological activity [3]. The flavonoids have been shown to be very potent antioxidants, because they have a high scavenging activity [4,5] and a capacity to complex iron [6]. In addition, the flavonoids are also potent antibacterial, antiviral, anticancer, immune-stimulant, hepatoprotector, antithrombotic, and anti-inflammatory agents [7,8].

The importance of the chemical structure of the flavonoids, particularly the presence of a double bond at C_2–C_3 and a hydroxyl group at C_3 on the C ring, in relation with their antioxidant activity in biological systems has been a matter of much controversy [9–12]. Previous studies have revealed that the presence of two hydroxyl groups at C_3, and C_4, on the B ring is the most important structural feature for determining the antioxidant activity of these compounds [13,14].

The objective of this study was to analyse the structure–antioxidant activity relationship of four flavonoids, i.e. luteolin, quercetin, eriodictyol, and taxifolin, which differ in the position of the hydroxyl group at C_3 and in that of the double bond at C_2–C_3 (Fig. 1), by using the retinal cells as a model of neuronal cells. We demonstrate that oxidative stress, induced in the presence of ascorbate/Fe^{2+}, is highly reduced in the presence of the flavonoids. Eriodictyol, which has no double bond at C_2–C_3 and no hydroxyl group

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Abbreviations: BME, basal medium of Eagle; $DCFH_2$, 2′,7′-dichlorodihydrofluorescein; LDH, lactate dehydrogenase; PC, partition coefficient; Rf, retardation factor; ROS, reactive oxygen species; TBA, thiobarbituric acid; and TBARS, thiobarbituric acid-reactive substances.

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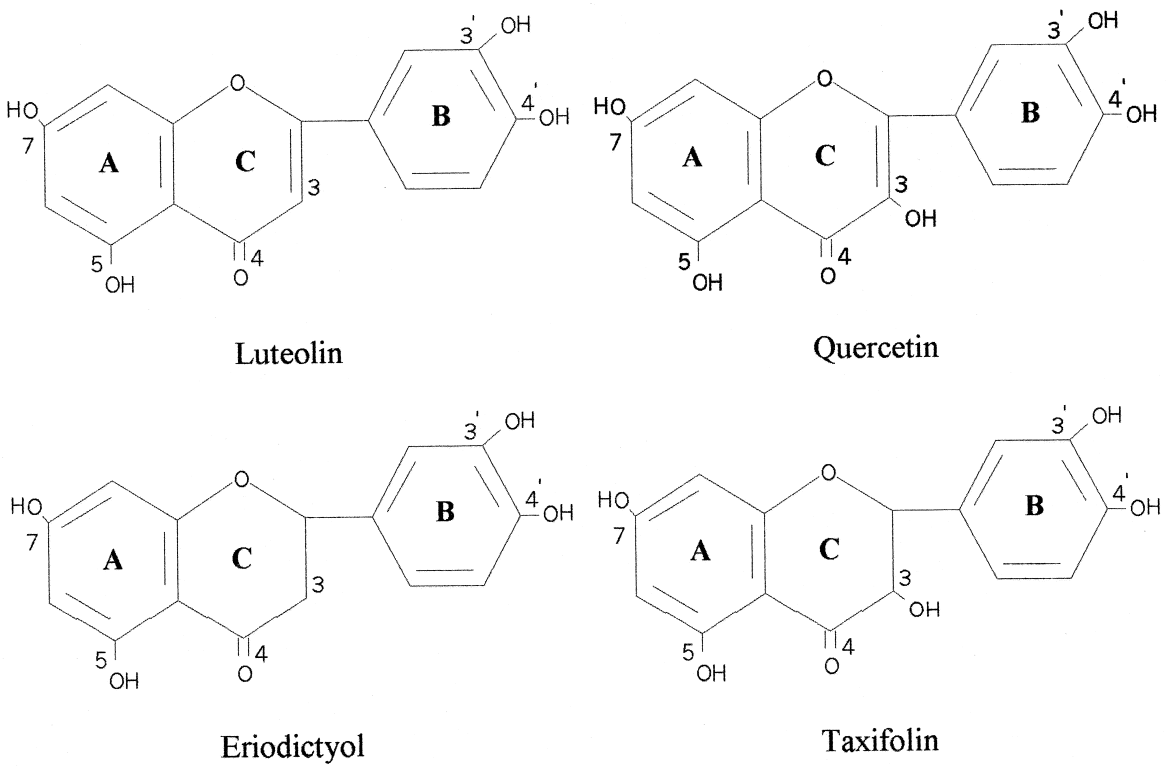

