Dietary flavonoids as intracellular substrates for an erythrocyte trans-plasma membrane oxidoreductase activity

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The plasma membrane oxidoreductase (PMOR) activity, which mainly utilises ascorbate as intracellular electron donor, represents a major mechanism for cell-dependent reduction of extracellular oxidants and might be an important process used by the erythrocytes to keep a reduced plasma environment. We previously reported that in human erythrocytes, myricetin and quercetin act as intracellular substrates of a PMOR showing a novel mechanism whereby these flavonoids could exert beneficial effects under oxidative stress conditions. Here, we evaluated the ability of different flavonoids (quercetin, myricetin, morin, kaempferol, fisetin, catechin, luteolin, apigenin, acacetin, rutin, taxifolin, naringenin, genistein) and of two *in vivo* O-methylated metabolites of quercetin (isorhamnetin and tamarixetin) to be substrates of PMOR, by comparing their antioxidant capacity (i.e. direct interaction with the oxidant ferricyanide or with the free radical 1,1-diphenyl-2-picryl-hydrazil) with their ability to penetrate the erythrocytes and donate electrons to the PMOR. The results obtained indicate that, although most of the flavonoids display significant antioxidant activities, only those (quercetin, myricetin, fisetin) that combine the cathecol structure of the B ring (responsible for the reducing activity) with the 2,3 double bond and 4-oxo function of the C ring (responsible for the uptake by erythrocytes) can act as intracellular substrates for PMOR. It is of note that the metabolites of quercetin enter erythrocytes and donate electrons to the PMOR as the parent compound. The present data show a relationship between the flavonoid structures and their ability to provide electrons to the PMOR, suggesting an additional mechanism whereby dietary flavonoids may exert beneficial effects in man.

Flavonoids: Trans-plasma membrane oxidoreductase: Ferricyanide: 1,1-Diphenyl-2-picryl-hydrazil

It is now well established that diets rich in fruits and vegetables are protective against the oxidative effects of reactive oxygen species, which are formed *in vivo* during the cellular aerobic metabolism and can cause damage to various cellular components such as DNA, proteins, lipids, etc (Steinmetz & Potter, 1991*a,b*; Keli *et al.* 1996; Ness & Powles, 1997; Ross & Kasum, 2002). Despite the cells being well equipped with antioxidant defence systems, the accumulation of unrepaired products may be critical to the development of several important pathologies.

Flavonoids are polyphenol compounds, widely distributed in plant foods, which may exert beneficial effects in various diseases, including cancer, CVD and neurodegenerative disorders (Steinmetz & Potter, 1991*a,b*; Richter *et al.* 1999). Many of the biological actions of flavonoids have been attributed to their antioxidant properties (Afanas'ev *et al.* 1989; Bors *et al.* 1990; Rice-Evans *et al.* 1997); more recently, it has been proposed that flavonoids and their metabolites may exert their effects by displaying modulatory actions in cells (Williams *et al.* 2004).

We have previously reported that flavonoids quercetin and myricetin are efficiently taken up by human erythrocytes and can act as substrates for the plasma membrane oxidoreductase (PMOR) activity, suggesting another mechanism whereby flavonoids can exert their protective effects (Fiorani *et al.* 2002). Indeed, this enzyme activity represents an important means to defend the cells against extracellular oxidative stressors (May

et al. 1996), and since the erythrocytes are constantly exposed to oxidative stress, it might have a major role in maintaining a reduced plasma environment (Kennett & Kuchel, 2003).

The aim of the present study was to evaluate the ability of several flavonoids (Fig. 1), commonly present in fruits and vegetables (flavonols: quercetin, myricetin, morin, kaempferol, fisetin; flavanol: catechin; flavones: luteolin, apigenin, acacetin, rutin; flavanones: taxifolin and naringenin; isoflavone: genistein) and of two reported *in vivo* quercetin metabolites, isorhamnetin and tamarixetin (Spencer *et al.* 2003*a*), to interact with human erythrocyte plasma membrane and induce extracellular reduction of oxidants.

Materials and methods

Materials

Flavonoids, 1,10-phenanthroline, ethyl acetate and 1,1-diphenyl-2-picryl-hydrazil (DPPH·) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Isorhamnetin and tamarixetin were purchased from Extrasynthese (Z.I. Lyon Nord, Geney, France).

