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Correlation between mammalian cell cytotoxicity of flavonoids and the redox potential of phenoxyl radical/phenol couple

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Flavonoids exhibit prooxidant cytotoxicity in mammalian cells due to the formation of free radicals and oxidation products possessing quinone or quinomethide structure. However, it is unclear how the cytotoxicity of flavonoids depends on the ease of their single-electron oxidation in aqueous medium, i.e., the redox potential of the phenoxyl radical/phenol couple. We verified the previously calculated redox potentials for several flavonoids according to their rates of reduction of cytochrome *c* and ferricyanide, and proposed experimentally-based values of redox potentials for myricetin, fisetin, morin, kaempferol, galangin, and naringenin. We found that the cytotoxicity of flavonoids (*n*=10) in bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) and murine hepatoma (line MH-22a) increases with a decrease in their redox potential of the phenoxyl radical/phenol couple and an increase in their lipophilicity. Their cytotoxicity was decreased by antioxidants and inhibitors of cytochromes P-450, α-naphthoflavone and isoniazide, and increased by an inhibitor of catechol-*O*-methyltransferase, 3,5-dinitrocatechol. It shows that although the prooxidant action of flavonoids may be the main factor in their cytotoxicity, the hydroxylation and oxidative demethylation by cytochromes P-450 and *O*-methylation by catechol-*O*-methyltransferase can significantly modulate the cytotoxicity of the parent compounds.

Key words: flavonoids, antioxidants, oxidative stress, cytotoxicity

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INTRODUCTION

Flavonoids are universally recognized antioxidants which can protect the cell from the oxidative stress, *i.e.,* neutralise the damaging effect of reactive oxygen species (ROS). However, at high concentrations, flavonoids and other polyphenols may be cytotoxic, causing an increase in mitochondrial permeability, cytochrome *c* release, the activation of caspases, an increase in levels of p53 and p21, the suppression of Bcl-2, apoptosis induction, and necrotic cell death (Bolton *et al.,* 2000; Inayat-Hussain *et al.,* 2001; Morin *et al.,* 2001; Salvi *et al.,* 2002; Shen *et al.,* 2004; Lee *et al.,* 2011).

There are several mechanisms of the cytotoxicity of flavonoids, including the inhibition of topoisomerases (Webb & Ebeler, 2004; Bandele *et al.,* 2008) and kinases (Gamet-Payrastre *et al.,* 1999; Hou & Kumamoto, 2010), and their prooxidant action (Mioshi *et al.,* 2007; Sharma *et al.,* 2007, and references therein). The prooxidant action is evidenced by an increase in ROS levels and lipid peroxidation under the action of flavonoids and other polyphenols, which is prevented by antioxidants and transition metal-chelating agents (Sergedienė *et al.,* 1999; Galati *et al.,* 2002; Nemeikaitė-Čėnienė *et al.,* 2005). The prooxidant cytotoxicity may arise due to the formation of quinone- or quinomethide-type oxidation products of polyphenols that arylate reduced glutathione (GSH) and other cellular nucleophiles (Metodiewa *et al.,* 1999; Boersma *et al.,* 2000; Awad *et al.,* 2002; Lu *et al.,* 2006). The single-electron oxidation of polyphenols to phenoxyl radicals may lead to the cooxidation of NAD(P)H and other cellular reductants with the concomitant ROS formation (Galati *et al.,* 1999,2002). In the cell, flavonoids and other polyphenols can be oxidized by the transition metals, mitochondrial bc_1 complex and cytochrome *c* (Rich & Bendall, 1980; Rich, 1982; Yoshino & Murakami, 1998; Moini *et al.,* 1999), and during the reductive Fe^{2+} mobilization from ferritin (Hynes & Coinceanainn, 2002). Flavonoids also autooxidize in cell growth medium with the production of extracellular H_2O_2 (Nemeikaitė-Čėnienė *et al.,* 2005; Robaszkiewicz *et al.,* 2007).

The structural factors determining the prooxidant cytotoxicity of flavonoids are not well understood. One may expect that the cytotoxicity of flavonoids, if determined by their prooxidant action, may increase with a decrease in the redox potential of the semiquinone/hydroquinone (or phenoxyl radical/phenol) couple (E_7 ($Q^{\bullet-}/QH_2$)). This relationship has been demonstrated for a related group of antioxidants, polyhydroxybenzenes (Nemeikaitė-Čėnienė *et al.,* 2005; Grellier *et al.,* 2008). It reflects the relative ease of formation of prooxidant oxidation products or ROS by hydroquinones and other polyphenols, because the rates of their oxidation by cytochromes, Fe^{3+}, ferritin, or oxygen increase with a decrease in their E_7 ($Q^{\bullet-}/QH_2$) (Rich & Bendall, 1980; Rich, 1982; O'Brien, 1991; Hynes & Coincemainn, 2002). However, the values of E_7 ($Q^{\bullet-}/QH_2$) have been determined by pulse-radiolysis for a few flavonoids only, and for several other flavonoids redox potentials based

