FEBS Letters 462 (1999) 392–396 FEBS 22993

# Prooxidant toxicity of polyphenolic antioxidants to HL-60 cells: description of quantitative structure-activity relationships

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Received 23 September 1999; received in revised form 8 November 1999

Edited by Gunnar von Heijne

**Abstract Polyphenolic antioxidants exhibited a dose-dependent toxicity against human promyelocytic leukemia cells (HL-60). Their action was accompanied by malondialdehyde formation, and was partly prevented by desferrioxamine and the antioxidant *N,N′*-diphenyl-*p*-phenylene diamine. This points to a prooxidant character of their cytotoxicity. A quantitative structure-activity relationship (QSAR) has been obtained to describe the cytotoxicity of 13 polyphenolic antioxidants belonging to three different groups (flavonoids, derivatives of gallic and caffeic acid): log $cL_{50}$ (μM) = (2.7829 ± 0.2339)+(1.2734 ± 0.4715) $E_{p/2}$ (V)−(0.3438 ± 0.0582) log *P* ($r^2$ = 0.8129), where $cL_{50}$ represents the concentration for 50% cell survival, $E_{p/2}$ represents the voltammetric midpoint potential, and *P* represents the octanol/water partition coefficient. Analogous QSARs were obtained using enthalpies of single-electron oxidation of these compounds, obtained by quantum-mechanical calculations. These findings clearly point to two important characteristics determining polyphenol cytotoxicity, namely their ease of oxidation and their lipophilicity.**



*Key words:* Flavonoid; Polyphenol; Antioxidant; Cytotoxicity; Oxidative stress

## 1. Introduction

Polyphenolic compounds (flavonoids, catechols, derivatives of gallic acid) are widely distributed in edible plants, and considered to be dietary antioxidants. The latter property is derived from their free radical and activated oxygen scavenging activities [1]. Some of these compounds are currently used as antioxidant food additives, e.g. esters of gallic acid [2].

The ability of polyphenols to protect cells from 'oxidative stress' has been demonstrated [3,4]. In addition, these compounds show a wide and contradictory spectrum of action, involving antitumor, antiviral, antibacterial, cardioprotective, pro- and antimutagenic activity [1,5–9]. The antiproliferative effects or apoptosis induced by flavonoids and other polyphenols on several tumor cell lines were explained in terms of topoisomerase [5] or phosphatidylinositol 3-kinase [6] inhibition, cell cycle arrest [7], accumulation of the tumor suppression protein p53 [8], or enhancement of expression of c-*fos* and c-*myc* genes [9].

A possible, although insufficiently investigated mechanism of polyphenol cytotoxicity may be related to their prooxidant properties, since the same polyphenol compounds could behave as both antioxidants and prooxidants, depending on concentration and free radical source [10]. Flavonoids autoxidize in aqueous medium, and may form highly reactive $OH^{\bullet}$ radicals in the presence of transition metals [11]. Besides, polyphenols and flavonoids may act as substrates for peroxidases and other metalloenzymes, yielding quinone- or quinomethide-type prooxidant and/or alkylating products [12–14]. The suggestion about the prooxidant character of polyphenol cytotoxicity is supported by the formation of activated oxygen species during gallic acid-induced apoptosis [15], and by the enhancement of gallic and caffeic acid-induced apoptosis by non-toxic concentrations of copper ions [16].

In this work, we have found that the toxicity of polyphenolic oxidants belonging to three different groups (flavonoids and derivatives of gallic and caffeic acid) towards human promyelocytic leukemia cells (HL-60) is associated with the prooxidant events. Further, we have established a quantitative structure-activity relationship (QSAR), linking the polyphenol cytotoxicity to their ease of oxidation (voltammetric midpoint potential, enthalpy of single-electron oxidation), and lipophilicity.

## 2. Materials and methods

### 2.1. Materials

Flavonoids, gallic acid and its esters, caffeic and chlorogenic acid, desferrioxamine and *N,N′*-diphenyl-*p*-phenylene diamine (DPPD) were obtained from Sigma or Aldrich, and used as received.

