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Inhibition of free radical initiated peroxidation of human erythrocyte ghosts by flavonols and their glycosides

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Received 2nd February 2004, Accepted 17th March 2004
First published as an Advance Article on the web 13th April 2004

The *in vitro* peroxidation of human erythrocyte ghosts was used as a model to study the free radical-induced damage of biological membranes and the protective effect of flavonols and their glycosides, *i.e*., quercetin (Q), quercetin galactopyranoside (QG), quercetin rhamnopyranoside (QR), rutin (R), morin (MO), kaempferol (K) and kaempferol glucoside (KG). The peroxidation was initiated by a water-soluble free radical initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPAD) at physiological temperature, and monitored by oxygen uptake. Kinetic analysis of the peroxidation process demonstrates that these flavonols and their glycosides are effective antioxidants against AMPAD-initiated oxidative damage of human erythrocyte ghosts, and that the flavonols bearing *ortho*-dihydroxyl groups possess significantly higher antioxidant activity than those bearing no such functionalities and the glycosides are less active than their parent aglycones.

Introduction

Epidemiological and biological studies have provided various lines of evidences that free radical induced oxidative damage of cell membranes, DNA and proteins might play a causative role in aging and several degenerative diseases, such as cancer, atherosclerosis and cataracts.[1–4] Therefore, inhibition of oxidative damage by supplementation of antioxidants becomes an attractive therapeutic strategy to reduce the risk of these diseases.[5–7] Flavonoids, including flavones, flavanone, flavonols, flavanols and isoflavones, are polyphenolic compounds which are widespread in foods and beverages and possess a wide range of biological activities,[8] of which antioxidation has been extensively explored.[9–17] We have recently found that flavanols isolated from green tea leaves are good antioxidants against free radical initiated lipid peroxidation in solution,[18] in micelles,[19–21] in human red blood cells,[22] in human low density lipoprotein,[23] and in rat liver microsomes,[24] and that the antioxidant activity of these flavanols depends significantly on the structure of the molecules and the initiation conditions.[18–24] It was also found that these green tea flavonols might interact with α-tocopherol (vitamin E) synergistically to enhance the antioxidant activity.[25] Therefore, it is of interest to extend these researches to other dietary flavonoids and their glycosides to study the structure–activity relationship since many dietary flavonoids exist in the form of glycosides.[26] We report herein a quantitative kinetic study on the antioxidation effect of a set of typical flavonols and their glycosides, *i.e.*, quercetin (Q), quercetin galactopyranoside (QG), quercetin rhamnopyranoside (QR), rutin (R), morin (MO), kaempferol (K) and kaempferol glucoside (KG) (Fig. 1), against peroxidation of human erythrocyte ghosts in phosphate buffered saline (PBS, pH = 7.4) under atmospheric oxygen. The peroxidation was initiated by a water-soluble azo-initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPAD) which is a widely used free radical initiator for biological systems. It was found that all of these flavonols and their glycosides are effective antioxidants against AMPAD-induced peroxidation of human erythrocyte ghosts, and that the flavonols bearing *ortho*-dihydroxyl groups possess significantly higher antioxidant activity than those bearing no such functionalities, and the glycosides are less active than their parent aglycones. The mechanistic details of their antioxidative action are discussed.