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Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; cL_{50}, concentration of agent for 50% cell death; COMT, catechol-*O*-methyltransferase; DPPD, *N,N'*-diphenyl-*p*-phenylene diamine; $E_7(Q^{\bullet-}/QH_2)$, redox potential of semiquinone/hydroquinone (phenoxyl radical/phenol) couple at pH 7.0; $E_7(Q^{\bullet-}/QH_2)_{(calc)}$, calculated redox potential of semiquinone/hydroquinone (phenoxyl radical/phenol) couple at pH 7.0; GSH, reduced glutathione; k_{app}, apparent first order rate constant; *k*, second order rate constant; *P*, octanol/water partition coefficient; QSAR, quantitative structure-activity relationship; ROS, reactive oxygen species

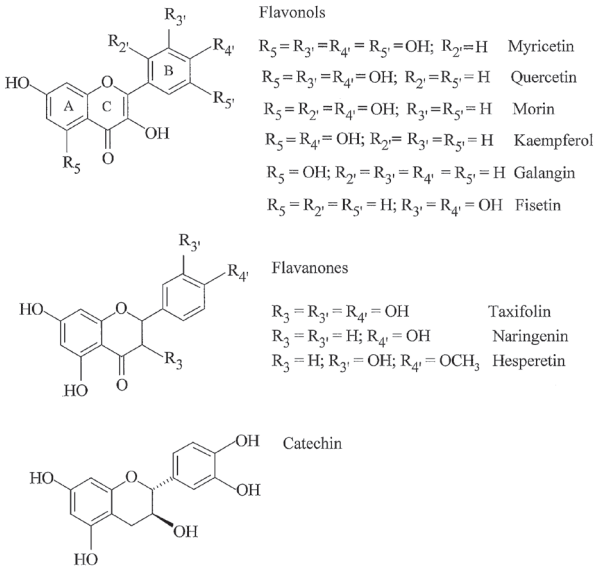


Figure 1. Structural formulae of flavonoids studied.

on calculations are available (Jovanovic *et al.,* 1998, and references cited therein). This hampers further studies in this direction.

In this study, we examined the reactivity of a series of flavonoids (Fig. 1) and model hydroxybenzenes possessing a broad range of E_7 ($Q^{\bullet-}/QH_2$) values towards the single-electron oxidants cytochrome *c* and ferricyanide. The obtained dependences enabled us to propose experimentally-based E_7 ($Q^{\bullet-}/QH_2$) values for several flavonoids. Further, we demonstrated an increase in the mammalian cell cytotoxicity of flavonoids upon a decrease in their potential of the phenoxyl radical/phenol redox couple.

MATERIALS AND METHODS

Chemicals. Flavonoids (Fig. 1), cytochrome *c*, other enzymes and chemicals were obtained from Sigma-Aldrich and used as received.

Kinetic studies. The kinetic experiments were carried out spectrophotometrically in 0.1 M K-phosphate buffer (pH 7.0), containing 1 mM EDTA, at 25°C. The reduction of cytochrome *c* by myricetin, fisetin, and morin was monitored using a DX.17MV stopped-flow spectrophotometer (Applied Photophysic) assuming $\Delta\varepsilon_{550}$ = 20 $mM^{-1}cm^{-1}$. The concentrations of flavonoids and cytochrome *c* after mixing were equal to 20–100 μM and 4.0 μM, respectively. The apparent first order rate constants (k_{app}) were obtained from the analysis of the kinetics of the absorbance increase according to a single-exponent fit, using the software supplied by Applied Photophysics. The second order rate constants (k) were calculated from the slopes of the linear dependences of k_{app} on the reductant concentration. Several experiments were performed with 500 μM cytochrome *c*, and 20 μM of each flavonoid, using a 0.2-cm optical path cell. For other compounds, the bimolecular reaction rate constants were calculated from the initial rates of reduction of the excess cytochrome *c* (500–150 μM) by 20 μM polyphenol, using a Hitachi-557 spectrophotometer and a 0.2-cm optical path cell. When the initial reaction rates were measured, corrections were introduced in several cases for the absorbance of polyphenol oxidation products at 550 nm: $\Delta\varepsilon_{550}$=0.8 mM^{-1} cm^{-1} (catechin), $\Delta\varepsilon_{550}$=0.28 mM^{-1} cm^{-1} (taxifolin, caffeic acid), and $\Delta\varepsilon_{550}$=0.2 mM^{-1} cm^{-1} (ethylgallate, catechol). The absorbance of the oxidation products at 550 nm was determined after the oxidation of the polyphenols by excess ferricyanide.

The reduction of ferricyanide by flavonoids and hydroxybenzenes was monitored at 420 nm using excess ferricyanide (final concentrations, 0.4–1.8 mM) over the reductant (final concentrations, 0.05–0.2 mM). Additional measurements were performed by monitoring the kinetics of the decrease of the absorbance of the reductant at 370 nm (myricetin, quercetin, morin), 365 nm (kaempferol, fisetin), and 320 nm (hesperetin). The reaction rate constants above 10 M^{-1} s^{-1} were determined using a stopped-flow spectrophotometer. Because the oxidation of polyphenols is sometimes accompanied by the slower formation of secondary products (Terland *et al.,* 2006), only the initial stage of the processes was analyzed according to a single-exponent fit. For the slowly reacting compounds, the reaction rates were measured using a Hitachi-557 spectrophotometer. In those cases, the k_{app} values were determined according to the Guggenheim method from the plots ln ΔA *vs.* t, where ΔA is the absorbance changes at equal time intervals (Connors, 1990). The second order rate constants were calculated from the plots k_{app} *vs.* ferricyanide concentration.

