Comparative Analysis of Molecular Properties and Reactions With Oxidants for Quercetin, Catechin and Naringenin

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Abstract

Flavonoids, a large group of secondary plant phenolic metabolites, are important natural antioxidants and regulators of cellular redox balance. The present study addressed the evaluation of the electronic properties of some flavonoids belonging to different classes: quercetin (flavonols), catechin (flavanols), and naringenin (flavanones) and their interactions with oxidants in the model systems of DPPH reduction, flavonoid autoxidation and chlorination. According to our *ab initio* calculations, the high net negative excess charges of the C rings and the small positive excess charges of the B rings of quercetin, catechin, and naringenin make these parts of flavonoid molecules attractive for electrophilic attack. The 3'-OH group of the B-ring of quercetin has the highest excess negative charge and the lowest energy of hydrogen atom abstraction for the flavonoids studied. The apparent reaction rates ($M^{-1}s^{-1}$, 20 ºC) and the activation energies (kJ/mol) of DPPH reduction are 4000±1000 and 23.0±2.5 in the case of quercetin, 1100±200 and 32.5±2.5 in the case of catechin, respectively. The stoichiometry of the DPPH – flavonoid reaction was 1:1. The activation energies (kJ/mol) of quercetin and catechin autoxidations were 50.8±6.1 and 58.1±7.2, respectively. Naringenin was not oxidized by the DPPH radical and air oxygen (autoxidation) and the flavonoids studied effectively prevented HOCl-induced hemolysis due to direct scavenging of hypochlorous acid (flavonoid chlorination). The best antioxidant quercetin has the highest value of HOMO energy, the planar structure and the optimal electron orbital delocalization on all phenolic rings due to C2=C3 double bond in the C ring (absent in catechin and naringenin).

Highlights

The 3'-OH group of quercetin has the lowest energy of hydrogen abstraction.

The C2=C3 bond provides planarity and electron orbital delocalization in quercetin.

The stoichiometry of the radical DPPH reduction by quercetin or catechin is 1:1.

Naringenin did not reduce DPPH and was not autoxidized.

Quercetin, catechin and naringenin effectively prevented HOCl-induced hemolysis.

1. Introduction

Oxidative stress, a result of overproduction of reactive oxygen/nitrogen/chlorine species and/or impairment in the cellular antioxidative defense system, has been considered as an important mechanism of cyto- and genotoxicity. There is a growing interest in searching for exogenous natural and synthetic antioxidants as therapeutic and pharmacological agents [1–5]. Flavonoids, a large group of secondary plant phenolic metabolites (phenolic acids, phenolic diterpenes, flavonoids, tannins, and coumarins being among them), are important natural antioxidants, free radical scavengers and regulators of cellular redox balance and redox signaling cascades [1–6]. Flavonoids are abundant components of human diet and about 10,000 flavonoids have been found in different natural plant sources. The total

amount of flavonoids consumed is estimated at hundreds of mg/day [3]. The basic flavonoid structure consists of two phenyl groups joined by a three carbon atom bridge. Flavonoids are classified into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones according to their chemical structures.

The molecular structure and mechanisms of antioxidative, cytoprotective and regulative activities of flavonoids as well as their potential use (tested both *ex vivo* and *in vivo*) are widely discussed [1–7]. Several mechanisms of action are involved in biological properties of flavonoids such as direct free radical scavenging, transition metal ion chelation, indirect upregulation of cellular antioxidant defense enzymes (such as glutathione S-transferase or UDP-glucuronosyl transferase), activation of survival genes, regulation of a number of signaling pathways and mitochondrial function, modulation of inflammatory responses, and anti-microbial action [5, 6]. Flavonoids directly bind many proteins and activate, inhibit, upregulate, or downregulate such cascades as protein kinases, AMPK, MAPK, NF-*k*B, the TGFβ-2/PI3K/AKT pathways, p53-mediated apoptotic events, the NF-E2-related transcription factor (NRF2)-mediated activation of genes (the Nrf2-Keap1 pathway), the major regulator of cytoprotective responses to oxidative stress [7, 8]. The flavonoids quercetin, kaempferol, and epicatechin, weak acids with a hydrophobic character, considerably prevent cellular reactive oxygen species (ROS) production by mitochondria ($IC_{50} \approx 1–2$ μM), inhibit redox enzymes, NAD(P)H oxidases, xanthine oxidases, monooxygenases, cyclooxygenases, and lipooxygenases [9].