### 2.2. Cytotoxicity studies

HL-60, human promyelocytic leukemia cells, obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and incubated in a humidified atmosphere with 5% $CO_2$ at 37°C. Cells were passaged every 4 days. In cytotoxicity experiments, 2 ml cell suspension ($1.0\times10^5$/ml) was incubated in the presence of various concentrations of polyphenols for 24 h. The cell viability was determined by an automated bioluminescent assay for cellular ATP [17], and expressed as the ratio of the ATP content in polyphenol-exposed

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*Abbreviations:* $cL_{50}$, concentration of agent for 50% cell survival; *P*, octanol/water partition coefficient; $E_{p/2}$, voltammetric midpoint potential; $E^1$, single-electron oxidation potential; ΔHf, enthalpy of reaction; DPPD, *N,N′*-diphenyl-*p*-phenylene diamine


PII: S0014-5793(99)01561-6

to unexposed cells. The stock solutions of polyphenols were prepared in dimethylsulfoxide (DMSO). The concentration of DMSO in cultivation media did not exceed 0.2%, and did not affect the cell viability.

The lipid peroxidation in the cells was monitored by detecting the formation of malondialdehyde, using the thiobarbituric acid test [18]. Briefly, cells were washed by cold physiological saline and disrupted by sonication. To the disrupted cells (1.4 ml) was added 0.2 ml of 10% trichloroacetic acid and 0.4 ml 0.6 M thiobarbituric acid, and tubes were heated in boiled water. Upon cooling and subsequent centrifugation (8000 rpm, 20 min), the absorbance of supernatant was read at 532 nm by a Hitachi 557 spectrophotometer, and the amount of malondialdehyde was calculated using $\Delta\varepsilon = 156\ mM^{-1}\ cm^{-1}$.

### 2.3. Cyclic voltammetry studies of polyphenols

Cyclic voltammograms of quercetin and derivatives of gallic acid were recorded with a PA-2 polarograph provided with 4103 X-Y recorder (Laboratorni Pristroje, Czech Republic), in 0.1 M K-phosphate (pH 7.0) containing 1 mM EDTA, at 25°C under anaerobic conditions. A glassy carbon SU-1500 electrode served as a working electrode, a saturated calomel electrode (SCE, +244 mV vs. NHE) was used as the reference, and a platinum electrode (surface area 56 $mm^2$) was used as an auxiliary electrode. The cyclic voltammograms were recorded at potential scan rate 100 mV $s^{-1}$ as described previously [13]. The voltammetric midpoint potentials ($E_{p/2}$) were calculated from anodic ($E_{pa}$) and cathodic ($E_{pc}$) peak potentials, $E_{p/2} = (E_{pa} + E_{pc})/2$, for the reversible and quasi-reversible processes. For irreversible systems characterized by the absence of cathodic peak potentials, $E_{p/2}$ was determined as a potential where current is half the anodic peak current [13].

### 2.4. Quantum-mechanical and partition coefficient calculation, statistical analysis

Semiempirical PM3, ab initio HF/3-21G* and density functional theory (DFT) B3LYP/6-311G** calculations on compounds studied in this work were carried out with the use of the GAUSSIAN 94 package (Gaussian Inc., Pittsburgh, PA, USA). Calculations were performed on polyphenols and the single- and two-electron oxidized forms of these polyphenols specified below. In all cases the geometry was optimized with respect to all degrees of freedom. The PM3 optimized geometries of the species studied served both to calculate PM3 thermochemical data and as starting geometries for the ab initio HF/3-21G* optimization. The HF/3-21G* optimized geometries were then used for higher level DFT B3LYP/6-311G** single-point calculations. The enthalpies of reactions (ΔHf) were calculated using the Hess law from Eqs. 1–3, where $QH_2$ denotes polyphenol, $QH^{\bullet}$ or $Q^{\bullet -}$ denote its single-electron oxidized forms, Q denotes its two-electron oxidized form, and Hf denotes the heat of formation:

