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METAL-CHELATING PROPERTIES, ELECTROCHEMICAL BEHAVIOR, SCAVENGING AND CYTOPROTECTIVE ACTIVITIES OF SIX NATURAL PHENOLICS

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Chelation, electrochemical, antioxidant and cytoprotective properties of six phenolics – cynarin and caffeic, chlorogenic, ferulic, protocatechuic and rosmarinic acids were studied on the following models: (i) chelation of transition metals, (ii) quenching of the diphenylpicrylhydrazyl radical (DPPH), (iii) determination of half-wave potential, (iv) erythrocytres or mitochondrial membranes damaged by *tert*-butyl hydroperoxide (*t*BH) and (v) a primary culture of rat hepatocytes intoxicated by Cu(II) and Fe(III) or *t*BH. All phenolics suppressed cell membrane damage induced by transition metals or *t*BH. The protectivity correlated with their capacity to bind transition metals, to scavenge DPPH radical and with the value of half-wave potentials. In *in vitro* assays, the most promising was rosmarinic acid.

INTRODUCTION

Protocatechuic (PA), ferulic (FA) and caffeic acid (CA) (C_6–C_3) are secondary metabolites in all higher plant genera[1,2]. Their precursor is phenylalanine with the exception of rosmarinic acid (RA) where one of the two C_6–C_3 structural units is derived from tyrosine. In plants, these acids occur mostly as esters, for instance caffeic acid as chlorogenic acid (CHA) and cynarin (CY), or bound to biopolymers. One of the physiological functions of these acids is the protection of plant tissues against the toxic effects of radicals. They represent an important group of dietary phytochemicals. In the literature there is a plethora of data assessing the chemoprotective potential of phenolic acids[3]. However, the results reflect different experimental approaches and methodologies and in some cases they are hard to compare. In this paper, we examine a series of six phenolics (PA, CA, FA, CHA, RA, CY) using methods: evaluation of their chelation activity, scavenging of $HO^{\cdot}$ radical, reduction ability, inhibition of lipid peroxidation, and cytoprotectivity in order to correlate chemoprotective behaviour with structures.

MATERIALS AND METHODS

Phenolic Acids and Reagents

Protocatechuic acid (3,4-dihydroxybenzoic acid; PA), *trans*-ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid; FA) chlorogenic acid (3-caffeoylquinic acid; CHA) and quercetin (QE) were obtained from Sigma-Aldrich (Germany). *trans*-Caffeic (3-(3,4-dihydroxyphenyl)-2-propenoic acid; CA) and (*R*)-(+)-rosmarinic (O-caffeoyl-(3,4-dihydroxyphenyl)lactic acid; RA) acids were isolated from *Prunella vulgaris*[4]. Cynarin (1,5-dicafeoylquinic acid; CY) was kindly donated by Dr. J. Slanina (Brno, Czech Republic). *tert*-Butyl hydroperoxide (*t*BH) was from Merck (Germany). Metalcaptase® 150 (penicillamin) was from Heyl (Germany), Desferal was from CIBA-GEIGY (Switzerland). All other chemicals used were obtained from Pliva-Lachema (Czech Republic) and Sigma-Aldrich. All were of analytical grade.

Spectrophotometric Equilibrium Measurements

Interactions of phenolics (PA, CA, FA, CHA, RA and CY), the arising complexes with transition metals (Cu(II) and Fe(III)), and stoichiometry were studied spectrophotometrically with metal ion concentrations ($Cu(ClO_4)_2 \cdot 6H_2O$, $FeCl_3$) 2×10^{-6} – 5×10^{-4} M, and ligand concentration of 5×10^{-5} M at pH 7.4. Phosphate buffer solution (0.2 M) – saline (0.15 M NaCl) was used in order to match reaction conditions used for cell culture experiments. The composition of the complex was determined from the absorbance/metal ion concentration relationship $A = f(c_{Metal})c_{Ligand}$.