$K_3Fe(CN)_6$, $FeCl_3$, citric acid, NaH_2PO_4 and acetonitrile (HPLC grade) were Carlo Erba products (Milan, Italy).
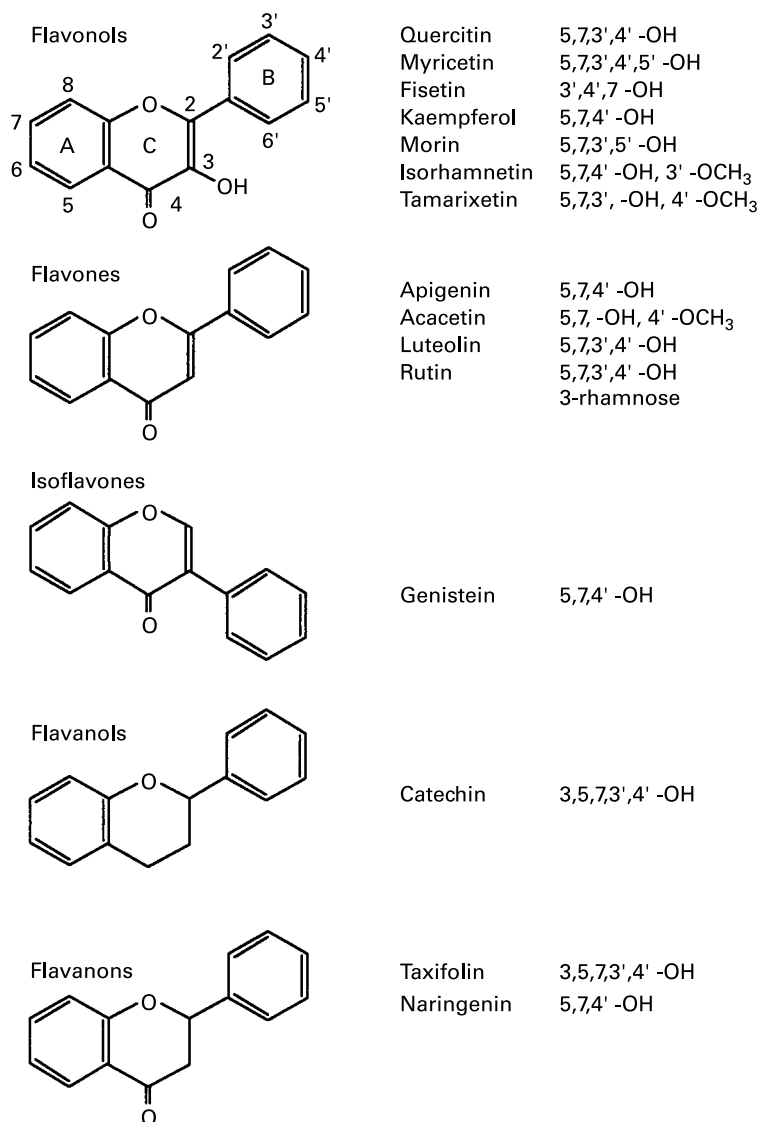
Flavonols		
Quercitin	5,7,3',4' -OH	
Myricetin	5,7,3',4',5' -OH	
Fisetin	3',4',7 -OH	
Kaempferol	5,7,4' -OH	
Morin	5,7,3',5' -OH	
Isorhamnetin	5,7,4' -OH, 3' -OCH₃	
Tamarixetin	5,7,3', -OH, 4' -OCH₃	

Isorhamnetin 5,7,4' -OH, 3' -OCH$_3$
Tamarixetin 5,7,3', -OH, 4' -OCH$_3$

Flavones
Apigenin 5,7,4' -OH
Acacetin 5,7, -OH, 4' -OCH$_3$
Luteolin 5,7,3',4' -OH
Rutin 5,7,3',4' -OH
3-rhamnose

Isoflavones
Genistein 5,7,4' -OH

Flavanols
Catechin 3,5,7,3',4' -OH

Flavanons
Taxifolin 3,5,7,3',4' -OH
Naringenin 5,7,4' -OH

Fig. 1. Structures of the flavonoids.

Methods

Measurement of chemical ferricyanide reduction by different flavonoids. To a 1 mM-ferricyanide (FIC) solution in PBS (pH 7·4) were added different amounts of the flavonoid solution (final concentrations 2·5–500 μM). After standing for 30 min at 37°C (a time long enough to reach the reaction thermodynamic equilibrium), the ferrocyanide (FOC) formation was measured as reported by Avron & Shavit (1963), using 1,10-phenanthroline as an indicator and measuring absorption at 510 nm ($\varepsilon = 10\,500/$ M per cm). The percentage of FOC formation (% of reduction of the 1 mM-FIC solution) was plotted against the sample concentration to obtain the EC_{50}, defined as the flavonoid concentration required to obtain 50 % of the maximal FIC-reducing activity.

Measurement of scavenging activity on 1,1-diphenyl-2-picryl-hydrazil. The free radical-scavenging activity of flavonoids against the DPPH free radical was measured using a modified version of the method of Mallors & Tappel (1966). Briefly, to 0·850 ml of 100 μM-DPPH ethanolic solution were added different amounts of flavonoid solutions in ethanol (final concentration 1–150 μM) in 1 ml cuvettes. After standing in the dark for 10 min (a time long enough to reach the reaction thermodynamic equilibrium), the absorbance (Abs) at 517 nm was measured. Controls containing ethanol instead of the flavonoid solution and blanks containing ethanol instead of DPPH solution were also made. The DPPH-scavenging activity was calculated according to the following formula: DPPH scavenging activity (%) = $(Abs_{control} - Abs_{sample})/Abs_{control} \times 100$. The percentage of scavenging activity was plotted against the sample concentrations to obtain the EC_{50}, defined as the flavonoid concentration required to obtain 50 % of the maximal scavenging activity.