Fig. 1. Chemical structures of four flavonoids, i.e. luteolin, quercetin, eriodictyol, and taxifolin.

at C_3 (Fig. 1), was found to be the most efficient flavonoid in preventing the formation of ROS in the retinal cells, suggesting its higher antioxidant activity.

2. Materials and methods

2.1. Compounds

Quercetin, NADH, and BME were purchased from Sigma; luteolin, eriodictyol, and (±)-taxifolin were from Extrasynthèse; trypsin was from GIBCO; fetal bovine serum was from BioChrom KG; and $DCFH_2$-diacetate was from Molecular Probes. All other reagents were of analytical grade.

2.2. Culture of chick retinal cells

Primary cultures of retina were prepared from 8-day-old chick embryos, as described previously [15]. The retinas were dissected free from other ocular tissues and dissociated with 0.1% trypsin in a Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution for 15 min at 37°. The digested tissue was centrifuged at 500 *g* for 1 min, and the pellet was resuspended in BME containing Earle's salts and L-glutamine, buffered with 25 mM HEPES and 25 mM $NaHCO_3$, and supplemented with 5% fetal calf serum (heat-inactivated), penicillin (100 U/mL), and streptomycin (100 μg/mL). Then, the tissue was dissociated mechanically with a glass pipette and the retinal cells were further cultured in BME-supplemented medium, as described above. The cells were plated at a density of 0.76×10^6 cells/cm^2 on poly-L-lysine (0.1 mg/mL)-coated coverslips for fluorescence measurements or at a density of 0.53×10^6 cells/cm^2 on poly-L-lysine-coated 6-multiwell Costar plates for the other measurements. The cells were maintained in culture for 5–6 days in an atmosphere of 95% air and 5% CO_2. A cell preparation similar to the one used in this work was shown to contain a significant percentage of amacrine-like neurons and neurons resembling bipolar cells, and only a few glial cells [16–20].

2.3. Induction of oxidative stress

The oxidant pair ascorbate/Fe^{2+} was used to induce oxidative stress in retinal cells in culture. After removal of the culture medium, the retinal cells were washed twice and incubated in Na^+ medium, containing (in mM): 140 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 1 NaH_2PO_4, 5.6 glucose, and 20 HEPES, pH 7.4, at 37°, for 10 min. Ascorbate (5 mM)/Fe^{2+} (100 μM) was incubated in Na^+ medium (pH 7.4), for 15 or 20 min, at 37°. In this study, we used the oxidant pair ascorbate/Fe^{2+} because an increase in free iron concentration occurs in many neuropathological situations as a result of a disruption of iron homeostasis. Furthermore, free iron can be reduced by ascorbate that exists at a high (millimolar) concentration in the nervous tissue, generating hydroxyl radicals by the Fenton reaction [1,2,21].

Incubation with the flavonoids was performed simultaneously with ascorbate/Fe^{2+} (20 min) or during a preincubation in Na^+ medium (2 hr), followed by the induction of oxidative stress (15 min). A preincubation of the flavonoids for 2 hr ensured the permeation into the plasma membrane [10,22]. The four flavonoids studied were used at the following range of concentrations: luteolin (20–50 μM), quercetin (10–30 μM), eriodictyol (10–30 μM), and taxifolin (30–60 μM). Under control conditions, the cells were incubated in the absence of ascorbate/Fe^{2+}.