Scavenging Activity of DPPH Radical

The antioxidant activity of test compounds was determined on the basis of the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (ref.[5]) Aliquots of 0.75 mL of a methanolic solution containing test compound (0.01–0.10 mM) were added to 1.5 mL of methanolic DPPH solution (20 mg.L^{-1}). Absorbance at 517 nm was determined after 5 min, and percent inhi-

bition activity IC_{50} values denote the concentration of sample required to scavenge 50 % DPPH radicals.

Cyclic Voltammetry

Cyclic voltammetry was carried out with Potentiostat/Galvanostat Model 273 (EG&G, Princetown, USA). A three-electrode system with a working and auxiliary electrode of glassy carbon and platinum and a saturated calomel reference electrode (SCE) was used. The test compounds were dissolved to concentrations of 1×10^{-4} M in sodium-phosphate buffer of pH 7.4. The solutions were purged with Ar and held under constant Ar flux during the electrochemical experiments. The cyclic voltammograms (CV) were recorded at 21 ± 2 °C. Half-wave potentials (E_{pa}) were measured at a scan rate of 200 mV.s^{-1} unless otherwise indicated. The working electrode was polished with aluminium oxide prior to each electrochemical measurement, because polymeric film formation inactivated the electrode after each scan. Since the surface cannot be reproduced identically each time, the E_{pa} value depended on each pre-treatment and for a given compound varied up to 0.05 V between measurements.

Animals

Three-month-old male Wistar rats with a body weight of 190–210 g were used for the preparation of erythrocytes, hepatocytes and liver mitochondria. Rats were supplied with food and water *ad libitum* and exposed to a 12 h light-dark cycle.

Haemolysis of Rat Erythrocytes

Rat erythrocytes were separated from heparinized blood by centrifugation (500 × *g*; 15 min; 22 °C). Erythrocytes (10 % v/v) were incubated in phosphate buffer saline (PBS) buffer (pH 7.4) with the test compound (0.1, 0.25 and 0.5 mM) and *t*BH (1.2 mM) for 4 h at 37 °C. After incubation the erythrocyte haemolysis was measured at 540 nm as released haemoglobin[6].

Lipid Peroxidation Assay

The mitochondrial fraction was prepared from rat liver homogenate and its protein concentration was determined by the Lowry method[7]. Mixtures of mitochondrial suspension (0.8 mL; 4 mg total protein/mL) and test compounds dissolved in DMSO (0.1 mL; 0.1–25.0 mM) were incubated in a shaking water bath at 37 °C and lipid peroxidation was induced by 0.02 mL TBH, final concentration 1.5 mM (ref.[8]) for 1 h. The products of lipid peroxidation (TBARS) were determined by reaction with thiobarbituric acid[9].

Rat Hepatocyte Culture

Rat hepatocytes were isolated by two-step collagenase perfusion of liver[10]. The cells were then resuspended in William's medium E, supplemented with glutamine (2 mM), streptomycin (0.1 g/L), penicillin G (10 000 U/L), dexamethasone (1 µM) and insulin (0.1 µM), and washed by centrifugation (50 × *g*; 1 min). Using the trypan blue exclusion test, cell viability was ~90%. Cell suspensions in medium supplemented with 10 % bovine serum were seeded on collagen type I pre-coated 24-well plates at a density of 1×10^5 cells/cm^2 and incubated at 37 °C in an atmosphere containing 5 % CO_2. The medium was changed for a serum-free one after 3 h. Rat hepatocytes were preincubated for 4 h with phenolic acids (0.025–1.0 mM), then exposed to toxic concentrations of 20 µM Fe(III) or Cu(II) for 20 h, respectively. For *t*BH intoxication (0.5 mM, 90 min), cells were preincubated for 20 h with test compounds. After the experiment, the culture medium was removed and assayed for extracellular lactate dehydrogenase activity (LDH) and thiobarbituric acid reactive substances (TBARS). The level of intracellular reduced glutathione (GSH) was determined according to Sedlak and Lindsay (1968) (ref.[11]).