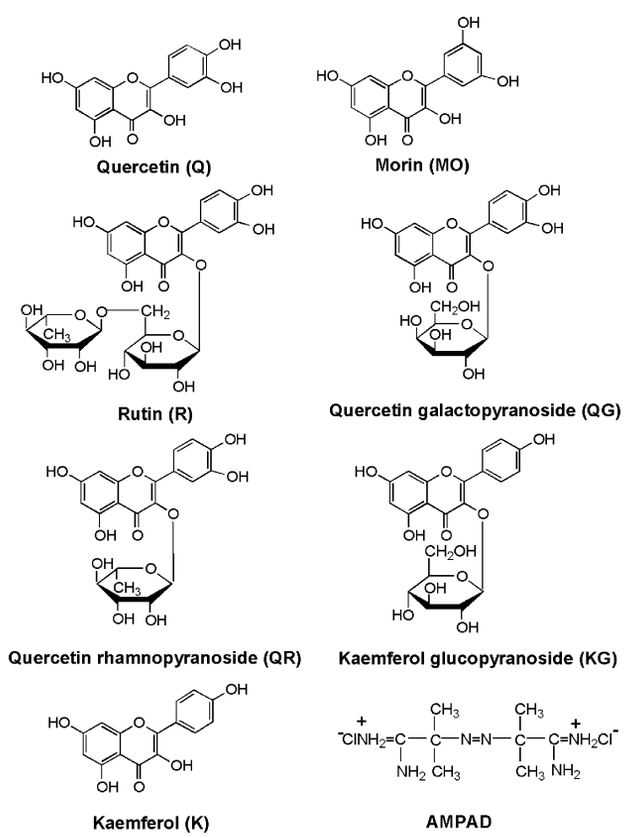


Fig. 1 Molecular structures.

Results

The peroxidation of human erythrocyte ghosts was initiated by the water-soluble azo-initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPAD) and monitored by oxygen uptake. Fig. 2 shows oxygen uptake curves recorded during the AMPAD-induced peroxidation of the erythrocyte ghost in the absence and in the presence of exogenous quercetin (Q). In the absence of Q the oxygen uptake did not take place immediately as in the case of lipid peroxidation conducted in model membranes,[27,28] but was still inhibited for *ca*. 22 minutes (Fig. 1,

DOI: 10.1039/b401550a

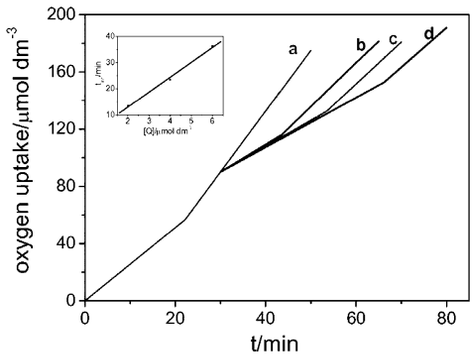


Fig. 2 Oxygen uptake curves recorded during the AMPAD-initiated and quercetin (Q) inhibited human erythrocyte ghost peroxidation in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [ghost] = 1.7 mg cm^{-3}; $[AMPAD]_0$ = 10.0 mmol dm^{-3}. (a) Native erythrocyte ghost containing 0.98 μmol dm^{-3} of TOH; (b) $[Q]_0$ = 2.0 μmol dm^{-3}; (c) $[Q]_0$ = 4.0 μmol dm^{-3}; (d) $[Q]_0$ = 6.0 μmol dm^{-3}. The inset shows the concentration dependence of the inhibition time (t_{inh}).

line a), demonstrating the presence of endogenous antioxidants in the erythrocyte ghost, such as α-tocopherol and β-carotene,[29] which can trap the propagating radicals to inhibit the peroxidation. The oxygen uptake rate during the inhibition period is designated as R_{inh}. After the inhibition period the oxygen uptake became faster, indicating depletion of the endogenous antioxidants. The turning point from the inhibition period to the restoration of oxygen uptake refers to the inhibition time, t_{inh}. The slope of the oxygen uptake curve after the inhibition period represents the intrinsic peroxidation rate, R_p, of the erythrocyte ghost in the absence of antioxidants. After a short time of the inhibition period quercetin (Q) of different concentrations was added. It was found that addition of Q produced a new inhibition period and this inhibition time is proportional to the concentration of Q (Fig. 2, lines b–d and the inset). It demonstrated that the peroxidation of the erythrocyte ghost was inhibited dose-dependently by Q in the absence of endogenous antioxidants. Other flavonols also showed the dose-dependent inhibition for the peroxidation (data not shown).

Fig. 3 shows representative oxygen uptake curves recorded during the AMPAD-induced peroxidation of the erythrocyte ghost in the presence of the same concentration (2.0 μM) of different flavonols which were added after the depletion of endogenous antioxidants. It is seen that after depletion of the endogenous antioxidants addition of these flavonols produced new inhibition periods, indicating that the erythrocyte ghost peroxidation could be inhibited by all of these flavonols in the absence of endogenous antioxidants. The inhibition time, t_{inh}, is appreciably different for different flavonols and follows the