Cell culture cytotoxicity studies. Cultures of bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) and murine hepatoma (line MH-22) were grown and maintained at 37°C in Eagle's medium or in DMEM medium, respectively, supplemented with 10% fetal bovine serum and antibiotics, as described (Nemeikaitė-Čėnienė *et al.,* 2005; Grellier *et al.,* 2008). In the cytotoxicity experiments, cells (2.5×10^4/ml FLK, and 3.0×10^4/ml MH-22a) were seeded on 18×18 mm glass slides in 5-ml flasks either in the presence or in the absence of compounds, and were grown for 24 h. Then, the slides were rinsed 3-4 times with phosphate buffered saline and stained with Trypan blue. The cells adherrent to the slides were counted under a light microscope. Typically, they did not accumulate Trypan blue and their viability was 98.5–99.3%. Stock solutions of poorly soluble compounds were prepared in dimethyl sulfoxide. Its concentration in cultivation media did not exceed 0.2%, and did not affect cell viability. The experiments were conducted in triplicate.

Log P calculation, statistical analysis. The octanol/water partition coefficients of flavonoids (log *P*) were calculated using the ACD/ChemSketch software (version 4.02, Advanced Chemistry Development, Toronto, Ontario, Canada), while statistical and multiparameter regression analysis was performed using Statistica (version 4.3, Statsoft Inc., 1993).

RESULTS

Kinetics of reduction of cytochrome *c* and ferricyanide by flavonoids and hydroxybenzenes

Among the flavonoids examined (Fig. 1), the redox potentials for quercetin, taxifolin, catechin, and hesperetin were obtained directly from pulse-radiolysis studies, whereas the values of E_7 ($Q^{\bullet-}/QH_2$) for other flavonoids (Table 1) are based on calculations (Jovanovic *et al.,* 1998). In order to verify the calculated E_7 ($Q^{\bullet-}/QH_2$) values, we examined the reactions of the flavonoids and a number of model polyhydroxybenzenes with the E_7 ($Q^{\bullet-}/QH_2$) values from 0.33 V to 0.73 V (Table 1),

Table 1. Kinetic and thermodynamic characteristics of flavonoids and hydroxybenzenes.
Redox potentials of phenoxyl radical/phenol couples of flavonoids and hydroxybenzenes at pH 7.0 ($E_7(Q^{\bullet-}/QH_2)$) (Wardman, 1989; Jovanovic *et al.,* 1998; Lin *et al.,* 1998), their bimolecular rate constants of reduction of cytochrome *c* and ferricyanide (*k*), and their redox potentials of phenoxyl radical/phenol couples calculated according to Eqn. (1) ($E_7(Q^{\bullet-}/QH_2)_{(calc.)}$), standard error of estimate, ±0.04 V).

Compound	$E_7(Q^{\bullet-}/QH_2)$ (V)[a]	*k* ($M^{-1}s^{-1}$)		$E_7(Q^{\bullet-}/QH_2)_{7(calc.)}$ (V)
		cytochrome *c*	ferricyanide[c]	
Quercetin	0.33	390±20[b]	1460±150	0.36
Fisetin	(0.33)	220±30	2200±200	0.36
Myricetin	(0.36)	1030±50; 970±60[b]	7500±700	0.31
t-Butylhydroquinone	0.46	5.9±0.3; 5.6±0.5[b]	117±17.0	0.50
Hydroquinone	0.48	11.6±0.9[b]	170±5.0	0.48
Taxifolin	0.50	1.0±0.1[b]	32.0±3.0	0.56
Catechol	0.53	2.1±0.2	65.5±5.0	0.54
Ethylgallate	0.56	7.3±0.5[b]	200±20	0.49
Catechin	0.57	2.0±0.5	35.6±2.0	0.55
Morin	(0.60)	90.5±5.0; 98.0±8.0[b]	800±50	0.40
3,4-Dioxybenzoic acid	0.60	3.3±0.2	7.1±0.6	0.57
Galangin	(0.62)	0.03±0.004	0.69±0.15	0.72
Ferrulic acid	0.69	0.04±0.003	10.0±0.7	0.66
Hesperetin	0.72	0.01±0.002	0.5±0.04	0.75
p-Methoxyphenol	0.73	0.005±0.001	1.8±0.15	0.73
Kaempherol	(0.75)	92.0±7.0[b]	1400±100	0.39
Naringenin	–	≤0.002	0.3±0.03	0.79

[a]The calculated E^2_7 values for flavonoids (Jovanovic *et al.,* 1998) are shown in parentheses. [b]From Nemeikaitė-Čėnienė *et al.,* 2005. [c]Determined from the absorbance changes at 420 nm.

with two single-electron oxidants, cytochrome *c* and ferricyanide. According to the model of an ‚outer-sphere' electron-transfer, one may expect a linear dependence of log (rate constant) on the difference between the redox potential of a single-electron oxidant and a series of homologous reductants, *e.g.,* polyphenols, if the reactions are endothermic or modestly exothermic (Marcus & Sutin, 1985). A similar approach, based on linear log (rate constant) *vs.* redox potential dependences was used in the determination of unknown values of single-electron reduction potentials for nitroaromatic compounds (Čėnas *et al.,* 2009; Uchimiya *et al.,* 2010, and references therein).