Human erythrocytes. Human venous blood (in heparin) from healthy volunteers was obtained by venepuncture. The erythrocytes were used immediately after sampling. The blood was centrifuged at 1861·5 g for 10 min at 4°C. After removal of plasma, buffy coat, and the upper 15 % of the packed erythrocytes, the erythrocytes were washed twice with cold PBS (150 mM-NaCl, 5 mM-Na$_2$HPO$_4$, in deionised water, adjusted to pH 7·4) and then re-suspended as described below.

Incubation of human erythrocytes with flavonoids. A stock solution (20 mM) of each flavonoid was prepared in dimethyl

sulfoxide and then diluted 1:2 with PBS. Packed erythrocytes (10 %, v/v) were incubated in PBS at 37°C for 10 min in the presence of the flavonoids (flavonols: quercetin, myricetin, morin, kaempferol, fisetin; flavanol: catechin; flavones: luteolin, apigenin, acacetin, rutin; flavanones: taxifolin and naringenin; isoflavone: genistein) and of two reported *in vivo* quercetin metabolites, isorhamnetin and tamarixetin. After this time, the suspensions were immediately centrifuged at 1861·5 *g*, the erythrocytes were washed twice with at least 50 vol. of PBS and then processed as reported below.

Measurement of ferricyanide reduction by human erythrocytes. FIC reduction was estimated as reported by Avron & Shavit (1963). After exposure to the flavonoid, erythrocytes were washed twice with PBS and re-suspended (10 %, v/v) in PBS + 2·5 mM-adenosine containing 1 mM-FIC (potassium salt), dissolved immediately before use. The suspensions were incubated for 30 min at 37°C and then centrifuged at 1861·5 *g* at 4°C. The resulting supernatant fractions were assayed for their FOC content using 1,10-phenanthroline as an indicator and measuring absorption at 510 nm ($\varepsilon = 10\ 500$/M per cm).

Extracellular and intracellular content of flavonoids. The extracellular and intracellular content of flavonoids was measured as described by Ferrali *et al.* (1997), with slight modifications, by performing ethyl acetate extractions. To measure the extracellular concentration of flavonoids, the supernatant fraction obtained at the end of the incubation time was extracted three times with ethyl acetate. For the assessment of intracellular flavonoid content the erythrocyte lysates were extracted three times with ethyl acetate. The absorbance of the clear upper phase was measured spectrophotometrically at the wavelength corresponding to the maximal absorption spectrum (morin 380 nm; quercetin, myricetin, isorhamnetin, tamarixetin, kaempferol 378 nm; fisetin 370 nm; rutin 340 nm; apigenin 350 nm; luteolin 336 nm; acacetin, genistein 330 nm; taxifolin 325 nm; naringenin 320 nm; catechin 285 nm). The extra- and intracellular concentrations of the flavonoids were obtained from the corresponding calibration curve, performed in ethyl acetate (morin, $\varepsilon_{380} = 8300$/M per cm; quercetin, $\varepsilon_{378} = 26000$/M per cm; myricetin, $\varepsilon_{378} = 18900$/M per cm; isorhamnetin, $\varepsilon\varepsilon_{378} = 48700$/M per cm; tamarixetin, $\varepsilon_{378} = 39300$/M per cm; kaempferol, $\varepsilon_{378} = 22500$/M per cm; fisetin, $\varepsilon_{370} = 22700$/M per cm; apigenin, $\varepsilon_{350} = 15200$/M per cm; luteolin, $\varepsilon_{336} = 68900$/M per cm; acacetin, $\varepsilon_{330} = 16600$/M per cm; genistein, $\varepsilon_{330} = 2500$/M per cm; taxifolin, $\varepsilon_{325} = 3200$/M per cm; naringenin, $\varepsilon_{320} = 2200$/M per cm; catechin $\varepsilon_{285} = 4·700$/M per cm). In all experiments, ethyl acetate extraction of a control sample was performed (either erythrocyte or supernatant fraction), in order to look for possible interferences with erythrocyte constituents. The results obtained showed that the absorbance values of the erythrocyte control samples were identical to those of an ethyl acetate solution.

The percentage of haemolysis was evaluated in the same samples by measuring the Hb concentration *v.* the total Hb content. The extent of lysis was not different from controls and never higher than 0·5 %.

High-performance liquid chromatography analysis of quercetin and its methylated metabolites. After incubation of the human erythrocyte suspensions with 50 μM-quercetin, tamarixetin or isorhamnetin as described earlier, the samples were centrifuged at 1861·5 *g* for 5 min and the supernatant fractions were collected. The packed erythrocytes were then extensively washed with PBS and lysed with cold bi-distilled water. Both samples

(extracellular milieu and erythrocyte lysate) were extracted three times with ethyl acetate. All the samples were taken to dryness by rotary evaporation and re-dissolved in dimethyl sulfoxide and diluted with bi-distilled water just before HPLC analysis. HPLC analysis of quercetin and its methylated derivatives was performed by using a 25 × 4·6 mm Discovery C18 (5 μm; Supelco, Bellefonte, PA, USA) equipped with a Supelguard Discovery C-18 guard column (2 cm × 4 mm, 5 μm). A modified version of the analytical HPLC method from Day *et al.* (2000) was used. Solvent A (0·1 % trifluoroacetic acid) and B (acetonitrile) were run at a flow rate of 1 ml/min. The running gradient was adjusted to 17 % B (2 min), increasing to 25 % B (5 min), 35 % B (8 min), 50 % B (5 min) and then 100 % B (10 min), followed by a re-equilibration at 17 % B (15 min). The injection volume was 100 μl and the eluate was monitored at 270 nm.