2.4. Measurement of the extent of lipid peroxidation

The extent of lipid peroxidation in the retinal cells was evaluated by measuring the production of TBARS, as determined by the TBA method. Thiobarbituric acid reacts with substances named TBARS (oxidative degradation products that react with TBA), including MDA (malondialdehyde). This method was previously revealed to be quite efficient in the determination of peroxidation in retinal cells, because is simple, quick, requires a minimum of handling, and shows a good correlation with other methods used to evaluate peroxidation [18–20,23].

After the induction of lipid peroxidation, the reaction was stopped by lowering the temperature to 0–4°, placing the wells on ice, and removing the incubation medium and adding 1 mL of ice-cold 15 mM Tris, pH 7.4. The cells were further scraped and 0.8 mL of cell suspension was added to 2 mL of a TBA–TCA–HCl–BHT reagent, containing 0.375% TBA (w/v), 0.25 N hydrochloric acid (HCl), and 6.8 mM 2,6-di-*tert*-butyl-4-methylphenol (BHT), still on ice. Then, the samples were placed in a boiling waterbath for 15 min, removed, and allowed to cool at 0–4°, in ice. The samples were centrifuged at 3000 rpm for 15 min (SIGMA-302 K centrifuge), the supernatant was collected, and the absorbance was measured at 530 nm. An absorption coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ was used for calculating the amount of TBARS produced, expressed as nmol TBARS/mg protein. The amount of protein was measured by the Sedmak method [24].

The efficacy of the flavonoids, luteolin, quercetin, eriodictyol, and taxifolin, was evaluated by determining the percentage of inhibition of TBARS production, as compared to the maximal oxidation of the cells (oxidant condition), in the absence of the flavonoids by using the formula:

$$\%\ \text{inhibition} = 100\% - \frac{\text{TBARS antioxidant condition–TBARS control}}{\text{TBARS oxidant condition–TBARS control}} \times 100\%$$

2.5. Measurement of oxidative stress

The extent of oxidative stress in retinal cells in culture was measured by following the oxidation of $DCFH_2$ to the fluorescent DCF [25,26]. After incubation with the flavonoids for 2 hr, the cells were loaded with 20 μM $DCFH_2$-DA for 15 min in the dark. Loading of the probe was renewed for each experiment. Then, the retinal cells were exposed to ascorbate/Fe^{2+} for 15 min. After rinsing, cell fluorescence was measured in Na^+ medium, at 37°, with excitation at 502 nm and emission at 550 nm, using a SPEX Fluorolog spectrometer equipped with a thermostatted waterbath. The increments in DCF fluorescence, after 15-min oxidation, were recorded for 5 min and expressed as arbitrary units above the initial values.

2.6. Analysis of retinal cell viability

The effect of oxidative stress, induced by ascorbate/Fe^{2+}, on the viability of cultured chick retinal cells was evaluated by determining the leakage of the cytoplasmic enzyme LDH to the extracellular medium. The viability of retinal cells was also determined after preincubation with the flavonoids for 2 hr, followed by incubation with ascorbate/Fe^{2+} for 20 min. LDH activity was measured spectrophotometrically by following the conversion of NADH to NAD^+, at 340 nm, as described elsewhere [20]. LDH leakage to the extracellular medium was expressed as a percentage of total LDH activity (intracellular plus extracellular).

2.7. Evaluation of the degree of hydrophobicity of the flavonoids

The degree of hydrophobicity of the flavonoids was examined by measuring the partition coefficients (PC) and the retardation factors (Rf). The PC were measured by using an *n*-octanol/HEPES system. The flavonoids were dissolved in *n*-octanol at a concentration of 200 μM, and 5 mL of each flavonoid solution was shaken with 100 mL HEPES (20 mM, pH 7.4) for 10 min, at 19 ± 1°. Then, the two phases were separated by centrifugation. The PC values were calculated by using the formula [27]:

$$PC = \log C_O/C_H$$

where C_O and C_H are the concentrations of flavonoids in *n*-octanol and HEPES, respectively, measured by HPLC/diode array.