The bimolecular rate constants (*k*) of the reduction of cytochrome *c* by hydroxybenzenes and flavonoids, determined in the present study or taken from our previous work (Nemeikaitė-Čėnienė *et al.,* 2005), are given in Table 1. In stopped-flow experiments, the reactions followed the first order kinetics. The rate constants determined using either excess polyphenol over cytochrome *c* or excess cytochrome *c* over polyphenol were identical within an experimental error. Supporting the previous observations (Nemeikaitė-Čėnienė *et al.,* 2005), the reactivity of hydroxybenzenes and flavonoids increased with a decrease in their experimentally determined E_7 ($Q^{\bullet-}/QH_2$) values, with $\Delta \log k/\Delta E_7$ ($Q^{\bullet-}/QH_2$)=–11.15±1.40 V^{-1} (r^2=0.876, F(1,9)=63.42) (Fig. 2A). One may note that the reactivities of fisetin and myricetin were close to those expected from their calculated E_7 ($Q^{\bullet-}/QH_2$) values, whereas the reactivity of galangin was lower, and those of morin and kaempferol were much higher than expected (Fig. 2A).

The reduction of ferricyanide by all the polyphenols examined except hydroquinone and *t*-butylhydroquinone was accompanied by the formation of oxidation products absorbing at 420 nm and above. It diminished the expected amplitude of absorbance

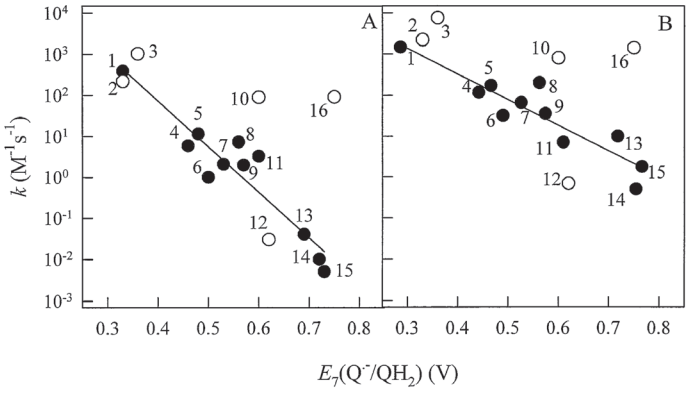
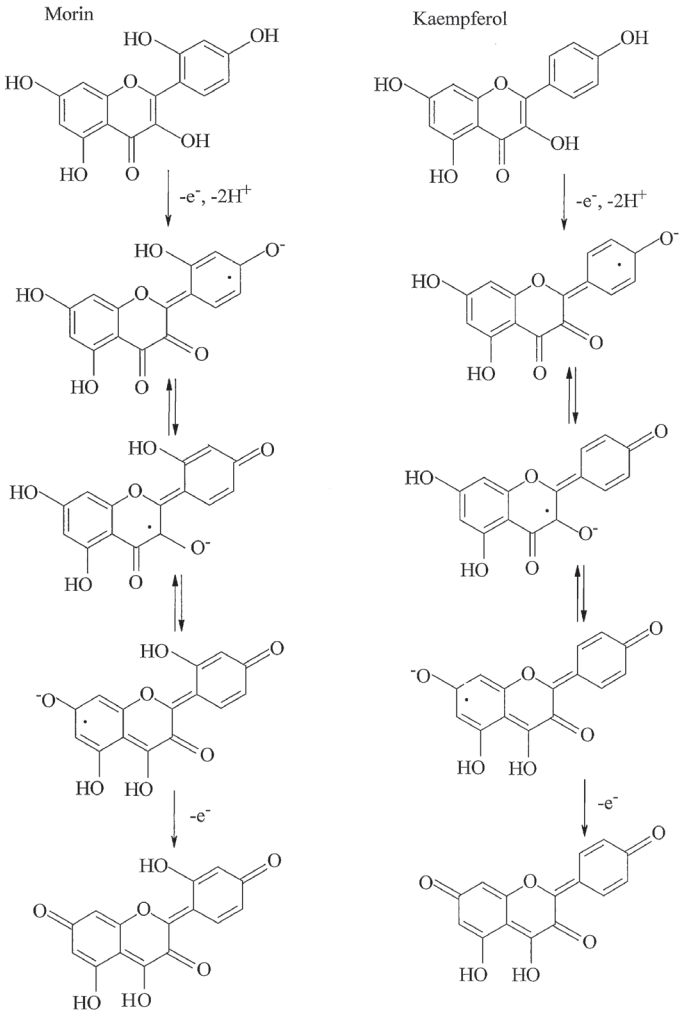