Results and discussion

In vitro *ferricyanide-reducing activity*

The direct interaction of the various flavonoids (belonging to the family of flavonol, flavanol, flavone, flavanone and isoflavone) with an oxidant was investigated by monitoring the formation of its reduction product. For this purpose, increasing concentrations (2·5 to 500 μM) of the flavonoids were incubated in PBS containing 1 mM-FIC and FOC formation was detected spectrophotometrically after 30 min. As reported in Fig. 2, quercetin and myricetin, followed by taxifolin, rutin and fisetin, displayed a high FIC-reducing activity.

These compounds are characterised by the presence of a B-ring catechol group (dihydroxylated B-ring) capable of readily donating hydrogen (i.e. electron). The substitution of 3′-OH or 4′-OH of quercetin with a methyl group, as in isorhamnetin or tamarixetin, respectively, only slightly decreased the FIC-reducing activity. A somewhat greater decrease was noticed by changing
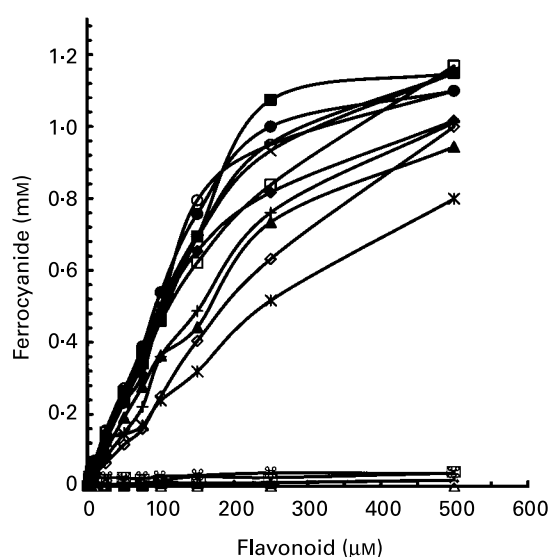

Fig. 2. Ferricyanide reduction by various flavonoids. Ferricyanide (1 mM) in PBS (pH 7·4) was incubated for 30 min at 37°C with the indicated concentrations of quercetin (○), myricetin (●), fisetin (□), luteolin (▲), isorhamnetin (■), taxifolin (•), kaempferol (×), tamarixetin (◆), morin (+), catechin (✳), rutin (−), apigenin (△), acacetin (⌘), genistein (❖) and naringenin (⊠). Then, ferrocyanide formation was assayed (for details, see p. 339).

the 3′,4′ orthodihydroxy arrangement of the B ring to 3′,5′ meta-dihydroxy, as in morin. Kaempferol, a flavonol identical to quercetin, but having no hydroxyl group at the 3′ position in the B ring, displayed an even lower activity. The 2,3 double bond in the C ring does not appear to play a major role for FIC-reducing activity, since taxifolin displayed remarkably similar effects to quercetin. However, catechin, which differs from taxifolin lacking the 4-oxo group in the C ring, showed a strong decrease in FIC-reducing activity. The substitution of 3-OH of quercetin with 3-O-rutinoside, as in rutin, decreased the reducing activity by about 10 %, whereas a 50 % reduction was observed upon dehydroxylation in the 3-position, as in luteolin. Finally, dehydroxylation at both the 3- and 3′-positions led to a complete loss of activity, as in apigenin, acacetin (4′-methoxyapigenin) and naringenin, the latter lacking 2,3 double bonds as well. The isoflavone genistein was also inactive.

Table 1 reports the EC_{50} values as calculated from the curves illustrated in Fig. 2.

Taken together, these results emphasise the relevance of the catechol-like structure of the B ring in association with the 3-OH group on the expression of maximal FIC-reducing activity, and show that the substitution of 3-OH with 3-O-rutinoside as in rutin, or the methylation of 3′ or 4′-OH as in isorhamnetin or tamarixetin only slightly reduce the antioxidant activity of these compounds.

It is of note that the very high reducing activities displayed by most flavonoids tested have also been observed by other authors. As reported by Cao *et al.* (1997) the flavonoids that contain multiple OH substitutions (i.e. myricetin, quercetin, luteolin, etc) showed peroxyl radical-scavenging activity several times higher than Trolox, an α-tocopherol analogue. They reported that the stoichiometric factor (i.e. the number of peroxyl radicals trapped per molecule of antioxidant) of these flavonoids is about 6–9, whereas the stoichiometric factor of Trolox is 2. Moreover, other authors (Firuzi *et al.* 2005) evaluated the antioxidant activities of flavonoids by 'ferric-reducing antioxidant power' measurement. They showed that quercetin, fisetin and myricetin appeared the most active compounds in the ferric-reducing antioxidant power assay and they were 3·02, 2·52 and 2·28 times more active than Trolox, respectively.