The Rf were determined by TLC on silica gel plates (Merck, G 60) by using a solvent system consisting of chloroform:ethyl acetate:acetic acid (16:8:1) [28]. The plates were pre-run with the solvent and allowed to dry prior to the application of the sample. A volume of 2 μL of each flavonoid solution (200 μM) was applied on the silica plates.

2.8. Statistical analysis

The results are the means ± SEM of triplicates from 2–4 independent experiments. Statistical values ($P < 0.05$) were determined by the unpaired two-tailed Student's *t*-test or by the one-way ANOVA Tukey–Kramer post-test for multiple comparisons.

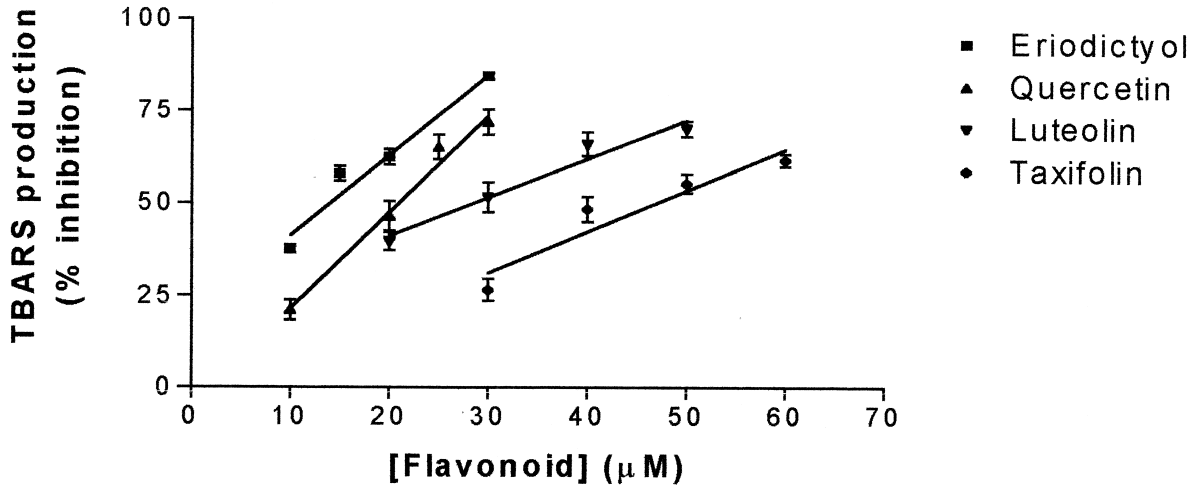

Fig. 2. Dose-dependent effect of eriodictyol, quercetin, luteolin, and taxifolin on the extent of lipid peroxidation in retinal cells in culture. The flavonoids eriodictyol (10–30 μM), quercetin (10–30 μM), luteolin (20–50 μM), and taxifolin (30–60 μM) were incubated simultaneously with ascorbate (5 mM)/Fe^{2+} (100 μM) in Na^+ medium, pH 7.4, at 37° for 20 min. The extent of lipid peroxidation was measured by the TBA method. The results were expressed as the percentage of inhibition of TBARS production in comparison to retinal cells incubated in the presence of the oxidant pair, but in the absence of the flavonoids, and are the means ± SEM of triplicates from three to five experiments.

3. Results

3.1. Effect of flavonoids on ascorbate/Fe^{2+}-induced lipid peroxidation in cultured retinal cells

Fig. 2 shows the dose-dependent changes in TBARS production induced by eriodictyol, quercetin, luteolin, and taxifolin in retinal cells submitted to ascorbate/Fe^{2+}. Incubation of retinal cells with ascorbate (5 mM) and Fe^{2+} (100 μM) for 20 min significantly increased the production of TBARS (11.23 ± 0.28 nmol TBARS/mg protein) as compared to the control, in the absence of ascorbate/Fe^{2+} (0.49 ± 0.02 nmol TBARS/mg protein).