Figure 2. The dependence of reduction rate constants of cytochrome *c* (A) and ferricyanide (B) on the redox potentials of phenoxyl radical/phenol couple of polyphenols.
First order regression lines are drawn through the values of bimolecular reaction rate constants (*k*) of polyphenols with experimentally determined E_7 ($Q^{\bullet-}/QH_2$) values (solid circles). The reactivity of flavonoids with the calculated values of E_7 ($Q^{\bullet-}/QH_2$) (blank circles) is shown for comparison. Numbering of compounds, their *k* and E_7 ($Q^{\bullet-}/QH_2$) values are taken from Table 1: quercetin (1), fisetin (2), myricetin (3), *t*-butylhydroquinone (4), hydroquinone (5), taxifolin (6), catechol (7), ethylgallate (8), catechin (9), morin (10), 3,4-dioxybenzoic acid (11), galangin (12), ferrulic acid (13), hesperetin (14), *p*-methoxyphenol (15), kaempferol (16), and naringenin (17).



Scheme 1. Charge delocalization in flavonol anion-radicals

decrease at 420 nm. However, the absorbance changes at 420 nm during ferricyanide reduction by myricetin, quercetin, morin, kaempferol, fisetin, and ethylgallate followed the first order kinetics for more than 6–8 reaction half-times. In this time scale, the kinetics of oxidation of myricetin, quercetin, morin, kaempferol, and fisetin monitored at their λ_{max}, 365–370 nm, followed the first order as well. Their k_{app} values were identical within the experimental error to those obtained at 420 nm. On the other hand, the biphasic absorbance changes at 420 nm during the reduction of ferricyanide by taxifolin, catechin, and catechol were more pronounced (not shown). In those cases, only the initial stages of the process, 3–4 reaction half-times, were analyzed according to a single-exponent fit. The oxidation of other slowly reacting polyphenols also followed the first order kinetics. The data of Table 1 show that the reactivity of hydroxybenzenes and flavonoids towards ferricyanide increased with a decrease in their experimentally determined E_7 ($Q^{\bullet-}/QH_2$) values with $\Delta\log k/\Delta E_7$ ($Q^{\bullet-}/QH_2$)=–7.47±1.11 V^{-1} (r^2=0.835, F(1,9)=45.41) (Fig. 2B). Again, like in the reduction of cytochrome *c* (Fig. 2A), the reactivities of morin and kaempferol were much higher than expected (Fig. 2B). It shows that these deviations are not caused by the specificity of the particular flavonoids towards cytochrome *c*, but most probably by the overestimation of calculated E_7 ($Q^{\bullet-}/QH_2$) values for morin and kaempferol (Jovanovic *et al.,* 1998).

Calculation of E_7 ($Q^{\bullet-}/QH_2$) for flavonoids according to the kinetic data

For the calculation of unavailable values of E_7 ($Q^{\bullet-}/QH_2$) for flavonoids we used the geometric average of cytochrome *c* and ferricyanide reduction rate constants (Table 1) as a correlation parameter:

$$\log k=(6.00\pm1.07) - (9.30\pm1.07)\ E_7\ (Q^{\bullet-}/QH_2) \quad (1)$$

(r^2=0.893, F(1,9)=75.42).

The E_7 ($Q^{\bullet-}/QH_2$) values calculated according to Eqn. (1) (E_7 ($Q^{\bullet-}/QH_2)_{(calc.)}$, Table 1) are sufficiently close to the experimentally determined redox potentials, with an average difference of ±40 mV. In comparison, the experimental error in the determination of redox potential by pulse-radiolysis is ±10–15 mV (Jovanovic *et al.,* 1998; Wardman, 1989).

Concerning the obtained E_7 ($Q^{\bullet-}/QH_2)_{(calc.)}$ values for flavonoids, the calculated redox potentals for naringenin and galangin (Table 1) are close to that of resorcinol, 0.81 V, which reflects the ease of oxidation of the resorcinol group in the A-ring of flavonoids (Jovanovic *et al.,* 1998). The E_7 ($Q^{\bullet-}/QH_2)_{(calc.)}$ for myricetin and fisetin (Table 1) are close to their previously calculated values (Jovanovic *et al.,* 1998). In contrast, the E_7 ($Q^{\bullet-}/QH_2)_{(calc.)}$ values for morin and kaempferol (Table 1) are much more negative than those suggested previously (Jovanovic *et al.,* 1998). In our opinion, the previous calculations (Jovanovic *et al.,* 1998) could have underestimated the effects of charge delocalization in flavonol anion-radicals, which may result in their stabilization and a decrease in their E_7 ($Q^{\bullet-}/QH_2$) values (Scheme 1). The quinone/quinomethide tautomerisation of flavonoid oxidation products was experimentally demonstrated in quercetin oxidation (Boersma *et al.,* 2000; Awad *et al.,* 2002). This way of stabilization is not characteristic for other groups