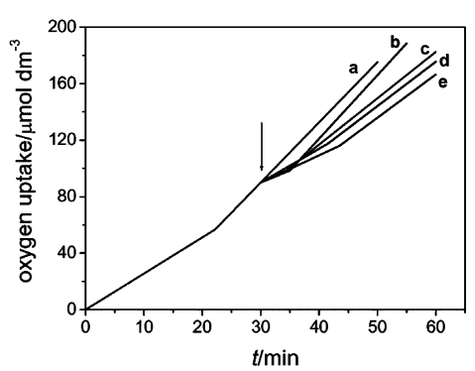


Fig. 3 Representative oxygen uptake curves recorded during the AMPAD-initiated and flavonol (FOH) inhibited erythrocyte ghost peroxidation in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [ghost] = 1.7 mg cm^{-3}; $[AMPAD]_0$ = 10.0 mmol dm^{-3}; $[FOH]_0$ = 2.0 μmol dm^{-3}. The arrow shows the time of addition of FOHs. (a) Native erythrocyte ghost containing 0.98 μmol dm^{-3} of TOH; (b) KG; (c) MO; (d) R; (e) Q. Curves for other FOHs are not shown for clarity.

efficacy sequence of Q > R > QG ~ QR > K ~ KG ~ MO (curves for QG, QR and K are not shown for clarity).

When the flavonol was added before the AMPAD-initiation the intrinsic inhibition period of the native erythrocyte ghost was remarkably prolonged and the overall inhibition time was approximately equal to the sum of the intrinsic inhibition time of the native erythrocyte ghost and the inhibition time induced by the flavonol when it was used after depletion of the endogenous antioxidants (compare Figs. 3 and 4). The efficacy sequence is Q > R > QG ~ QR > K ~ KG ~ MO (see Table 1, *vide infra*), which is the same as that obtained when the flavonols were used after depletion of the antioxidants in the native erythrocyte ghost. This suggests that the flavonols serve as chain-breaking antioxidants independently and they do not have synergistic interaction with the intrinsic antioxidants, *e.g.*, α-tocopherol (TOH), in the native erythrocyte ghost (*vide infra*).

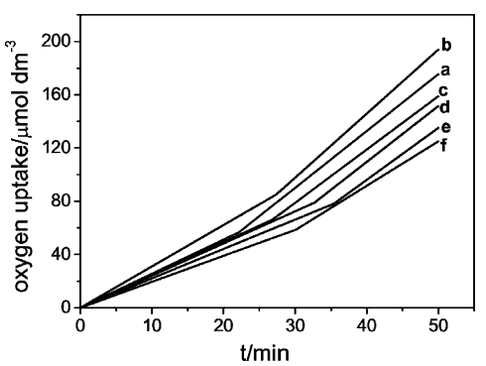


Fig. 4 Representative oxygen uptake curves recorded during the AMPAD-initiated and flavonol (FOH) inhibited erythrocyte ghost peroxidation in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [ghost] = 1.7 mg cm^{-3}; $[AMPAD]_0$ = 10.0 mmol dm^{-3}; $[FOH]_0$ = 2.0 μmol dm^{-3}. The FOHs were added before the initiation. (a) Native erythrocyte ghost containing 0.98 μmol dm^{-3} of TOH; (b) KG; (c) MO; (d) R; (e) Q; (f) QG. Curves for other FOHs are not shown for clarity.

Discussion

It has been proved that lipid peroxidation in model biomembranes follows the same classical rate law for autoxidation as that in homogeneous solutions,[27] and that α-tocopherol (TOH) is the principal lipid-soluble chain-breaking antioxidant in biomembranes.[30] The rate of oxygen uptake during the peroxidation of membrane lipids can be expressed as:[19,27]

$$-d[O_2]/dt = R_p = (k_p/(2k_t)^{1/2})R_i^{1/2}[LH] \quad (1)$$

where k_p and k_t are rate constants for the chain propagation and termination respectively, LH represents a lipid molecule with an abstractable hydrogen, *i.e.*, polyunsaturated fatty acids in the erythrocyte ghost, and R_i is the apparent rate of initiation:

$$R_i = 2k_g \times e[R–N=N–R] \quad (2)$$

Although the radical generation rate of AMPAD is known as 1.3~1.4 × 10^{-6} [AMPAD] s^{-1} at 37 °C for protein-containing solutions and liposomal dispersions,[31,32] the cage effect parameter e varies appreciably on the medium and the concentration of the antioxidant and the initiator.[32] Therefore, the R_i value is generally determined by the inhibition period and/or by the decay rate of TOH (eqns. (6) and (7), *vide infra*).