In the presence of the flavonoids, incubated simultaneously with ascorbate/Fe^{2+}, the production of TBARS decreased significantly in a dose-dependent manner, as observed by the sharp increase in the percentage of inhibition (Fig. 2). Determination of the concentration of the flavonoids sufficient to inhibit the production of TBARS by 50% (IC_{50} values) revealed the following order of antioxidant efficacy: eriodictyol (IC_{50} = 13.89 ± 0.66 μM) > quercetin (IC_{50} = 20.74 ± 0.64 μM) > luteolin (IC_{50} = 28.56 ± 1.73 μM) > taxifolin (IC_{50} = 46.49 ± 1.11 μM). All the IC_{50} values were found to be statistically different ($P < 0.001$, ANOVA statistical analysis).

We also determined the antioxidant effect of the flavonoids when preincubated for 2 hr before the induction of oxidative stress by using the IC_{50} values determined in Fig. 2. Under these conditions, eriodictyol (14 μM), quercetin (21 μM), luteolin (29 μM), and taxifolin (46 μM) also reduced the production of TBARS to 32.39 ± 1.64%, 28.76 ± 2.08%, 28.18 ± 2.13%, and 24.24 ± 3.17%, respectively. Data obtained in the presence of taxifolin, shown to inhibit TBARS production to a lower extent, were statistically different as compared to eriodictyol only ($P < 0.05$). In these experiments, oxidative stress in the absence of the flavonoids induced the formation of 10.42 ± 0.67 nmol TBARS/mg protein, significantly different as compared to the control (0.48 ± 0.05 nmol TBARS/mg protein).

3.2. Inhibitory effect of flavonoids on the extent of oxidative stress determined by $DCFH_2$

Oxidative stress was also analysed by following the increase in DCF fluorescence upon exposure to ascorbate/Fe^{2+}. Fig. 3 shows that oxidative stress induced a significant increase in the fluorescence intensity of DCF (approximately 1.9-fold) as compared to the control ($P < 0.001$). Preincubation of the flavonoids at the IC_{50} values determined in Fig. 2 reduced ascorbate/Fe^{2+}-induced DCF fluorescence by about 6.4-, 2.9-, 2-, and 3.9-fold in the presence of eriodictyol (14 μM), quercetin (21 μM), luteolin (28 μM), and taxifolin (46 μM), respectively (Fig. 3). Moreover, quercetin, taxifolin, and particularly eriodictyol were shown to significantly decrease the fluorescence of DCF as compared to the control (Fig. 3), suggesting their capacity to reduce basal ROS production in cultured retinal cells.

3.3. Analysis of cell viability after incubation with ascorbate/Fe^{2+} and the flavonoids

Table 1 shows the percentage of LDH leakage, a measurement of retinal cell viability. After oxidation of the retinal cells or incubation with the flavonoids, the percentage of LDH release to the extracellular medium did not change significantly in comparison to control cells (4.39 ± 0.36% LDH), indicating the maintenance of the integrity of the plasma membrane. Therefore, no toxic effects were