1,1-Diphenyl-2-picryl-hydrazil scavenging activity

As a further approach to estimate the relative potency of the tested flavonoids in reactions involving electron donation, we used the DPPH˙ assay, widely employed for the assessment of antioxidant activity. DPPH˙, a stable organic free radical, displays a characteristic absorption maximum between 515 and 517 nm that diminishes in the presence of compounds reducing it to its hydrazine form by hydrogen–electron donation. The different kinetic behaviour of antioxidants is an important factor in the evaluation of the radical-scavenging activity (Bandonienè & Murkovic, 2002). Fig. 3 (EC_{50} values are shown in Table 1) illustrates the scavenging activity of the tested flavonoids after a 10 min incubation in ethanol in the presence of DPPH˙ (100 μM). Complete conversion of DPPH˙ to its hydrazine derivative was observed at much lower concentrations of the flavonoids than those necessary to fully reduce FIC, an event probably ascribable to the different concentration of the oxidising substrate employed in the two experimental conditions. The results obtained were nevertheless similar to those previously described for the FIC-reduction assay, with some important exceptions. Quercetin, myricetin and fisetin most effectively scavenged the DPPH˙ radical, confirming previous results showing that the scavenging activity of flavonoids is related to the presence of (i) a 3′- 4′ dihydroxylated B ring, (ii) the 2,3-double bond in conjugation with a 4-oxo function in the C ring and finally (iii) the additional presence of both 3- and 5- hydroxyl groups (Williams *et al.* 2004). It should be noted, however, that in contrast with the results obtained measuring FIC reduction, the substitution of 3′ or 4′-OH with a methyl group (for example, isorhamnetin or tamarixetin) or 3-OH with 3-O-rutinoside (for example, rutin) substantially reduced the scavenging activity. The remaining compounds, either inactive or poorly active in the FIC assay, produced similar outcomes in the DPPH˙ assay.

Human erythrocyte ferricyanide-reducing activity promoted by different flavonoids

FIC is a mild oxidant that does not cross the cell membrane and accepts electrons from the PMOR activity (Himmelreich & Kuchel, 1997; van Duijn *et al.* 1998). Although the physiological electron donors for this enzyme are ascorbic acid and NADH (May, 1999; May *et al.* 1999), our recent findings indicate that quercetin and myricetin are also potent electron donors for the PMOR in human erythrocytes (Fiorani *et al.* 2002, 2003). Experiments were therefore performed to determine the potency of the tested flavonoids in supporting the ability of human

Table 1. Flavonoid concentrations required to obtain 50 % of the maximal effect (EC_{50} values) concerning ferricyanide reduction and 1,1-diphenyl-2-picryl-hydrazil (DPPH˙)-scavenging activity*

Flavonoid	EC_{50} ferricyanide reduction (μm)	EC_{50} DPPH˙-scavenging activity (μm)
Quercetin	96·5	6·23
Myricetin	97·3	6·37
Fisetin	114·8	6·75
Rutin	116·1	9·67
Luteolin	160·0	12·79
Isorhamnetin	108·0	12·94
Taxifolin	118·3	13·83
Kaempferol	197·1	18·4
Catechin	282·6	18·55
Tamarixetin	112·0	18·56
Morin	159·4	27·23

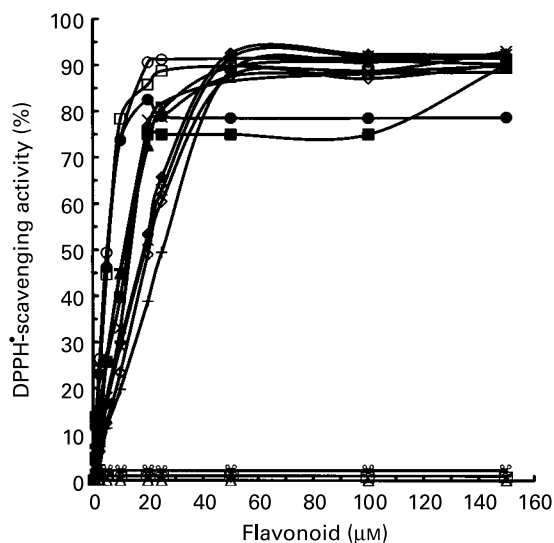* The values have been calculated from the curves reported in Figs. 2 and 3 respectively.