$$2H^+,\ 1e^-\ \text{transfer}:\ \Delta Hf_1 = Hf(Q^{\bullet -}) - Hf(QH_2), \quad (1)$$

$$1H^+,\ 1e^-\ \text{transfer}:\ \Delta Hf_2 = Hf(QH^{\bullet}) - Hf(QH_2), \quad (2)$$

$$2H^+,\ 2e^-\ \text{transfer}:\ \Delta Hf_3 = Hf(Q) - Hf(QH_2). \quad (3)$$

The octanol/water partition coefficients ($P$) for flavonoids and other polyphenols were calculated using an ACD logP (version 1) software, a generous gift of Advanced Chemistry Development Inc. (Toronto, Ont., Canada). The multiparameter regression analysis was performed using Statistica (version 4.3) software (StatSoft Inc., 1993).

## 3. Results

The presence of flavonoids and other polyphenols in growth media caused concentration-dependent decreases in cell viability. The concentrations of compounds for 50% decreasing cell viability ($cL_{50}$) are given in Table 1. The action of polyphenols was accompanied by prooxidant events, since several polyphenols at concentrations corresponding to 80–90% decrease in cell viability (300 μM quercetin, 800 μM taxifolin or 250 μM butylgallate) stimulated lipid peroxidation. Typically, malondialdehyde concentration increased from $0.3 \pm 0.1$ nmol/$10^6$ cells in control, to $1.0 \pm 0.28$ nmol/$10^6$ cells. Second, the toxicity of taxifolin to HL-60 cells was markedly prevented by the antioxidant DPPD [19] and the iron chelator desferrioxamine, the latter preventing the Fenton reaction (Fig. 1). Analogous effects of DPPD and desferrioxamine were observed for quercetin and butylgallate toxicity as well (data not shown). Another characteristic of polyphenol action is that their cytotoxicity increases upon an increase in their lipophilicity (octanol/water partition coefficient, $P$), which is most clearly evidenced by comparison of a series of gallic acid derivatives (Fig. 2, Table 1). This finding closely resembles the previously observed order of efficiency of gallic acid derivatives in preventing proliferation of the mouse B cell lymphoma cells [20].

Since the cytotoxicity of polyphenols to HL-60 cells is at least partly related to prooxidant events, it is of interest to examine the dependence of cytotoxicity on the energetics of single- and two-electron oxidation of polyphenols. In order to describe QSARs, we have compiled the available values of single-electron oxidation potentials ($E^1$) for flavonoids and polyphenols determined by pulse radiolysis [21,22] (Table 1).

Table 1
The concentrations of polyphenols for 50% survival of HL-60 cells ($cL_{50}$), the potentials of their single-electron oxidation ($E_7^1$), voltammetric midpoint potentials ($E_{p/2}$), and their octanol/water partition coefficients ($P$)

| Number | Compound | $cL_{50}$ (μM) | $E_7^1$ (V) | $E_{p/2}$ (V) | log $P$ |
|---|---|---|---|---|---|
| 1 | Quercetin | 100 ± 30 | 0.60[a] | 0.29[c,d] | 2.74 |
| 2 | Morin | 250 ± 40 | 0.95[a] | 0.34[c] | 1.97 |
| 3 | Kaempferol | 125 ± 20 | 0.95[a] | 0.39[c] | 2.69 |
| 4 | Taxifolin | 600 ± 150 | | 0.37[c,e] | 1.22 |
| 5 | Hesperetin | 500 ± 100 | 0.72[a] | 0.59[c,e] | 2.3 |
| 6 | Naringenin | 700 ± 100 | | 0.76[c,e] | 2.59 |
| 7 | Gallic acid | 750 ± 200 | | 0.29[d,e] | 0.91 |
| 8 | Methylgallate | 400 ± 100 | 0.56[b] | 0.28[d,e] | 1.54 |
| 9 | Ethylgallate | 160 ± 40 | | 0.28[d,e] | 2.07 |
| 10 | Butylgallate | 110 ± 30 | | 0.28[d,e] | 3.13 |
| 11 | Octylgallate | 30 ± 10 | | 0.27[d,e] | 5.26 |
| 12 | Caffeic acid | 800 ± 150 | 0.54[b] | 0.36[c] | 2.47 |
| 13 | Chlorogenic acid | ≥ 2000 | | 0.37[c] | 0.69 |