Statistical Analysis

Values were expressed as mean ± s.e.m. Each experiment was performed in triplicate and measured three times. Student *t*-test was used for statistical analysis.

RESULTS

The interaction of phenolic compounds with Cu(II), Fe(III) and stoichiometry of formed complexes was studied spectrophotometrically using molar ratios method at pH 7.4. Except for FA, all phenolics formed Cu(II) complexes; apart from PA and FA they formed Fe(II) complexes. As for Fe(III) complexes, they are formed by RA, CHA, CY. The formation of complexes was manifested by the appearance of an absorption band in the region 308–375 nm. The dependence of absorbance on the concentration of metal ions shows: (i) the composition of the complex depends on the metal/ligand ratio and there are two types of complexes formed with the stoichiometry metal : ligand 1 : 1 and 1 : 2; (ii) complexes of both types are stable. Estimation of the stability constant carried out for a 1 : 1 complex from equimolar solutions ($c_M \cong c_L$) considering the difference between the curves of a quantitative and of an actual reaction yields the constants in the range 2×10^5 – 5×10^6 for all ligands and both metals, see Table 1. After the addition of EDTA to all complexes, the spectra of non-complexed phenolics were restored.

The scavenging of the DPPH radical is a simple model reaction providing relevant information about the ability of acids to scavenge free hydroxyl radicals. The adventitious data are then correlated with their antilipoperoxidative properties *in vitro* models. However there are conflicting data on phenolic acids in the literature. The order of scavenging effectiveness found here was: CA > RA > CY > CHA > PA >> FA >> QE (Table 2). Statistically significant different scavenging effect of phenolic acids compared to quercetin can be explained by the presence of a carboxyl group rather than by the number and position of phenolic groups. We found similar IC_{50} for CA and RA. These differ from Chen *et al.*[12] who published that RA was significantly better than CA.

The half-wave oxidation potentials (E_{pa}) provide evidence for reduction properties. Low values imply strong reducing power. The anodic current reflects the concentration of the antioxidant and the number of electrons transferred in the reduction. This can be used for quantification. The low values of anodic potentials for CA, RA, and CHA were determined. They were similar to the value of QE (Table 2).

Phenolics were able to prevent lipid peroxidation of erythrocytes. The protective effect was dose dependent. While at 0.1 mM the most effective acids were PA and CA, at 0.25 mM it was RA and CA which showed the highest efficiency. CY and CHA were least effective over the whole concentration range (Table 3).

The results for antilipoperoxidative activity of phenolics on rat mitochondria damaged by *t*BH are shown in Table 4. RA was markedly active (RA > CA > CY > CHA > FA = PA).

The cytoprotective effect of phenolic acids was followed on primary rat hepatocyte cultures intoxicated by transition metals, Cu(II) and Fe(III), and *t*BH. The level of TBARS as a parameter of lipid peroxidation damage (Table 5), the level of extracellular LDH as a parameter of cellular damage (Table 6) and of intracellular GSH as a parameter of redox cell status (Table 7) were also measured. Cytoprotective effect of phenolic acids on metal intoxicated hepatocytes was compared with that of therapeutically used metal chelators, desferal and penicillamin. All phenolic acids were able to decrease the level of lipid peroxidation on all three models. On hepatocytes intoxicated by Fe(III), CA, RA and CY (0.5 and 1.0 mM) decreased lipid peroxidation comparably to desferal, but desferal was more effective at lower concentrations (0.1 mM). Phenolic acids protected hepatocytes intoxicated by Cu(II) better than penicillamin (RA > CA = CHA = CY = PA > FA), but CHA and CY (1.0 mM) stimulated TBARS production. A surprisingly potent protective effect of PA (1.0 mM) was found. On a model of hepatocytes intoxicated by *t*BH, all tested compounds inhibited the production of TBARS (RA > CA > CY = PA = FA > CHA).