Direct antiradical/antioxidant capacities of flavonoids are related to the redox properties of their easily oxidized phenolic hydroxyl groups and conjugated rings. Bors et al. were the first to claim three partial structures contributing to the radical-scavenging activity of flavonoids: the 3',4' - *ortho*-dihydroxyl structure in the B ring (catechol structure) as a radical target site (a), the C2 = C3-double bond with conjugation to the 4-oxo group in the C ring which is necessary for delocalization of an unpaired electron from the B ring (b), and the hydroxyl substituent at position 3 of the C ring which is necessary for enhancement of radical-scavenging activity (c) [10] (Fig. 1). The important role of intramolecular H-bonding was simultaneously claimed (d) [11, 12]. Quercetin, which belongs to the class of flavonols, contains all the four chemical structures that determine high antioxidant activity [13].

Recently we suggested that the biochemical effects of some flavonoids (quercetin, catechin, naringenin) were partially connected with modification of the membrane bilayer structure, fluidity, and hydration, as well as with flavonoid ability to regulate mitochondrial membrane permeability and to prevent oxidative stress in membrane compartments [14, 15]. In our experiments, the flavonoids considerably inhibited membrane lipid peroxidation (the IC_{50} values were equal to 9.7 ± 0.8 μM, 8.8 ± 0.7 μM, and 46.8 ± 4.4 μM in the case of quercetin, catechin and naringenin, respectively) and decreased glutathione oxidation in mitochondria and erythrocytes treated with tert-butyl hydroperoxide [14, 15]. The capacity of flavonoids (e.g. quercetin, baicalin, luteolin, hesperetin, gallocatechin gallate, epigallocatechin gallate, and scutellarein,) to inhibit key proteins involved in coronavirus infective cycle has recently been reviewed [16].

The mechanisms of direct antioxidant activity of polyphenols may be clarified via: 1. hydrogen atom transfer (HAT) and proton-coupled electron transfer (PCET) (the proton and electron are simultaneously transferred to the radical in one kinetic step), 2. single electron transfer followed by-proton transfer (SET-PT) (it is a two-step mechanism), 3. sequential proton loss electron transfer (SPLET) (it is a reverse mechanism with respect to SET-PT, relatively stable polyphenol anion formation after the proton loss is the first step of this mechanism), and 4. formation of stable adducts with radicals [17, 18]. In nonpolar environments (e.g., lipid bilayer), PCET is the only active process of free radical scavenging by flavonoids, and under nonacidic conditions, SPLET is the major mechanism and both the mechanisms take place in reactions against peroxyl or DPPH radicals [11, 19]. Simultaneously, flavonoids display pro-oxidant toxic effects associated with ROS generation via the catechol group oxidation to semiquinone radicals and quinones. The semiquinone radicals and quinones arylate protein thiols and form adducts with reduced glytathione [4].

Earlier the calculations of the structure and thermochemical parameters of flavonoids were widely performed using the methods of quantum chemistry [8, 17, 20–22]. The more reactive sites in quercetin molecule (Fig. 1), one of the most abundant and studied flavonoids, are the 4’- and 3’-OH groups (the catechol group) of the ring B, as well as the 3-OH group of the ring C through direct H-atom transfer were found. Participation of the 7-OH group, as an orientator of the reaction and not as a direct H-donor in radical scavenging, was revealed. The *o*-quinone (for quercetin unhydrate) and *o*-quinone and *p*-quinone (for quercetin dehydrate) derived from the 3’- and 4’- OH groups were suggested as the main oxidation products of quercetin [17]. It was supposed that the quercetin semiquinone radical arising after removal of the hydrogen atom of the 4’-OH group was the most stable radical and favored to deactivate other radicals via proton and/or electron transfer [18].