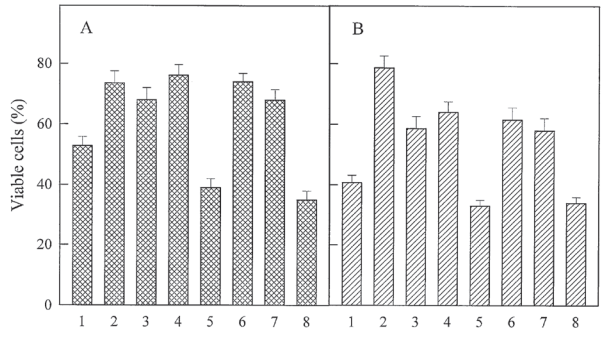


Figure 3. Toxicity of myricetin and galangin in MH-22a cells, and the effects of antioxidants, prooxidants, and cytochrome P-450 and COMT inhibitors.
Concentration of myricetin, 60 μM (A), concentration of galangin, 300 μM (B), additions: none (1), 2.5 μM DPPD (2), 300 μM desferrioxamine (3), 100 U/ml catalase (4), 20 μM BCNU (5), 5.0 μM α-naphthoflavone (6), 1.0 mM isoniazide (7), and 5.0 μM 3,5-dinitrocatechol (8). n = 3, P<0.02 for 2-4,6,7 against 1, and P<0.05 for 5, 8 against 1.

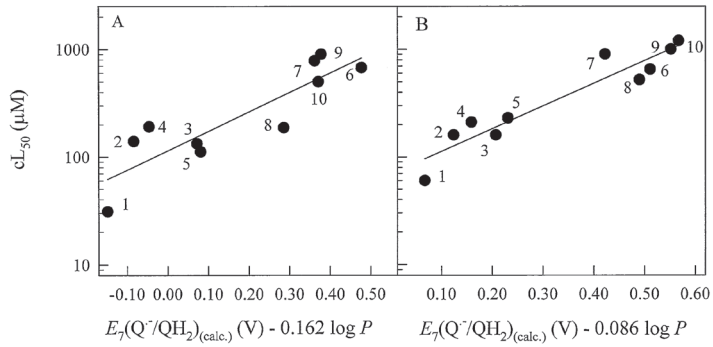


Figure 4. QSARs in flavonoid cytotoxicity in FLK (A) and MH-22a (B) cells. Dependence of flavonoid cytotoxicity on their calculated redox potential of phenoxyl radical/phenol couple, E_7 $(Q^{\bullet-}/QH_2)_{(calc.)}$ and octanol/water partition coefficient, log *P*, according to multiparameter Eqs. (2,3). Numbering of compounds and their E_7 $(Q^{\bullet-}/QH_2)_{(calc.)}$ values are taken from Table 2: myricetin (1), quercetin (2), fisetin (3), kaempferol (4), morin (5), catechin (6), taxifolin (7), galangin (8), hesperetin (9), and naringenin (10).

of flavonoids. The obtained low E_7 $(Q^{\bullet-}/QH_2)$ values for morin and kaempferol are in line with their voltammetric characteristics. Although morin and kaempferol do not possess an electrochemically reversible catechol group in B-ring, their electrochemical oxidation is reversible, with the voltammetric midpoint potentials at pH 7.4 being equal to 0.34 V and 0.39 V, respectively, which is close to the midpoint potential of quercetin, 0.29 V (Jorgensen & Skibstedt, 1998). This may be explained by the formation of quinomethides as two-electron oxidation products (Scheme 1). In contrast, an analogue of kaempferol, the flavone apigenin, which lacks 3-OH group in C-ring, is oxidized irreversibly with the peak potential at 0.71 V (Jorgensen & Skibstedt, 1998). Thus, the values of E_7 $(Q^{\bullet-}/QH_2)$ of flavonoids obtained in this work may be considered as reasonable approximations.

Prooxidant mammalian cell cytotoxicity of flavonoids

The prooxidant action of quercetin, myricetin, and several polyhydroxybenzenes in FLK and HL-60 cells has been confirmed before (Nemeikaitė-Čėnienė *et al.,* 2005). In this work we examined the action of myricetin and galangin in MH-22a cells. The latter compound is of considerable interest because it does not possess OH- groups in B-ring (Fig. 1). The cytotoxicity of both compounds was partly decreased by the antioxidant *N,N'*-diphenyl-*p*-phenylene diamine (DPPD) and the iron-chelating agent desferrioxamine, and potentiated by the prooxidant 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), which inactivates the antioxidant enzyme glutathione reductase and depletes intracellular GSH (Öllinger &Brunmark, 1991) (Fig. 3A, B). The protective effect of exogenous catalase (Fig. 3A, B) shows that the formation of extracellular H_2O_2 contributes to their cytotoxicity, evidently due to their oxidation in cell growth media (Morita *et al.,* 2003; Robaszkiewicz *et al.,* 2007). The cytotoxicity was also partly decreased by the inhibitor of cytochromes P-450 1A1/A2, α-naphthoflavone, and the inhibitor of cytochrome P-450 2E1, isoniazide, and potentiated by an inhibitor of catechol-*O*-methyltransferase (COMT), 3,5-dinitrocatechol (Moridani *et al.,* 2002a; 2002b) (Fig. 3A, B). It points to a common prooxidant cytotoxicity mechanism of both compounds.