Assessed by the LDH parameter, all phenolics were able to decrease cellular damage on all three models as well (Table 6). Hepatocytes intoxicated by Fe(III) were protected by phenolic acids in the following order: CY > CA > RA = CHA > PA > FA. CY and desferal were comparably effective at lower concentrations (0.1 mM). Similarly to TBARS parameter, phenolic acids protected hepatocytes intoxicated by Cu(II) better than penicillamin (RA = CY = CA > CHA > PA = FA). On a model of hepatocytes intoxicated by *t*BH all phenolic acids decreased the release of LDH in the medium (RA > CA > CY = CHA > PA > FA).

Phenolics were able to eliminate intracellular GSH decrease on all three models, see Table 7. Hepatocytes intoxicated by Fe(III) were protected by phenolic acids in this order: CY > CA > CHA > RA > PA > FA. As with the LDH parameter, CY protected comparably to desferal. In the case of Cu(II) intoxication, all phenolic acids except for FA protected intracellular GSH level similarly or better than penicillamin (CY > RA = CHA > CA = PA > FA). On hepatocytes intoxicated by *t*BH, phenolic acids eliminated the decrease of GSH (CHA > RA > CY > CA > PA > FA).

Table 1. Complexes of phenolic compounds with transition metals.

Compound	Absorption maximum (nm) Non-complexed	Cu(II) complex	Fe(II) complex Metal : Ligand Stability constant	Fe(III) complex
PA	289	308, light yellow 1 : 1, 1 : 2 4.35×10^{6}	–	–
CA	312	346, colourless 1 : 1, 1 : 2 1.68×10^{6}	358, colourless 1 : 1, 1 : 2 –	–
RA	325	372, light yellow 1 : 1, 1 : 2 2.44×10^{5}	354–360, blue 1 : 1, 1 : 2 2.25×10^{5}	372, blue 1 : 1, 1 : 2 –
CHA	324	372, light yellow 1 : 1, 1 : 2 6.58×10^{5}	372, blue 1 : 1, 1 : 2 6.78×10^{5}	375, blue 1 : 1, 1 : 2 8.75×10^{5}
CY	325	375, light yellow 1 : 1, 1 : 2 2.35×10^{6}	358, blue 1 : 1, 1 : 2 1.84×10^{6}	358, blue 1 : 1, 1 : 2 6.35×10^{5}

FA did not display chelating activity.

Table 2. IC_{50} values for the scavenging activity and redox potential of phenolic acids.

Compound	IC_{50} (μM)[a]	E_{pa} (V)
PA	8.87 ± 0.64[b]	0.46
CA	4.75 ± 0.25[b]	0.10
FA	37.10 ± 2.84[b]	0.39
RA	5.15 ± 0.15[b]	0.13
CHA	6.94 ± 0.47[b]	0.11
CY	5.00 ± 0.09[b]	0.33

IC_{50} and E_{pa} values for QE were 66.3 ± 3.7 μM and 0.16 V, respectively.
[a] IC_{50} values were determined graphically from dose-activity curves.
[b] Significantly more active than reference substance quercetin ($p < 0.01$).

Table 3. Effect of phenolic acids and cynarin on rat erythrocyte haemolysis induced by *t*BH.

Compound	Concentration (mM)		
	0.10	0.25	0.50
	Inhibitory activity (%)		
PA	64.3 ± 4.1	72.0 ± 6.4	83.2 ± 7.3
CA	55.8 ± 5.4	84.8 ± 8.5	86.7 ± 8.7
FA	30.7 ± 2.2	62.7 ± 4.8	73.3 ± 6.4
RA	37.1 ± 2.1	91.4 ± 9.0	91.7 ± 9.0
CHA	9.4 ± 0.9	16.5 ± 1.2	54.5 ± 3.9
CY	10.2 ± 1.5	17.2 ± 2.0	56.7 ± 4.5

Control value was 92.7 ± 9.1 and *t*BH (1.2 mM) value was 0.4 ± 0.0.