[a]vs. normal hydrogen electrode (NHE), pH 7.0 [21].
[b]vs. NHE, pH 7.0 [22].
[c]vs. NHE, pH 7.4 [13].
[d]vs. NHE, pH 7.4, present work.
[e]Irreversible.

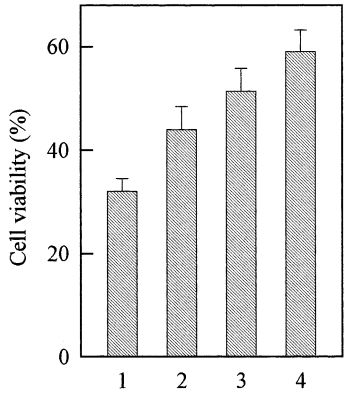


Fig. 1. The protective effects of DPPD (2.5 μM) and desferrioxamine (300 μM) against toxicity of 800 μM taxifolin to HL-60 cells. Additions: taxifolin (1), taxifolin+DPPD (2), taxifolin+desferrioxamine (3), taxifolin+DPPD+desferrioxamine (4), $n=4$, $P<0.05$ for 2–4 against 1. In the absence of flavonoid, DPPD and desferrioxamine did not affect cell viability.

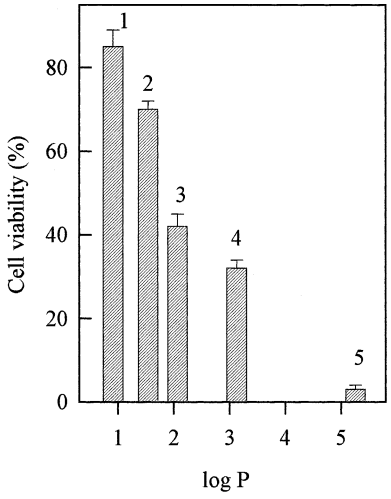


Fig. 2. The dependence of the toxicity of gallic acid derivatives to HL-60 cells on their octanol/water partition coefficient ($P$): gallic acid (1), methylgallate (2), ethylgallate (3), butylgallate (4), and octylgallate (5), $n=4$, concentration of each compound, 125 μM.

Next, we have determined the previously unknown voltammetric midpoint potentials ($E_{p/2}$) for several compounds investigated. Their values are given in Table 1 together with $E_{p/2}$ for other polyphenols taken from [13]. Finally, we have performed quantum-mechanical calculations of enthalpies of polyphenol oxidation (ΔHf). The calculated values of ΔHf increases upon increase in $E^1$ [22], and may serve as an alternative parameter to describe QSARs. The calculated values of ΔHf for various single- and two-electron oxidation products of flavonoids and polyphenols are given in Table 2. One should note that among several possible products of quercetin, morin and kaempferol oxidation, their *p*-quinomethide isomers (Fig. 3), characterized by the lowest values of ΔHf, were used for analysis.