In the presence of an antioxidant molecule, AH, the peroxyl radical can be trapped and a new antioxidant radical, $A^{\bullet}$, produced (eqn. (3)). If the $A^{\bullet}$ is a stabilized radical (*e.g.*, α-tocopheroxyl radical or vitamin C radical anion) which can promote the rate-limiting hydrogen abstraction reaction (eqn. (3)) and undergo fast termination reaction (eqn. (4)), the peroxidation would be inhibited.

Table 1 Inhibition of AMPAD-initiated peroxidation of human erythocyte ghosts by flavonols (FOHs)[a]

FOH	$R_p/10^{-8}$ mol dm^{-3} s^{-1}	$R_{inh}/10^{-8}$ mol dm^{-3} s^{-1}	t_{inh}/min	n	kcl_p[d]	kcl_{inh}[d]
TOH[b]	7.10 ± 0.48	4.27 ± 0.05	22.2 ± 0.18	2[c]	48.2	29.0
Q[e]	5.07 ± 0.26	3.20 ± 0.29	13.5 ± 0.70	1.2	34.5	21.8
R[e]	5.16 ± 0.20	4.06 ± 0.38	11.3 ± 1.00	1.0	35.1	27.6
QG[e]	4.57 ± 0.10	3.29 ± 0.10	8.9 ± 0.47	0.80	31.1	22.4
QR[e]	4.55 ± 0.46	3.11 ± 0.23	9.0 ± 0.50	0.81	30.9	21.1
K[e]	5.15 ± 0.60	4.34 ± 0.15	5.7 ± 0.30	0.51	35.0	29.5
KG[e]	7.14 ± 0.70	4.40 ± 0.40	5.1 ± 0.22	0.46	48.5	29.9
MO[e]	5.47 ± 0.53	3.28 ± 0.61	4.5 ± 0.11	0.40	37.2	22.3
Q[f]	6.50 ± 0.29	3.68 ± 0.48	35.4 ± 2.8		44.2	25.0
R[f]	6.98 ± 0.58	4.03 ± 0.41	32.7 ± 1.5		47.5	27.7
QG[f]	5.55 ± 0.50	3.25 ± 0.75	30.1 ± 0.5		37.2	22.1
QR[f]	5.44 ± 0.85	3.19 ± 0.75	30.1 ± 0.58		37.0	21.7
K[f]	8.01 ± 0.17	5.18 ± 0.03	27.3 ± 0.7		54.5	35.2
KG[f]	7.94 ± 0.03	5.78 ± 0.33	26.8 ± 0.9		54.0	39.3
MO[f]	6.67 ± 0.04	4.11 ± 0.16	26.8 ± 1.4		45.3	27.9

[a] The reaction conditions and initial concentrations of the substrates are identical with those described in the legends of Figs. 3 and 4. Data are the average of three determinations and the SDs are shown in the table. [b] Intrinsic TOH (0.98 μmol dm^{-3}) and other antioxidants in the native erythrocyte ghost. [c] Assuming the inhibition period was produced by TOH and each TOH molecule traps two peroxyl radicals, see text. [d] $R_i = 1.47 \times 10^9$ mol dm^{-3} s^{-1} calculated from eqn. (6) by taking the initial concentration of TOH in native erythrocyte ghosts as 0.98 μmol dm^{-3}. [e] FOH added after the depletion of the intrinsic antioxidants (see Fig. 3). [f] FOH added before the initiation (see Fig. 4).

$$LOO^{\cdot} + AH \xrightarrow{k_{inh}} LOOH + A^{\cdot} \quad (3)$$

$$LOO^{\cdot} + A^{\cdot} \xrightarrow{fast} LOOA \quad (4)$$

where k_{inh} is the rate of inhibition, representing the activity of the antioxidant. During the inhibition period the rate of peroxyl formation by the initiation equals the rate of peroxyl trapping, therefore

$$R_i = R_{inh} = k_{inh} \times n[AH] \times [LOO^{\bullet}] \quad (5)$$

where n is the stoichiometric factor that designates the number of peroxyl radicals trapped by each antioxidant molecule and is given by:

$$n = R_i t_{inh}/[AH]_0 \quad (6)$$

From eqns. (3)–(5) we have eqn. (7):

$$-d[AH]/dt = R_i/n \quad (7)$$

The n value of TOH is generally taken as 2 [30,32] according to eqns. (3) and (4), hence R_i can be determined from the inhibition period (eqn. (6)) or from the decay rate of TOH (eqn. (7)).