Fig. 3. 1,1-Diphenyl-2-picryl-hydrazil (DPPH˙)-scavenging activity by various flavonoids. DPPH˙ (100 μM) in ethanol was incubated for 10 min at 37°C with the indicated concentrations of quercetin (○), myricetin (●), fisetin (□), luteolin (▲), isorhamnetin (■), taxifolin (×), kaempferol (◇), tamarixetin (◆), morin (+), catechin (✳), rutin (−), apigenin (△), acacetin (⌘), genistein (✿) and naringenin (⊠) dissolved in ethanol. The decrease of the absorbance at 517 nm was then measured and the scavenging activity was expressed as percentage of the absorbance of the control DPPH˙ solution.

erythrocytes to reduce extracellular oxidants. For this purpose, erythrocytes were first incubated for 10 min with a 50 μM concentration of each flavonoid, centrifuged and extensively rinsed, resuspended in PBS supplemented with adenosine and 1 mM-FIC and finally incubated for 30 min at 37°C. Formation of FOC was subsequently measured in the supernatant fraction. It is important to note that this response, as previously shown for quercetin (Fiorani *et al.* 2002), is mediated by the activity of the PMOR and not by the flavonoids released from the cells. For this purpose, erythrocytes were incubated with 50 μM-flavonoids (listed earlier; p. 339) for 10 min, washed twice with at least 50 vol. of PBS and then incubated (10 %, v/v) with PBS plus adenosine. After 30 min, the erythrocyte suspensions were centrifuged and flavonoid content was assayed either in the supernatant fractions and in erythrocytes after ethyl acetate extractions, as detailed earlier (p. 340). The results obtained consistently demonstrated that none of the flavonoids was significantly released during post-treatment incubations (not shown; Fiorani *et al.* 2002). As illustrated in Fig. 4, quercetin and myricetin, followed by fisetin, isorhamnetin and tamarixetin were the most potent compounds in promoting the PMOR activity. Kaempferol, luteolin, taxifolin and morin were significantly less effective and the remaining flavonoids were inactive. For a correct interpretation of these results the cellular uptake of the different flavonoids was next assessed under equal loading conditions. Fig. 5 shows that quercetin, myricetin, kaempferol, fisetin, morin, luteolin, isorhamnetin, tamarixetin, apigenin and acacetin, were efficiently taken up by the erythrocytes and actually accumulated at levels above the initial extracellular loading concentration. The uptake of naringenin, genistein, taxifolin and finally catechin was remarkably lower and that of rutin was virtually undetectable.

Thus, all compounds displaying significant FIC-reducing activity (Fig. 2), and efficiently taken up by the erythrocytes
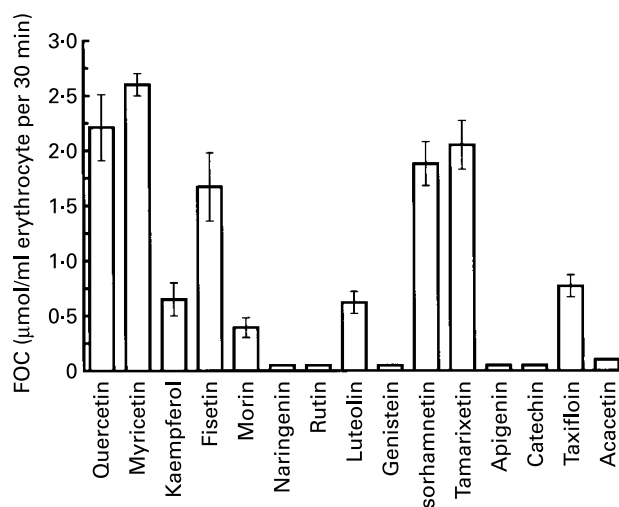

Fig. 4. Effect of various flavonoids on the rate of ferricyanide (FIC) reduction in human erythrocytes. Human packed erythrocytes were incubated for 10 min at 37°C in PBS (10 %, v/v) in the presence of 50 μM-flavonoid. After centrifugation the cells were washed twice with PBS and the packed erythrocytes were re-suspended in PBS (10 %, v/v) plus 2·5 mM-adenosine containing 1 mM-FIC. After 30 min of incubation at 37°C, the cell suspensions were centrifuged and the ferrocyanide (FOC) content was assayed as detailed on p. 340. Flavonoid-dependent FIC-reducing activity was determined upon subtraction of basal FIC-reducing activity detected in the untreated samples. All data are the means of at least three independent determinations, with standard deviations represented by vertical bars.

(Fig. 5), act in parallel as intracellular substrates for the PMOR activity. Examples are quercetin, myricetin, fisetin as well as the *in vivo* plasma metabolites of quercetin, isorhamnetin and tamarixetin. It is important to point out that the two latter compounds were not converted within the erythrocytes to their de-
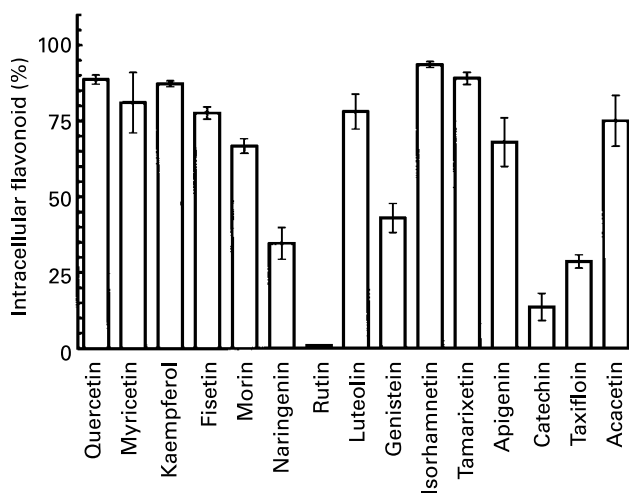