## 4. Discussion

The data of the present study, pointing to a prooxidant character of polyphenol cytotoxicity, are consistent with previous findings on the involvement of activated oxygen species in gallic and caffeic acid-induced apoptosis [15,16]. One may expect that the cytotoxicity of polyphenols, if related to 'oxidative stress', may increase upon decrease in the potential of their single-electron oxidation ($E^1$), i.e. the potential of semiquinone/catechol (or phenoxy radical/phenol) redox couple. For example, low-potential catechols or phenols will more rapidly autoxidize or be oxidized by hemoproteins [23], reduce transition metals or reductively mobilize iron from ferritin [24]. This approach is based on the analogy with prooxidant cytotoxicity of quinones and nitroaromatic compounds, which increases upon increase in their single-electron reduction potential [27,28], i.e. the rate of their single-electron enzymatic reduction initiating 'redox cycling' [26–28].

The analysis of data of Tables 1 and 2 reveals the absence of the correlations between polyphenol log $cL_{50}$ and the $E^1$, or $E_{p/2}$, or $\Delta Hf_{1-3}$ of the polyphenols, since $r^2$ varies in the range 0.0254–0.2011. The increase of cytotoxicity of gallic acid derivatives upon an increase in their lipophilicity (Fig. 2) prompted us to introduce log $P$ as the second parameter for the correlation studies. Further, it was assumed that $E^1$ for the derivatives of gallic acid (compounds 7–11, Table 1) is equal to 0.56 V, and that $E^1$ for chlorogenic acid is equal to 0.54 V, as for caffeic acid. The resulting Eq. 4 was character-

Table 2
Enthalpies of reaction of single-electron oxidation ($\Delta Hf_1$ and $\Delta Hf_2$) and two-electron oxidation ($\Delta Hf_3$) of flavonoids and polyphenols, calculated using DFT B3LYP/6-311G**//HF//3-21G* method. $\Delta Hf_{1-3}$ were obtained from Eqs. 1–3, and correspond to the formation of o-semiquinone or o-quinone products, unless specified otherwise.

| Number | Compound | $\Delta Hf_1$ (kcal/mol) ($-2H^+$, $-e^-$) | $\Delta Hf_2$ (kcal/mol) ($-H^+$, $-e^-$) | $\Delta Hf_3$ (kcal/mol) ($-2H^+$, $-2e^-$) |
|---|---|---|---|---|
| 1 | Quercetin[a] | 407.3 | 81.4 | 155.4 |
| 2 | Morin[a] | 408.9 | 83.9 | 164.7 |
| 3 | Kaempferol[a] | 414.4 | 88.4 | 162.5 |
| 4 | Taxifolin | 425.0 | 82.3 | 158.3 |
| 5 | Hesperetin | 429.5 | 89.6 | – |
| 6 | Naringenin | 425.0 | 90.8 | – |
| 7 | Gallic acid | 414.9 | 78.9 | 157.0 |
| 8 | Methylgallate | 416.2 | 78.3 | 156.4 |
| 9 | Ethylgallate | 416.2 | 78.2 | 156.2 |
| 10 | Butylgallate | 416.3 | 77.9 | 155.8 |
| 11 | Octylgallate | 416.2 | 78.2 | 156.2 |
| 12 | Caffeic acid | 417.6 | 81.2 | 160.5 |
| 13 | Chlorogenic acid | 418.8 | 80.8 | 160.0 |

[a] *p*-Quinomethide products (Fig. 3).