The kinetic chain length defines the number of chain propagations by each initiating radical and is given by eqns. (9) and (10) for inhibited and uninhibited peroxidations respectively. The kinetic data obtained from Figs. 2–4 are listed in Table 1.

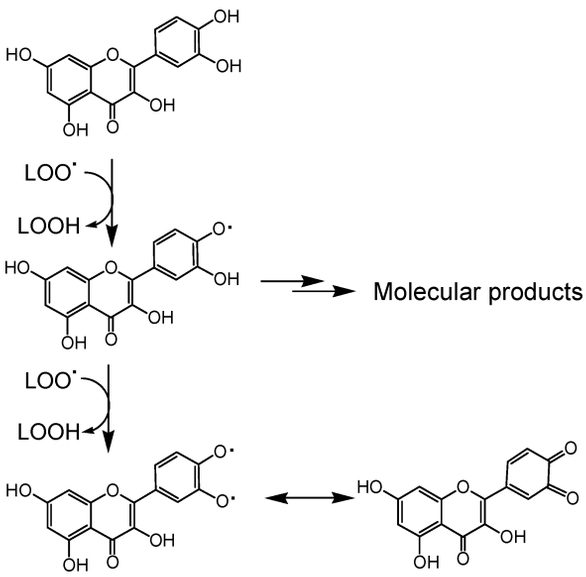
$$kcl_{inh} = R_{inh}/R_i \quad (8)$$

$$kcl_p = R_p/R_i \quad (9)$$

It can be seen from Figs. 2–4 and Table 1 that all of the flavonols (FOHs) produce clear inhibition periods, diminish the rate of peroxidation and shorten the kinetic chain lengths significantly in the absence of endogenous antioxidants, demonstrating that they are good antioxidants in the erythrocyte ghost. Comparison of the kinetic chain length of the erythrocyte ghost (kcl_p = 48.2) with that of linoleic acid in micelles (kcl_p = 22) [19] and in human low density lipoprotein (kcl_p = 18.9) [23] demonstrates that human erythrocyte ghosts are much more susceptible to the free radical induced peroxidation.

The stoichiometric factor of these flavonols is significantly smaller than 2, suggesting that although the flavonol can trap the propagating peroxyl radicals (eqn. (3)), the flavonol radical formed might be subject to other side reactions, such as dimerization of the radical, which may reduce the effectiveness of reaction (4). Based on the inhibition time and/or stoichiometric factor the antioxidant efficacy of the flavonols against the AMPAD-induced erythrocyte ghost peroxidation follows the sequence of Q > R > QR ~ QG > K > KG >MO. It is clearly seen that the antioxidative activity of Q and R is significantly higher than K and MO, *i.e.*, molecules bearing *ortho*-dihydroxyl functionalities are appreciably more active than those bearing no such functionalities. A similar result has been reported recently based on the kinetic electron spin resonance (ESR) study on the reaction of flavonoids with galvinoxyl radical in solution.[11,14] This is understood because the *ortho*-hydroxyl would make the oxidation intermediate, *ortho*-hydroxyl phenoxyl radical, more stable due to the intramolecular hydrogen bonding interaction as evidenced recently from both experiments [11,33] and theoretical calculations.[34] The theoretical calculation showed that the hydrogen bond in *ortho*-OH phenoxyl radical is *ca.* 4 kcal mol^{-1} stronger than that in the parent molecule catechol and the bond dissociation energy (BDE) of catechol is 9.1 kcal mol^{-1} lower than that of phenol and 8.8 kcal mol^{-1} lower than that of resorcinol.[34] In addition, it should be easier to further oxidize the *ortho*-OH phenoxyl radical and/or *ortho*-semiquinone radical anion to form the final product *ortho*-quinone as exemplified in Scheme 1. It is also seen that the glycosides QR, QG and KG are appreciably less active than their parent aglycones, because the glycosides are more hydrophilic which makes them more difficult to react with lipid peroxyl radicals inside the erythrocyte ghost. The lower activity of QG than Q in phospholipid bilayers has been reported previously.[16]