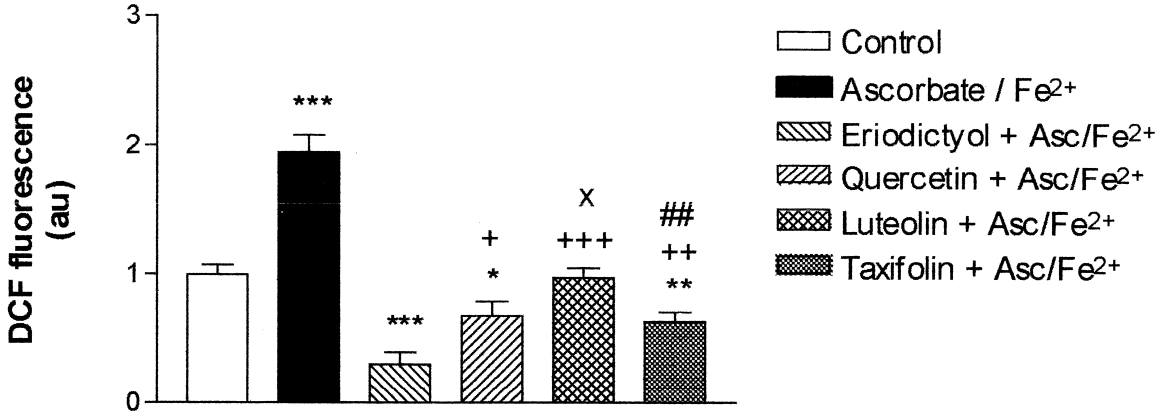

Fig. 3. Influence of the flavonoids on the production of intracellular ROS in retinal cells in culture. Retinal cells were preincubated with the flavonoids at the IC_{50} values determined in Fig. 2 (14 μM eriodictyol, 21 μM quercetin, 29 μM luteolin, and 46 μM taxifolin) for 2 hr in Na^+ medium, at pH 7.4 (37°), washed, and loaded with 20 μM $DCFH_2$ for 15 min in the dark. The cells were further exposed to 5 mM ascorbate/100 μM Fe^{2+} for 15 min. Control cells were incubated in the absence of the oxidant pair and the flavonoids. The results, expressed as arbitrary units (au) above the initial DCF fluorescence values, are the means ± SEM of duplicates from four independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to the control in the absence of ascorbate/Fe^{2+}. $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ as compared to eriodictyol. $^{x}P < 0.01$ as compared to quercetin. $^{\#\#}P < 0.01$ as compared to luteolin. The four flavonoids significantly inhibited ($P < 0.001$) the increase in DCF fluorescence induced by ascorbate/Fe^{2+}.

associated with ascorbate/Fe^{2+}-induced oxidation, as shown previously [29], or with the flavonoids.

3.4. Analysis of the degree of hydrophobicity of flavonoids

Because the antioxidant activity of flavonoids has been reported to be related to their capacity to interact and permeate the lipid bilayer [13,22], we also analysed the degree of hydrophobicity of these compounds. Table 2 shows the PC values for eriodictyol, quercetin, luteolin, and taxifolin by using the *n*-octanol/HEPES system at a temperature of 19 ± 1°. The results indicate the following order of affinity for the octanol phase: luteolin > quercetin > eriodictyol > taxifolin.

Because the PC values determined with octanol/HEPES may underestimate the partition of the molecules into the lipid bilayer, we also determined the Rf values for the four flavonoids by TLC to evaluate the interaction of the flavonoids with the biological membranes [28,30]. The Rf values obtained for eriodictyol, quercetin, luteolin, and taxifolin (Table 2) show that the relative affinity of the flavonoids for the silica, associated with the ability to establish hydrogen bonds, varies in the following order: taxifolin > luteolin > quercetin > eriodictyol.

Table 1
Analysis of retinal cell viability after ascorbate/Fe^{2+}-induced oxidative stress in the absence or presence of flavonoids

	LDH leakage (% of total)
Control	4.39 ± 0.36
Ascorbate/Fe^{2+}	5.43 ± 0.39
+Eriodictyol (14 μM)	3.66 ± 0.08
+Quercetin (21 μM)	4.64 ± 0.67
+Luteolin (29 μM)	3.20 ± 0.73
+Taxifolin (46 μM)	4.60 ± 0.35

The retinal cells were preincubated with the flavonoids, at the IC_{50} values, for 2 hr in Na^+ medium (37°). Then, the cells were rinsed and further incubated in the presence of ascorbate (5 mM)/Fe^{2+} (100 μM), pH 7.4, for 20 min. Control cells were incubated in the absence of the oxidant pair or the flavonoids. Cellular viability was determined by measuring the release of LDH to the extracellular medium. The results are the means ± SEM of triplicates from two independent experiments.

4. Discussion

Previous papers have described that flavonoids have the ability to form complexes with transition metal ions, causing them to behave either as antioxidants or pro-oxidants depending on the reaction conditions [5,31,32]. In this study, we show that 4 structurally related flavonoids, i.e. quercetin, luteolin, eriodictyol, and taxifolin, are efficient antioxidants against oxidative stress induced by ascorbate/Fe^{2+} in retinal cells in culture.