Quantitative structure-activity relationships (QSARs) in the cytotoxicity of flavonoids

The concentrations of flavonoids causing 50% death of FLK and MH-22a cells (cL_{50}), determined in the present work or taken from our previous studies (Nemeikaitė-Čėnienė *et al.,* 2005; Grellier *et al.,* 2008), and the previously obtained cL_{50} values of flavonoids in human promyelocytic leukemic cells (line HL-60) (Nemeikaitė-Čėnienė *et al.,* 2005; Grellier *et al.,* 2008) are given in Table 2, together with their E_7 $(Q^{\bullet-}/QH_2)_{(calc.)}$ values. Because the cytotoxicity of polyhydroxybenzenes increases with an increase in their lipophilicity (Nemeikaitė-Čėnienė *et al.,* 2005), the calculated octanol/water partition coefficients for flavonoids, log *P*, are also given in Table 2. The data analysis shows that the cytotoxicity of the flavonoids in general increases with a decrease in the energetics of their single-electron oxidation, but the dependence is relatively scatterred, with the coefficients $\Delta \log cL_{50}/E_7$ $(Q^{\bullet-}/QH_2)_{(calc.)}$ equal to 1.86±0.62 (FLK, r^2=0.527), 2.13±0.37 (MH-22a, r^2=0.805), and 2.28±0.72 (HL-60, r^2=0.665). There is no relationship between log cL_{50} and log *P* (r^2=0.275 (FLK), r^2=0.138 (MH-22a), and r^2=0.343 (HL-60)). On the other hand, a two-parameter analysis improved the regressions markedly, showing that the cytotoxicity of flavonoids increased with a decrease in their E_7 $(Q^{\bullet-}/QH_2)_{(calc.)}$ and with an increase in log *P*:

$$\log cL_{50}=(2.06\pm0.35)+(1.80\pm0.46)\ E_7\ (Q^{\bullet-}/QH_2)_{(calc.)} - (0.29\pm0.11)\ \log P,\ r^2=0.769,\ F(2,7)=11.67\ \text{(FLK)}, \quad (2)$$

$$\log cL_{50}=(1.84\pm0.20)+(2.10\pm0.26)\ E_7\ (Q^{\bullet-}/QH_2)_{(calc.)} - (0.18\pm0.06)\ \log P,\ r^2=0.916,\ F(2,7)=35.97\ \text{(MH-22a)}, \quad (3)$$

and

$$\log cL_{50}=(2.22\pm0.62)+(2.01\pm0.59)\ E_7\ (Q^{\bullet-}/QH_2)_{(calc.)} - (0.48\pm0.20)\ \log P,\ r^2=0.832,\ F(2,4)=9.88\ \text{(HL-60)}. \quad (4)$$

The dependence of the cytotoxicity of flavonoids in FLK and MH-22a cells on their E_7 $(Q^{\bullet-}/QH_2)_{(calc.)}$ and log *P* values is shown in Fig. 4A, B.

DISCUSSION

Although numerous studies on the prooxidant activity of flavonoids have been published (Mioshi *et al.,* 2007; Sharma *et al.,* 2007, and references therein), the relationship between their redox properties and the cytotoxicity has been addressed insufficiently so far. The available QSARs were based on the voltammetric oxidation potentials or on the calculated heats of formation of free radicals of flavonoids (Sergedienė *et al.,* 1999; Moridani *et al.,* 2003), which may not adequately reflect the energetics of single-electron oxidation of flavonoids in aqueous medium. In contrast, the values of E_7 $(Q^{\bullet-}/QH_2)$ are directly related to an „outer sphere" electron-transfer model (Marcus & Sutin, 1985), and well describe the reactivity of polyphenols towards single-electron oxidants (Rich & Bendall, 1980; Rich, 1982). Thus, their use in QSARs may more adequately characterize the cytotoxicity mechanism(s) with respect to the possible prooxidant action of flavonoids. Our kinetic studies (Fig. 2A, B, Table 1) enabled us to extend the number of flavonoids with experimentally-based E_7 $(Q^{\bullet-}/QH_2)$ values. Subsequently, we obtained QSARs for the first time, show-

Table 2. Cytotoxic characteristics of flavonoids.
The values of redox potentials of phenoxyl radical/phenol couples of flavonoids at pH 7.0 calculated according to Eqn. (1) ($E_7(Q^{\bullet-}/QH_2)_{(calc.)}$), the octanol/water partition coeficients of flavonoids (log *P*), and their concentrations for 50% survival (cL_{50}) of FLK, MH-22a, and HL-60 cells.