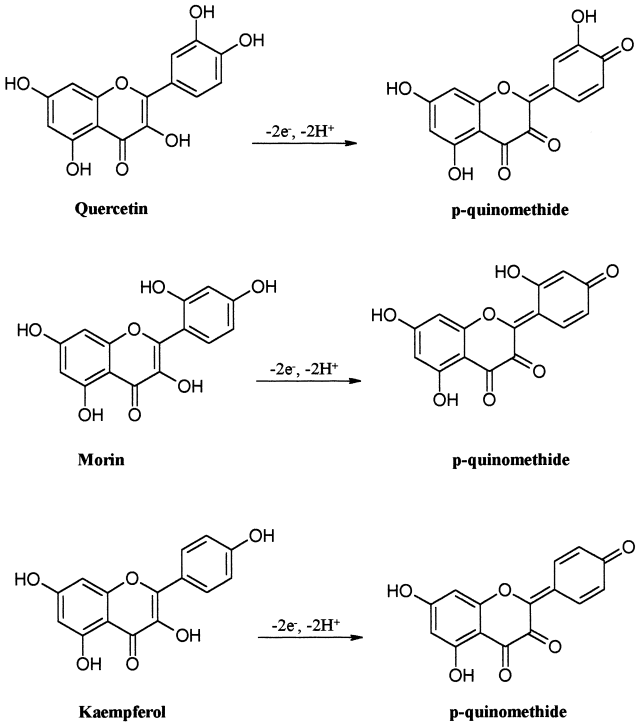


Fig. 3. Structures of two-electron oxidation products of quercetin, morin and kaempferol.

ized by $r^2 = 0.7590$, however, the dependence of log $cL_{50}$ on $E^1$ was relatively scattered:

$$\log cL_{50}\ (\mu M) = 3.574 \pm 0.4109 - (0.4781 \pm 0.5669)\ E^1\ (V)$$
$$-(0.3618 \pm 0.0733) \log P \quad (4)$$

In our understanding, this might be attributed to the evidently overestimated values of $E^1$ for morin and kaempferol (Table 1), calculated from the pulse radiolysis data [22]. Evidently, the pulse radiolysis data for morin, kaempferol and hesperetin are in marked contrast to their electrochemical characteristics, i.e. $E_{p/2}$ values and reversibility (Table 1).

Alternatively, we may observe a correlation between cytotoxicity, $E_{p/2}$ and log $P$, clearly pointing to an increase in cytotoxicity upon increase in ease of electrochemical oxidation of polyphenols, and their lipophilicity (Fig. 4):

$$\log cL_{50}\ (\mu M) = (2.7829 \pm 0.2339) + (1.2734 \pm 0.4715)\ E_{p/2}\ (V)$$
$$-(0.3438 \pm 0.0582) \log P\ \ (r^2 = 0.8129) \quad (5)$$

Although in general, the increase in $E_{p/2}$ parallels the increase in $E^1$, the $E_{p/2}$ values are not entirely adequate substitutes for $E^1$: (i) in reversible or quasi-reversible cyclic voltammograms where both $E_{pa}$ and $E_{pc}$ are present (corresponding to oxidation of polyphenols, and to re-reduction of quinone or quinomethide products, respectively), $E_{p/2}$ defines the energetics of a net two-electron transfer [13]; (ii) the irreversible electrooxidation characterized by the absence of $E_{pc}$ (isomerization of oxidation products into electroinactive species) may proceed at certain overvoltage, and $E_{p/2}$ (Table 1) may not represent the reversible redox potential described by the Nernst equation. On the other hand, the enthalpy of single-electron oxidation of polyphenols increases upon increase in $E^1$ [22]. For these reasons, we have used additional evidence for QSAR between the cytotoxicity and ease of (electro)oxidation of polyphenols, obtaining analogous correlations (Eqs. 6 and 7) between log $cL_{50}$, log $P$ and calculated enthalpies of single-electron oxidation of polyphenols ($\Delta Hf_1$ and $\Delta Hf_2$, Table 2) (data not shown):

$$\log cL_{50}\ (\mu M) = (-8.5079 \pm 4.8178) + (0.0281 \pm 0.0115)\cdot$$
$$\Delta Hf_1\ (kcal/mol) - (0.3310 \pm 0.0609)\ log\ P \quad (r^2 = 0.7977), \quad (6)$$

$$\log cL_{50}\ (\mu M) = (1.2965 \pm 1.4946) + (0.0234 \pm 0.0179)\cdot$$
$$\Delta Hf_2\ (kcal/mol) - (0.3483 \pm 0.07045)\ log\ P \quad (r^2 = 0.7254), \quad (7)$$