Since the electronic and molecular parameters are crucial for biochemical activity, the present study addressed parallel evaluation of the optimal molecular geometry and electronic properties of flavonoids and their reactions with free radicals/oxidants in the model systems of stable DPPH radical reduction and flavonoid autoxidations and chlorinations by hypochlorous acid. For analysis of the structure/activity relationships, we compared three flavonoid molecules representing different classes that are most abundant and possess high biochemical activity: quercetin (flavonols), catechin (flavanols or flavan-3-ols), and naringenin (flavanones) (Fig. 1). The chemical structure of lipophilic quercetin is characterized by the catechol (3’, 4’*o*-dihydroxy) group in the B ring, and the double bond in the C ring between C-2 and C-3 in conjunction with the 4-carbonyl group, as well as the 3-, 5- and 7- hydroxyl groups. The water - soluble catechin molecule does not have the 2, 3 double bond in conjunction with the 4-carbonyl group in the C ring and naringenin is a flavanon differing from the quercetin structure by the absence of the C2 = C3 double bond in the C ring and hydroxyl groups at the 3- position on the C ring and at the 3’- position on the B ring (Fig. 1). The parameters of quercetin, catechin, and naringenin molecules extend to a great number of flavonoid subclasses.

2. Materials And Methods

2.1. Chemicals

Quercetin, catechin, naringenin, 1,1-Diphenyl-2 picrylhydrazyl radical (DPPH), sodium hypochlorite (NaOCl), reduced glutathione, ascorbic acid, melatonin, and other chemicals were from Sigma-Aldrich (St Louis, MO, USA, or Steinheim, Germany). Methanol, ethanol and other organic solvents and reagents were of analytical grade, purchased from Reakhim (Moscow, Russia) and used without further purification. Flavonoids were used as freshly prepared stock solutions (15 mM) in ethanol. In preliminary experiments, we showed that ethanol at the concentration range used did not affect the parameters measured. All the solutions were made with water purified in the Milli-Q system.

2.2. Calculation of flavonoid molecular geometry and electronic properties

Flavonoid molecules were considered theoretically by semi empirical molecular orbital quantum chemical calculations. Preoptimization was performed by applying methods of molecular mechanics using MM + force field. The Austin Model 1 (AM1) semi-empirical method and the *Ab initio* method within Unrestricted Hartree-Fock (UHF) formalism in the self-consistent field approximation and algorithm Polak-Ribiere were considered to optimize fully the geometry of molecular systems [22, 23]. We performed all the calculations by using the HyperChem-8.0 software package (HyperCube, Inc.) for searching the conformations with minimum energy [http://www.hyper.com].

Energy of hydrogen atom abstraction was calculated as the difference of the Binding energies of the flavonoid molecule and the correspondent radical, formed after removal of hydrogen atom of each hydroxyl group.

2.3. Determination of the parameters of DPPH - flavonoid interaction

The radical scavenging activities of flavonoids against DPPH stable radical were evaluated by registration of the kinetics of its reduction. Briefly 10 µl of flavonoid at different concentrations (ethanol solutions) was added to 1990 µl of DPPH (100 µM in ethanol) at 10–40 ºC. The reduction of DPPH was monitored spectrophotometrically as its absorbance change at 520 nm using UV-VIS Spectrophotometer Jasco V-650 (Japan).

2.4. Flavonoid autoxidation and chlorination

Flavonoid autoxidation was monitored spectrophotometrically (UV-VIS Spectrophotometer Jasco V-650, Japan) over 3 h by recording progressive changes in the absorption spectra during flavonoid (50 µM) incubation in open tubes in the dark in ethanol / Na-phosphate buffer, pH 7.4, mixture (30% / 70%) at 10°C – 50°C under gentle stirring. We used phosphate buffered saline (PBS: 0.15 mol l^{-1} NaCl, 1.9 mmol l^{-1} KH_2PO_4, 8.1 mmol l^{-1} K_2HPO_4, pH 7.4). The prevention of flavonoid autoxidation by antioxidants was evaluated under incubation in the presence of 100 µM reduced gluthatione, ascorbic acid or melatonin.

For chlorination the flavonoids (50 μM) were incubated with 25, 50, 75, 100 μM hypochlorous acid (HOCl) in ethanol / Na-phosphate buffer, pH 7.4, mixture (30% / 70%) for 1 min at gentle stirring and 25°C and the absorption spectra were recorded.