Compound	$E_7(Q^{\bullet-}/QH_2)_{(calc.)}$	log *P*	cL_{50} (μM):		
	(V)		FLK	MK-22a	HL-60
Myricetin	0.31	2.83	31.0±4.0; 34.0±5.0[a]	60.0±3.0	20±5.0[a]
Quercetin	0.36	2.74	140±18[a]	160±25[b]	120±20[a]
Fisetin	0.36	1.78	133±15	160±20	–
Kaempferol	0.39	2.69	192±30; 185±25[a]	210±30	125±20[a]
Morin	0.40	1.97	112±15[a]	230±25; 250±35[b]	250±40[a]
Catechin	0.55	0.45	676±30	650±50	–
Taxifolin	0.56	1.22	780±120[a]	900±100[b]	600±150[a]
Galangin	0.72	2.68	188±20	520±50	–
Hesperetin	0.75	2.30	≥900; 750±100[a]	1000±100	500±100[a]
Naringenin	0.79	2.59	500±60	1200±100	700±100[b]

[a]From Nemeikaitė-Čėnienė *et al.,* 2005. [b]From Grellier *et al.,* 2008.

ing that the cytotoxicity of flavonoids in the three cell lines increases with a decrease in their E_7 $(Q^{\bullet-}/QH_2)_{(calc.)}$ values (Eqns. 2–4, Fig. 4A, B). Taken together with the protective effects of the antioxidants (Fig. 3A, B), this indicates that the oxidative stress may be a key factor for flavonoid cytotoxicity. An increase in the cytotoxicity of flavonoids with an increase in their lipophilicity (Eqsn. 2–4) points to the importance of their intracellular accumulation. The obtained QSARs and the effects of antioxidants, prooxidants, and inhibitors of cytochromes P-450 and COMT (Figs. 3,4) for flavonoids are similar to those observed in the action of polyhydroxybenzenes in the same cell lines (Nemeikaitė-Čėnienė *et al.,* 2005; Grellier *et al.,* 2008). It shows that both groups of polyphenolic antioxidants may share the same main mechanism(s) of prooxidant cytotoxicity.

On the other hand, the dependence of flavonoid cytotoxicity on their oxidation potential is not strongly expressed, because the coefficients $\Delta\log cL_{50}/\Delta E_7$ $(Q^{\bullet-}/QH_2)_{(calc.)}$ in Eqns. 2–4, 1.8–2.2 V^{-1}, are lower than those describing the cytotoxicity of polyhydroxybenzenes in the same cell lines, 5.1–6.9 V^{-1} (Nemeikaitė-Čėnienė *et al.,* 2005; Grellier *et al.,* 2008). The latter coefficients closely match the order of reactivity of polyphenols towards single-electron oxidants, $\Delta\log k/\Delta E_7$ $(Q^{\bullet-}/QH_2)$=~ –8.5 V^{-1} (Rich & Bendall, 1980; Rich, 1982). Thus, apart from the ease of formation of prooxidant oxidation products or ROS, the cytotoxicity of flavonoids may be affected by other factors. A possible explanation is the interconversion of flavonoids under the action of cytochromes P-450 and COMT (Duarte Silva *et al.,* 1997; Nielsen *et al.,* 1998; Lautala *et al.,* 2002; Lee *et al.,* 2005), which may attenuate the expected dependence of cytotoxicity of flavonoids on their E_7 $(Q^{\bullet-}/QH_2)$. Typically, *O*-methylation of catechols decreases their cytotoxicity, evidently due to an increase in their E_7 $(Q^{\bullet-}/QH_2)$, whereas their hydroxylation increases the autooxidation rate and cytotoxicity (Moridani *et al.,* 2002a; 2002b). Although myricetin is not a substrate for cytochromes P-450 (Nielsen *et al.,* 1998), and galangin does not possess hydroxy groups in B-ring, the data of Fig. 3A, B show that their cytotoxicity is modulated by inhibitors of cytochromes P-450 and COMT to a similar extent. Thus, *O*-methylation of myricetin by COMT (Lee *et al.,* 2005) may be followed by the subsequent oxidative demethylation of the reaction products by cytochromes P-450. In turn, cytochromes P-450 may convert galangin into kaempferol, and, subsequently, into quercetin (Fig. 1) (Duarte Silva *et al.,* 1997), which may be followed by *O*-methylation of quercetin by COMT (Lee *et al.,* 2005). This is in line with the sufficiently close cL_{50} values for galangin, kaempferol, and quercetin in FLK cells (Table 2). Hesperetin and naringenin (Fig. 1) may also undergo the cytochrome P-450-catalyzed conversion into eriodictyol (Hodek *et al.,* 2002), which may be also partly responsible for the similar cytotoxicity of the above flavanones (Table 2). Because flavonoids are *O*-methylated much faster than catechols (Lautala *et al.,* 2002), the action of COMT may decrease their cytotoxicity more efficiently. This may explain a less pronounced role of redox potential in the cytotoxicity of flavonoids as compared to that of polyhydroxybenzenes (Nemeikaitė-Čėnienė *et al.,* 2005; Grellier *et al.,* 2008).

CONCLUSIONS

Our studies have shown that the reactivity of flavonoids and related polyphenolic antioxidants with model single-electron oxidants may be a useful tool to characterize their E_7 $(Q^{\bullet-}/QH_2)$. Subsequently, this parameter may be used in the prediction of the cytotoxicity of flavonoids to the mammalian cell, which seems to be caused mainly by their prooxidant action. Another important factor to be considered is the reactions of flavonoids with cytochromes P-450 and COMT, which may significantly attenuate the dependence of the cytotoxicity of flavonoids on their E_7 $(Q^{\bullet-}/QH_2)$.

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