Table 4. Antilipoperoxidative effect of phenolic acids and cynarin on lipoperoxidation of rat mitochondria induced by *t*BH.

Compound	IC_{50} (mM)
PA	> 2.0
CA	0.59 ± 0.04
FA	> 2.0
RA	0.09 ± 0.01
CHA	2.37 ± 0.15
CY	1.96 ± 0.13

Table 5. Effect of phenolic acids, desferal and penicillamin on extracellular level of TBARS in rat hepatocyte primary cultures intoxicated by 20 μM Fe(III), 20 μM Cu(II) and 0.5 mM *t*BH.

Compound	Dose (mM)	Protection (%) Fe(III)	Cu(II)	*t*BH
PA	0.1	0.0 ± 0.9	77.9 ± 4.7	0.9 ± 0.1
	0.5	42.6 ± 2.1	88.9 ± 5.6	10.5 ± 0.9
	1.0	63.9 ± 3.4	99.6 ± 7.2	37.1 ± 1.7
CA	0.1	59.7 ± 2.8	88.3 ± 6.7	32.3 ± 2.4
	0.5	99.6 ± 5.8	88.5 ± 7.0	55.2 ± 3.4
	1.0	99.9 ± 7.0	93.4 ± 6.5	75.9 ± 5.1
FA	0.1	0.0 ± 0.7	49.9 ± 2.4	2.3 ± 0.4
	0.5	9.9 ± 1.2	79.3 ± 4.1	10.6 ± 1.1
	1.0	23.6 ± 1.8	89.7 ± 6.8	39.7 ± 3.1
RA	0.1	58.5 ± 3.1	96.4 ± 7.2	43.4 ± 3.2
	0.5	96.3 ± 4.9	99.9 ± 6.7	63.5 ± 5.2
	1.0	99.8 ± 6.4	100.1 ± 6.0	89.0 ± 7.6
CHA	0.1	2.4 ± 0.6	97.1 ± 5.4	9.8 ± 0.8
	0.5	86.8 ± 7.2	92.0 ± 6.1	14.9 ± 1.6
	1.0	89.6 ± 6.3	81.4 ± 4.5	25.0 ± 1.9
CY	0.1	75.1 ± 5.4	97.7 ± 4.9	7.7 ± 0.7
	0.5	99.3 ± 7.8	95.0 ± 4.2	16.0 ± 1.3
	1.0	99.7 ± 6.4	89.8 ± 9.3	35.3 ± 2.6
DES	0.1	97.7 ± 6.2		
	0.5	99.9 ± 7.0		
	1.0	96.1 ± 7.2		
PEN	0.1		3.7 ± 0.3	
	0.5		9.5 ± 0.8	
	1.0		30.3 ± 3.0	

Values are mean ± s.e.m. of three times repeated experiments.
TBARS control values were for intoxication by: Fe(III) 0.005 pmol/10^6 cells (0.10 ± 0.03 %), Cu(II) 0.005 nmol/10^6 cells (0.2 ± 0.08 %) and *t*BH 0.08 nmol/10^6 cells (2.1 ± 0.2 %).
TBARS toxicity values were for intoxication by: Fe(III) 5.17 nmol/10^6 cells (100.2 ± 1.1 %), Cu(II) 5.36 nmol/10^6 cells (100.0 ± 0.8%) and *t*BH 4.15 nmol/10^6 cells (100.8 ± 0.3 %).