Fig. 5. Flavonoid uptake by human erythrocytes. Human packed erythrocytes were incubated for 10 min at 37°C in PBS (10 %, v/v) in presence of 50 μM-flavonoid. After centrifugation, the cells were washed twice with at least 50 vol. of PBS, then lysed by re-suspending with 3 vol. of cold distilled water and maintained 10 min at 4°C. Flavonoids were extracted from either the haemolysate or supernatant fraction with ethyl acetate, as described on p. 340. All data are the means of at least three independent determinations, with standard deviations represented by vertical bars. The data are expressed as percentage of the flavonoid recovered in erythrocytes with regard to the total flavonoid content (extra + intracellular concentration).
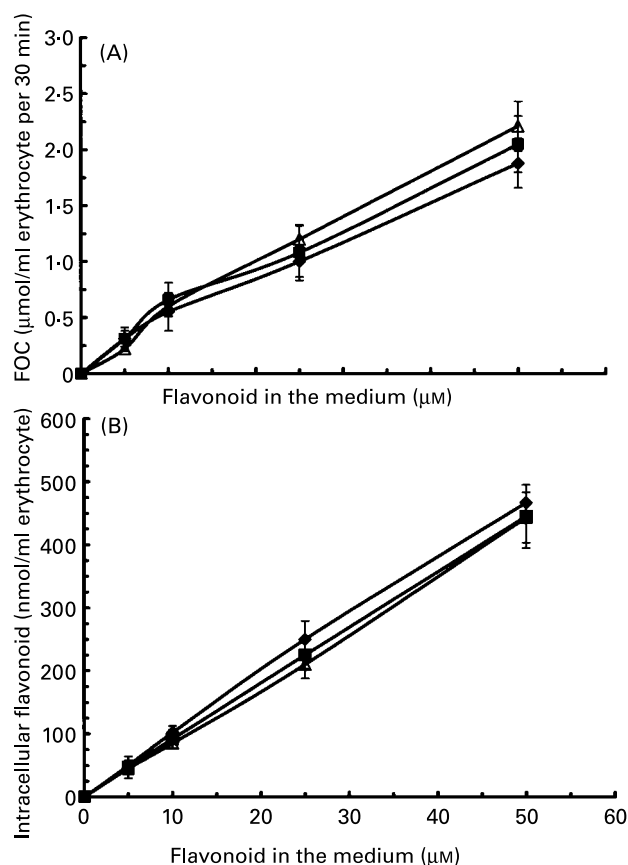
Fig. 6. Ferricyanide (FIC)-reducing activity (A) and flavonoid uptake (B) by human erythrocytes. Human packed erythrocytes were incubated for 10 min at 37°C in PBS (10 %, v/v) with 50 μM-flavonoid (isorhamnetin (■); tamarixetin (◆); quercetin (△)). After centrifugation the cells were washed twice with PBS and the packed erythrocytes were: (A) re-suspended at 10 % packed cell volume in PBS plus 2·5 mM-adenosine containing 1 mM-FIC and the ferrocyanide (FOC) formation was assayed as reported in the legend of Fig. 4, or (B) lysed by re-suspending with 3 vol. of cold distilled water and the flavonoid content was measured as described in the legend of Fig. 5. Flavonoid-dependent FIC-reducing activity was determined upon subtraction of basal FIC-reducing activity detected in the untreated samples. All data are the means of at least three independent determinations, with standard deviations represented by vertical bars.

methylated form (for example, to quercetin), an event that would explain the identical abilities to reduce extracellular oxidants mediated by the PMOR activity. This notion is clearly established by the HPLC analysis, which revealed that only isorhamnetin or tamarixetin are recovered inside the cell after exposure of erythrocytes to the methylated derivatives.

Fig. 6 (A) shows concentration-dependence curves of the FIC-reducing activity of isorhamnetin, tamarixetin and quercetin by human erythrocytes. As illustrated, the two methylated metabolites are able to act as intracellular substrates of the PMOR already at concentrations ≤ 5 μM with a rate quite similar to that of quercetin. Similarly, the intracellular uptake of these compounds was substantially identical (Fig. 6 (B)).