On the other hand, in the analogous two-parameter Eq. 8, the dependence of log $cL_{50}$ on $\Delta Hf_3$ was relatively scattered:

$$\log cL_{50}\ (\mu M) = (0.3445 \pm 4.594) + (0.0181 \pm 0.0288)\cdot$$
$$\Delta Hf_3\ (kcal/mol) - (0.3510 \pm 0.0695)\ log\ P \quad (r^2 = 0.7816). \quad (8)$$

Our findings imply that although prooxidant events are important in polyphenol cytotoxicity to HL-60 cells, it does not depend entirely on their thermodynamic parameters, and that an increase in polyphenol lipophilicity may also increase their cytotoxicity. Evidently, the polyphenols have to pass the cell membrane and enter the cell before exerting their toxic effects. This may be supported by the fact that irrespective of the thermodynamical parameter used ($E_{p/2}$, $Hf_1$ or $Hf_2$), the proportionality coefficient $\Delta$log $cL_{50}/\Delta$log $P$ varies insignificantly, in the range $-0.33$ to $-0.35$ (Eqs. 5–8). The toxicity of polyphenols is relatively insensitive to their redox potential, since the coefficient $\Delta$log $cL_{50}/\Delta E_{p/2}$ (Eq. 5) is close to 1.3 $V^{-1}$, whereas for redox cycling quinones and aromatic nitro

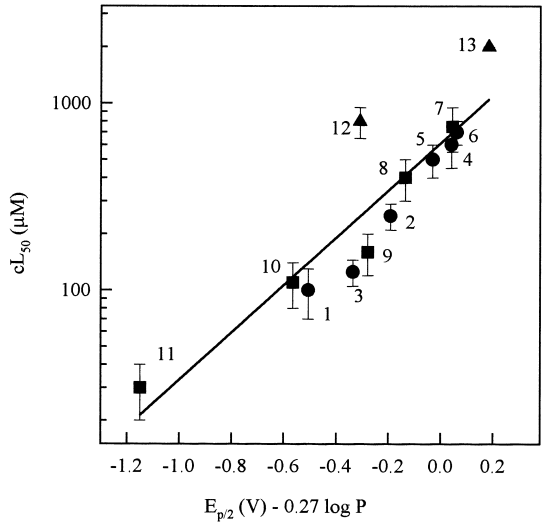


Fig. 4. The dependence of polyphenol cytotoxicity on their voltammetric midpoint potential ($E_{p/2}$) and octanol/water partition coefficient ($P$) according to a multiparameter Eq. 5. The numbers of compounds are taken from Table 1.

compounds, this coefficient is close to $-10\ V^{-1}$ [25–27]. Probably, other modes of polyphenol action may contribute to their toxicity as well. Nevertheless, our data provide a diagnostic tool for prediction of cytotoxicity of polyphenolic compounds. This is especially important in relation to increased toxicity of certain polyphenols, e.g. epigallocatechin gallate [9] or derivatives of gallic acid [20] to malignant and transformed cells as compared to their normal counterparts. Interestingly, cell malignization or transformation is often accompanied by a decrease in activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) [29], which increases the cell sensitivity to prooxidant compounds [29,30]. On the other hand, the use of polyphenolic compounds may represent a novel approach in the treatment of anthracycline-resistant acute myelogenous leukemia [17,31]. Our preliminary studies of a previously established anthracycline-resistant HL-60 cell line [17] indicate that it retains almost the same sensitivity to polyphenolic compounds (quercetin, $cL_{50} = 50 \pm 10$ μM; morin, $cL_{50} = 280 \pm 50$ μM; caffeic acid, $cL_{50} = 890 \pm 150$μM) as its anthracycline-sensitive counterpart (Table 1).

*Acknowledgements:* This work was supported in part by EC Grant IC15CT961004 and the Swedish Cancer Society. The generous assistance of the laboratory of Professor Christer Paul (KFC Novum, Sweden) is gratefully acknowledged. We thank Advanced Chemistry Development Inc. (Toronto, Ont., Canada) for their generous gift of ACD logP (version 1) software.

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