It is also seen from Table 1 that when the flavonol was added before the initiation the overall inhibition time is approximately equal to the sum of the inhibition time of the endogenous antioxidants (principally TOH) in the native erythrocyte ghost and that of the flavonol when it was added after depletion of the endogenous antioxidants. This fact suggests that the endogenous TOH and the exogenous flavonol might act independently, *i.e.*, the α-tocopherol regeneration reaction (eqn. (10)) did not take place between the flavonol (FOH) and α-tocopheroxyl radical (TO$^{\bullet}$). It has been reported previously that if the exogenous antioxidant, such as vitamin C and green tea polyphenols, could react with TOH the overall inhibition time when the antioxidant was added before the initiation would be significantly longer than the sum of the intrinsic inhibition time of the native



Scheme 1

biomembrane and the inhibition time induced by the antioxidant when it was used individually due to the corresponding α-tocopherol regeneration reaction.[25,31]

$$FOH + TO^{\bullet} \longrightarrow FO^{\bullet} + TOH \qquad (10)$$

Experimental

Materials

Quercetin galactopyranoside (QG), quercetin rhamnopyranoside (QR) and kaempferol glucoside (KG), were isolated from apple peel and green tea leaves respectively by extraction with methanol, water and ethyl acetate consecutively and chromatographic separation on a Sephadex LH-20 column with reference to the procedure reported previously.[35] Their structures and purity were confirmed by ^{1}H and ^{13}C NMR spectra and HPLC respectively. Quercetin (Q, from Sigma), rutin (R, from Aldrich), morin (MO, from Tokyo Kaset Kogyo) and kaempferol (K, from Fluka) were purchased with the highest purity available and used as received. 2,2′-Azobis(2-methylpropioamidine) dihydrochloride (AMPAD, from Aldrich), was used as received.

Human erythrocyte ghost preparation

Human erythrocyte ghosts were separated from heparinized blood of a healthy donor from the Central Blood Center, Red Cross of Gansu province. The cells were washed three times in 10 volumes of phosphate-buffered saline (PBS) at pH 7.4, which consisted of 137 mmol dm^{-3} of NaCl, 2.7 mmol dm^{-3} of KCl, 8.1 mmol dm^{-3} of Na_2HPO_4 and 1.5 mmol dm^{-3} of KH_2PO_4 in distilled water. The supernatant and buffy coat were carefully removed after each wash. The packed erythrocyte ghosts were then suspended in 30 volumes of an ice-cold hypotonic PBS (5 mmol dm^{-3}, pH 7.6) for lysis.[29] The hemoglobin-free ghosts were pelleted by centrifugation at 20000 × *g* at 4 °C for 20 min and further washed twice with the same hypotonic buffer. The protein concentration in the membranes was determined by the method of Lowry.[36]

Oxygen uptake measurements

The rate of oxygen uptake was measured in a closed glass vessel of *ca.* 2 ml in volume, thermostated at 37 ± 0.1 °C and provided with a magnetic stirrer, using a 5946-50 oxygen meter (Cole-Parmer Instruments, USA) which was able to record oxygen concentrations as low as 10^{-8} mol dm^{-3}. The erythrocyte ghosts were suspended in PBS (pH 7.4) to the final concentration of 1.70 mg cm^{-3} protein under air. Flavonols were dissolved in DMSO to the concentration of 2 mmol dm^{-3} as stock solutions. The final concentration of DMSO in the suspension was less than 0.1 (v/v) of the erythrocyte ghost suspension to avoid disturbance of the system. AMPAD was directly dissolved in PBS (pH 7.4) and injected into the erythrocyte ghost suspension to initiate the peroxidation. Every experiment was repeated three times and the results were reproducible within 10% experimental deviation.

α-Tocopherol (TOH) determination

TOH was extracted from the erythrocyte ghost by hexane–ethanol partitioning which yielded >97% of TOH,[32] and separated and determined by a Gilson model 702 liquid chromatograph using a Sychropack KPP-100 reversed-phase column and a Gilson Model 142 electrochemical detector by setting the oxidation potential at +700 mV as described previously.[23]

Conclusions

Flavonols and their glycosides are effective antioxidants against AMPAD-initiated peroxidation of human erythrocyte ghosts. The flavonols bearing *ortho*-dihydroxyl groups possess significantly higher antioxidative activity than those bearing no such functionalities and the glycosides are less active than their parent aglycones.

Acknowledgements

This work was supported by grants 20172025 and 20332020 from the National Natural Science Foundation of China.

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