Table 2
Partition coefficient (PC) and retardation factor (Rf) values for eriodictyol, quercetin, luteolin, and taxifolin

Flavonoids	PC values	Rf values
Eriodictyol	1.65 ± 0.05	0.43
Quercetin	2.04 ± 0.04	0.34
Luteolin	2.30 ± 0.06	0.27
Taxifolin	0.79 ± 0.02	0.20

PC values were determined by the *n*-octanol/HEPES (20 mM, at pH 7.4) system, and Rf values were determined by TLC on silica gel plates using a non-polar elution system consisting of chloroform:ethyl acetate:acetic acid (16:8:1). The flavonoids were tested at a concentration of 200 μM. The results of PC values are the means ± SEM of duplicates from three independent determinations. All values were found to be statistically different ($P < 0.001$, ANOVA statistical analysis).

Eriodictyol, characterised by not having a double bond at C_2–C_3 or a hydroxyl group at C_3 (Fig. 1) and by having a decreased ability to establish hydrogen bonds (Table 2), was shown to be the most efficient antioxidant, followed by quercetin, luteolin, and taxifolin. The antioxidant activity of the flavonoids was determined by their capacity not only to decrease the extension of lipid peroxidation, but also to reduce the production of ROS induced by ascorbate/Fe^{2+}.

Lipid peroxidation has been described to cause gradual changes in membrane structure, ultimately leading to the loss of membrane function and integrity [33, for review]. The extent of cellular oxidation induced by 5 mM ascorbate/100 μM Fe^{2+} was considered moderate, because a significant increase in the formation of ROS and in the extent of lipid peroxidation was observed in cells exposed to the oxidant pair (Figs. 2 and 3) without significative changes in retinal cell viability (Table 1).

Lower IC_{50} values obtained in the presence of eriodictyol or quercetin as compared to luteolin or taxifolin suggest their greater efficiency as antioxidants. We also observed that the antioxidant activity of the flavonoids did not correlate with the presence of the double bond at C_2–C_3 and/or with the hydroxyl group at C_3 [order of antioxidant activity: eriodictyol > quercetin ($C_2 = C_3$; 3-OH) > luteolin ($C_2 = C_3$) > taxifolin (3-OH)], suggesting that the changes in chemical structure are a requirement for the antioxidant activity upon ascorbate/Fe^{2+}-induced oxidative stress in cultured retinal cells.

The antioxidant activity of a compound can depend either upon the free radical species generated and/or the oxidants used in a given system [5,34,35]. In a previous study, Rice-Evans *et al*. determined the radical-scavenging abilities of these flavonoids by measuring their ability to scavenge 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation and the activity order was: quercetin > luteolin > taxifolin > eriodictyol [36]. Major differences between our results and the data obtained by Rice-Evans *et al*. may be related to the use of a more complex system when using the retinal cells in culture. In another study, using H_2O_2–Cu^{2+} as the generator of hydroxyl radicals, Cao *et al*. showed that eriodictyol and taxifolin have a high antioxidant capacity as compared to quercetin and luteolin [5]. These results were explained on the basis of a direct interaction of the flavonoids with the transition metals, thereby affecting the rate of hydroxyl radical generation. In accordance, Acker *et al*. verified that taxifolin complexes more strongly with Fe^{2+} than luteolin or quercetin [10].

Thus, the antioxidant activity of the flavonoids has been described to result from a combination of iron chelation and free radical-scavenging activities [37]. Although the identification of these two processes is not easy, by comparing the antioxidant efficacy of the flavonoids after a preincubation, which mainly reflects their ability to permeate the plasma membrane, and the simultaneous incubation of the flavonoids with the oxidant pair, which accounts for a certain ability of the flavonoids to chelate iron in solution, we can partly discriminate the contribution of iron chelation. Taxifolin showed a lower antioxidant efficacy in inhibiting the extent of lipid peroxidation induced by ascorbate/Fe^{2+} as compared to eriodictyol (Fig. 2). In fact, taxifolin was shown to be the flavonoid that established the weakest hydrophobic interactions and the strongest hydrogen bonds (Table 2), suggesting that it had a lower ability to permeate the plasma membrane.