DISCUSSION

Phenolic acids are contained in a number of agricultural and medicinal plants. They are respected phytochemicals with prebiotic, antioxidant, chelation and anti-inflammatory effects. Their toxicity is low and they are considered non-toxic. Pharmacologically best characterized is CA with its esters and RA. As for the antioxidant/scavenging activity of these compounds, there are wide ranging reports in the literature which rarely match. On the other hand, chelation effect of these substances, characterized by the efficacy to bind free copper and iron, have not been thoroughly studied so far. Our results show that in *in vitro* assays, phenolic acids displayed the same or higher cytoprotectivity against the ROS formation than desferal (Fe intoxication) or penicillamin (Cu intoxication). In the whole concentration range tested (0.1–1.0 mM), the most efficient substances were acids/their esters with a catechol group and a double bond in the side chain. A distinctly superior chelation effect of CA esters (i.e. CHA and CY) compared to CA was found in iron intoxication. In copper intoxication, tested compounds were better protectants than penicillamin. In erythrocytes and mitochondrial membranes damaged by *t*BH, CA and RA were more effective protectants than CHA, CY, PA, and FA. In this assay, the magnitude of the protective effect agreed with the IC_{50} of the DPPH quenching and with E_{pa} potentials of these substances.

Like many other substances, phenolic acids, too, display a bimodal effect. In the concentration range (0.1–1.0 mM) that we used, the acids behaved as antioxidants with chemoprotective effects. In contrast, PA at concentrations above 2.5 mM showed *in vitro* pro-oxidation activity linked to the decrease in reduced glutathione and increase in cellular TBARS[13]. Nakamura *et al.*[14] described hepatotoxic and nephrotoxic effect of PA in mice at doses of 500 mg/kg, linked to decrease in GSH. At higher concentrations of CA and FA, their pro-oxidation effect on Cu^{2+}-induced LDL oxidation has been described[15, 16] as well.

Table 6. Effect of phenolic acids, desferal and penicillamin on extracellular level of LDH in primary rat hepatocyte culture intoxicated by 20 μM Fe(III), 20 μM Cu(II) and 0.5 mM *t*BH.

Compound	Dose (mM)	Protection (%) Fe(III)	Cu(II)	*t*BH
PA	0.1	21.9 ± 2.1	34.1 ± 3.2	0.3 ± 0.3
	0.5	67.0 ± 5.6	48.5 ± 3.9	8.4 ± 1.2
	1.0	91.0 ± 7.3	60.8 ± 5.5	39.8 ± 2.9
CA	0.1	89.6 ± 7.3	37.1 ± 3.2	25.5 ± 2.4
	0.5	99.4 ± 8.4	80.6 ± 6.8	53.8 ± 4.3
	1.0	106.2 ± 8.6	85.1 ± 7.6	68.3 ± 5.7
FA	0.1	26.8 ± 2.1	30.7 ± 2.4	0.0 ± 0.2
	0.5	57.2 ± 4.3	40.2 ± 3.6	4.7 ± 0.6
	1.0	74.6 ± 6.3	54.0 ± 4.3	17.4 ± 1.8
RA	0.1	81.5 ± 4.3	32.5 ± 2.9	41.0 ± 3.8
	0.5	96.0 ± 8.3	77.5 ± 6.8	79.3 ± 6.5
	1.0	97.9 ± 7.8	104.0 ± 9.9	99.7 ± 8.7
CHA	0.1	78.7 ± 6.9	41.0 ± 3.2	28.3 ± 2.4
	0.5	103.4 ± 9.8	61.4 ± 5.3	38.1 ± 2.9
	1.0	112.0 ± 8.9	88.1 ± 7.2	53.5 ± 4.3
CY	0.1	101.6 ± 10.1	56.5 ± 4.3	19.8 ± 2.4
	0.5	107.0 ± 9.8	84.6 ± 6.8	42.2 ± 3.1
	1.0	104.8 ± 8.9	101.1 ± 8.3	48.8 ± 3.9
DES	0.1	101.5 ± 9.8		
	0.5	106.4 ± 9.2		
	1.0	101.5 ± 9.4		
PEN	0.1		0.0 ± 0.4	
	0.5		26.0 ± 2.0	
	1.0		37.9 ± 3.4	

Values are mean ± s.e.m. of three times repeated experiments.
LDH control values were for intoxication by: Fe(III) 10.34 ± 0.12 nkat/10^6 cells (25.8 ± 0.3 %), Cu(II) 9.57 ± 0.18 nkat/10^6 cells (20.9 ± 0.4 %) and *t*BH 7.57 ± 0.76 nkat/10^6 cells (14.9 ± 1.5 %).
LDH toxicity values were for: Fe(III) 40.04 ± 3.36 nkat/10^6 cells (100.4 ± 8.4 %), Cu(II) 45.7 ± 3.5 nkat/10^6 cells (100.5 ± 7.7 %) and *t*BH 50.83 ± 5.40 nkat/10^6 cells (100.4 ± 10.6 %).