Additional flavonoids, namely kaempferol, morin and luteolin, were also efficiently taken up by the erythrocytes (Fig. 5) but displayed poor direct (Fig. 2) or cell-mediated (Fig. 4) FIC reduction. Taxifolin was less efficiently taken up by the erythrocytes (Fig. 5) and, while more effective than kaempferol and luteolin in reducing FIC (Fig. 2), caused a similar erythrocyte-dependent reduction of extracellular oxidants (Fig. 4). Although

apigenin, acacetin, genistein and naringenin were accumulated in the erythrocytes in fairly high amounts (Fig. 5), these compounds were unable to reduce FIC chemically (Fig. 2) and in the cell-mediated assay (Fig. 4). Rutin was similar to quercetin in promoting chemical reduction of FIC (Fig. 2) but was not taken up by the erythrocytes (Fig. 5) and therefore unable to behave as intracellular substrates for the PMOR activity (Fig. 4). Finally, catechin did not permeate the cell membrane (Fig. 5) and thus was inactive in the cell-mediated assay (Fig. 4), while it was poorly effective in the chemical FIC-reduction assay (Fig. 2). It is of note that taxifolin, which shows a chemical FIC-reducing activity similar to that of quercetin (Fig. 2), contributes to a lesser extent to the PMOR activity (Fig. 4). This fact is quite probably linked to the poor uptake of taxifolin by human erythrocytes (Fig. 5). This flavonoid, as already mentioned, differs from quercetin at the 2,3 position, lacking in the double bond. Naringenin, which, as taxifolin, lacks a 2,3 double bond, penetrates the erythrocyte membrane with a percentage similar to taxifolin. However, naringenin does not display chemical FIC-reduction activity or DPPH·-scavenging ability. This fact is probably linked to the lack of 3- and 3′-hydroxyl groups. Catechin, which does not have either the 2,3 double bond or a 4-carbonyl group, penetrates the erythrocyte membrane in very small amounts.

Therefore, it could be attributed to the 2,3 double bond and to the 4-carbonyl group ring a pivotal role on flavonoid uptake by erythrocytes.

Kaempferol and morin, although efficiently taken up by erythrocytes, were remarkably less effective than quercetin in promoting reduction of extracellular FIC. This observation confirms the previous results, emphasising the notion that the cathecol plays a pivotal role in the biological response mentioned earlier.

In conclusion, in the present study the *in vitro* evidence of antioxidant capacity of various polyphenolic compounds was related with that obtained from a biological system represented by human erythrocytes. The results obtained show that the flavonoids, which possess the catechol structure in the B ring (responsible for the reducing activity), in conjunction with a 2,3 double bond and 4-oxo function in the C ring (which favour the uptake of flavonoid by erythrocytes) appear to be the most potent agents in acting as electron donors to the PMOR activity. On the other hand, the role of quinone and quinone methide should be also considered, oxidation products of quercetin (Awad *et al.* 2000, 2001), which may display cytotoxic effects (MacGregor & Jurd, 1978; Sahu & Washington, 1991; Bolton *et al.* 1998; Penning *et al.* 1999). However, according to the literature (Cao *et al.* 1997; Lee *et al.* 2003) the pro-oxidant activities of natural antioxidants are unlikely to be a significant problem 'in vivo', and most of the studies show that flavonoids exert beneficial effects against pathological conditions such as CVD, cancer (Steinmetz & Potter, 1991*a,b*) and neurodegenerative disorders. Therefore, diets rich in these phenolic compounds are now strongly recommended. However, some doubts on the physiological relevance of these results arise from the bioavailability of these compounds. In fact, it should be underlined that plasma concentrations reached after flavonoid consumption vary highly according to the nature of the polyphenol and to the food source. Data reported in the literature show that the plasma concentrations of total metabolites can vary from 0 to 4 μmol/l after consumption of 50 mg aglycone equivalents, and the relative urinary excretion ranged from 0·3 to 43 % of the ingested dose, depending on polyphenolic compounds (Manach *et al.* 2005).

Moreover, as pointed out by Kroon *et al.* (2004), most of the dietary polyphenols undergo extensive modifications during transfer across the small intestine and then again in the liver, so that the forms reaching the blood and tissues are in general neither aglycones (except for green tea catechins) nor the various glycosides as the dietary source (Kroon *et al.* 2004). Circulating glucuronides, sulfates, and *O*-methylated forms are believed to be those most likely to exert bioactivity and express beneficial effects in human subjects and animals (Spencer *et al.* 2001*a,b*, 2003*b*, 2004; Guglielmone *et al.* 2002; Schroeter *et al.* 2003). It should be considered, however, as pointed out by Spencer *et al.* (2004), that there is the possibility that both flavonoid and *O*-methylated flavonoid glucuronides may be de-conjugated by the action of β-glucuronidases present in human tissues such as liver or small intestine or during local conditions of inflammation. In this case, free aglycone or *O*-methylated forms will be released and may go on to express cellular effects. Indeed, glucuronidases are present in a number of tissues within the body (Tukey *et al.* 2000) and may be released by certain cells.

In the bloodstream, erythrocytes encounter a variety of oxidant stressors which can be both endogenous, from cellular generation of superoxide and H_2O_2 (van Dyke & Saltman, 1996) and exogenous in areas of inflammation. Therefore, the PMOR activity in erythrocytes can play a protective role by reducing extracellular oxidants. The fact that specific flavonoids, abundantly present in fruits and vegetables as well as their plasma metabolites, act as substrates for this membrane enzyme provides an efficient way to maintain a redox state in the plasma, supporting the statement of the beneficial effects of dietary flavonoids.

Acknowledgements

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