The incorporation of the flavonoids in cellular membranes is affected by electrostatic interactions, formation of hydrogen bonds with polar groups of the phospholipids, hydrophobic interactions with fatty acyl chains, and by the molecular geometry of the phospholipids [22,38]. Whereas in *n*-octanol/HEPES, hydrophobic interactions or van der Waals forces are the main factors involved in the separation of the flavonoids, according to their polarity, the preponderant factors in TLC on silica gel plates are the hydrogen bonds formed between the silica (SiO_2) and the flavonoids [27,28]. Thus, the order of affinity for the octanol phase (luteolin > quercetin > eriodictyol > taxifolin, Table 2) was different when compared to the order of affinity for the silica (taxifolin > luteolin > quercetin > eriodictyol, Table 2), suggesting that luteolin is the flavonoid that forms the strongest hydrophobic interaction with octanol, whereas taxifolin forms the strongest hydrogen bonds and least strong hydrophobic interactions. These differences may be explained by the planarity and electronic delocalisation of the molecules: luteolin or quercetin have a more planar structure than eriodictyol or taxifolin due to the presence or absence of the double bond at C_2–C_3 on the C ring [39]. Analysis of our data strongly suggests that the order of antioxidant efficacy of the flavonoids in the retinal cells is closely related to the Rf values (eriodictyol > quercetin > luteolin > taxifolin), i.e. an increased antioxidant efficiency is in the inverse order to their ability to establish hydrogen bonds. Furthermore, the higher antioxidant activity of eriodictyol can also result from its non-planar structure, which confers a higher flexibility to conformational changes, according to Arora *et al*. [40] and a higher permeation through the plasma membrane, as compared to the more rigid structure of quercetin or luteolin.

The extent of oxidative stress was also determined by following the changes in fluorescence of DCF in the presence of $DCFH_2$, a measurement of intracellular ROS production. Ascorbate/Fe^{2+} significantly increased the formation of intracellular free radicals in the retinal cells, as observed previously [25]. A high inhibition in the formation of intracellular ROS was observed after incubation in the presence of eriodictyol (14 μM), followed by taxifolin (46 μM), quercetin (21 μM), and luteolin (28 μM) (Fig. 3). Although the order of antioxidant activity as determined by DCF fluorescence (eriodictyol > quercetin > luteolin) was in accordance with the inhibition of TBARS, the observation that taxifolin had a higher antioxidant activity than luteolin was probably due to a higher hydrophobic interaction of luteolin with the plasma membrane, as evaluated by

the PC and Rf values (Table 2). Our results are in accordance with Oyama *et al*., who showed that quercetin and other flavonoids can reduce the oxidation of DCFH in both resting (basal conditions) and Ca^{2+}-loaded brain neurons [41]. Although the effects of the flavonoids in reducing DCF fluorescence were mainly associated with a decrease in intracellular ROS, we cannot exclude the possibility that the flavonoids can scavenge the DCF semiquinone free radical intermediate (oxygen radical) produced during the formation of the fluorescent product DCF.

In conclusion, eriodictyol was shown to be the most efficient flavonoid in inhibiting the oxidative stress induced by ascorbate/Fe^{2+}. The higher antioxidant activity of eriodictyol observed in our study may be associated with its capacity to inhibit the production of hydroxyl radicals in Fenton reaction and membrane permeability in retinal cells in culture [5,12]. This observation implicates eriodictyol as a highly potent neuroprotector in pathological conditions, in which the generation of free radical species is involved. These data may be relevant to establish natural flavonoids as potent antioxidants against oxidative stress-mediated cell injury associated with neuropathological conditions.

Acknowledgments

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