In conclusion it can be said: when evaluating a combined effect of phenolic acids on living organisms, several factors should be considered such as (i) results of *in vitro/in vivo* studies, (ii) size of the daily dose, (iii) bioavailability, and (iv) potential toxicity of phenolic acid metabolites.

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Table 7. Effect of phenolic acids, desferal and penicillamin on GSH level in primary rat hepatocyte culture intoxicated by 20 mM Fe(III), 20 mM Cu(II) and 0.5 mM *t*BH.

Compound	Dose (mM)	Protection (%)		
		Fe(III)	Cu(II)	*t*BH
PA	0.1	0.2 ± 0.1	0.6 ± 0.3	2.0± 0.3
	0.5	64.9 ± 4.4	13.2 ± 1.1	5.9 ± 0.5
	1.0	66.4 ± 6.3	16.2 ± 1.5	14.8 ± 1.9
CA	0.1	95.3 ± 8.3	0.9 ± 1.2	3.9 ± 1.4
	0.5	100.2 ± 8.5	12.1 ± 1.0	16.7 ± 1.1
	1.0	112.8 ± 8.6	10.8 ± 1.6	28.5 ± 2.7
FA	0.1	0.1 ± 1.1	0.5 ± 1.4	0.2 ± 0.2
	0.5	11.5 ± 1.1	6.8 ± 0.9	0.8 ± 0.8
	1.0	9.1 ± 1.3	3.5 ± 0.6	2.9 ± 0.8
RA	0.1	67.3 ± 5.3	21.1 ± 2.3	8.1 ± 0.8
	0.5	82.9 ± 4.6	34.9 ± 2.3	24.8 ± 2.1
	1.0	75.9 ± 6.8	68.2 ± 5.8	49.9 ± 3.7
CHA	0.1	33.6 ± 4.9	22.9 ± 2.9	6.7 ± 1.4
	0.5	108.9 ± 7.9	25.2 ± 2.1	15.9 ± 1.3
	1.0	94.7 ± 8.9	80.8 ± 7.2	51.35 ± 3.3
CY	0.1	95.6 ± 10.1	42.5 ± 4.3	8.2 ± 1.4
	0.5	100.9 ± 6.6	56.5 ± 5.4	18.2 ± 1.2
	1.0	101.8 ± 8.9	78.4 ± 7.3	45.8 ± 3.9
DES	0.1	101.5 ± 9.8		
	0.5	104.5 ± 8.5		
	1.0	101.5 ± 9.4		
PEN	0.1		7.7 ± 1.4	
	0.5		9.4 ± 1.3	
	1.0		3.9 ± 2.4	

Values are mean ± s.e.m. of three times repeated experiments.
GSH control values were for: Fe(III) 40.1 ± 2.6 nmol/10^6 cells (100.0 ± 6.4 %), Cu(II) 42.4 ± 2.5 nmol/10^6 cells (100.0 ± 5.9 %) and *t*BH 41.8 ± 3.0 nmol/10^6 cells (100.3 ± 7.2 %).
GSH toxicity values were for: Fe(III) 4.2 ± 0.3 nmol/10^6 cells (10.4 ± 0.8 %), Cu(II) 10.5 ± 0.8 nmol/10^6 cells (26.1 ± 1.9 %) and *t*BH 20.4 ± 2.1 nmol/10^6 cells (50.9 ± 5.3 %).

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