2.5. HOCl – induced hemolysis. Effects of the flavonoids

Erythrocytes were isolated from heparinized rat blood by centrifugation (1500 r.p.m., 5 min at 4°C). After removal of plasma and the leukocyte layer, erythrocytes were washed three times with cold (4°C) phosphate buffered saline (PBS, pH 7.4). The erythrocytes were used immediately after isolation. The care and procedures performed on rats were approved by the Ethics Committee of the Institute of Biochemistry of Biologically Active Compounds, National Academy of Sciences, Belarus (Protocol No. 22/15 of 01.10.2015) and have been followed by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

At pH 7.4, the solution of sodium hypochlorite (NaOCl) contains a mixture of HOCl and NaOCl at a ratio of approximately 1:1 and is subsequently referred to as HOCl [24]. The susceptibility of erythrocytes to hypochlorous acid-induced damage was measured as the rate of hemolysis. Erythrocyte suspension (hematocrit 0.05%) was mixed with different concentrations of HOCl (5–75 μM) in PBC, pH 7.4, at 25°C, and the kinetic curves of hemolysis were determined spectrophotometrically (UV-VIS Spectrophotometer Jasco V-650, Japan) from the changes in the optical density of suspension at 670 nm and 25 °C in the presence or absence of flavonoids. Erythrocyte suspensions were preincubated with flavonoids for 5 min before HOCl was introduced.

2.6. Statistical analysis

The results of the experiments were expressed as the means of four or five replicates ± SEM. The differences between the values for the parameters measured in groups were analyzed using the Student's t-test or nonparametric Mann-Whitney test depending on the normality of values distribution in the groups. The normality of distribution was determined by Shapiro-Wilk test. Statistical analysis was conducted using the GraphPad Prism 6.0 software package (https://www.graphpad.com/quickcalcs). The results were assumed to be statistically significant compared to the control group values when P value was less than 0.05.

3. Results

We made an attempt to better understanding of the biochemical reactivity of the flavonoids (quercetin, catechin and naringenin) and their radical-scavenging sites by comparing their molecular structure, properties and reactions with oxidants.

3.1. Molecular properties and geometry of flavonoids

We calculated the optimized geometry, excess charges of the atoms (Fig. 1), molecular and electronic parameters of quercetin, catechin and naringenin molecules: heat of formation, dipole moment, surface

area and etc., using the semi-empirical molecular orbital theory (Table 1). The AC and B rings in the quercetin molecule (and not in catechin and naringenin) were located in the same planes (Fig. 1). The torsion angle was C3-C2-B1’-B2’ ≈ 180° due to the double C2=C3 bond in the C ring of quercetin structure. The dipole moment of flavonoid molecules, according to our semi-empirical models, increased in the order: quercetin (0.99 D) < naringenin (1.6 D) < catechin (2.1 D) that correlate with polarity and water solubility of the flavonoids (Table 1).

Table 1
Calculated molecular parameters of naringenin, quercetin and catechin

Parameters	Naringenin	Quercetin	(+)-Catechin
AM1 UHF			
Number of electrons	102	112	110
Number of doubly occupied levels	51/51	56/56	55/55
Number of total orbitals	92	98	98
Total Energy, kcal/mol	-85033.7341	-99164.7732	-93070.1819
Binding Energy, kcal/mol	-3640.1191	-3717.6003	-3861.3785
Isolated Atomic Energy, kcal/mol	-81393.6150	-95447.1729	-89208.8034
Electronic Energy, kcal/mol	-513116.2131	-601021.9335	-597927.1805
Core-Core Interaction kcal/mol	428082.4789	501857.1603	504856.9985
Heat of Formation, kcal/mol	-153.7501	-216.3173	-211.2465
E (HOMO) α, eV	-9.310636	-8.827891	-9.042646
E (HOMO) β, eV	-9.296082	-8.997514	-9.061333
E (LUMO) α, eV	-0.591512	-1.085702	0.012489
E (LUMO) β, eV	-0.597053	-0.852294	0.089334
ΔE = E (HOMO) α – E (LUMO) α, eV	-9.902148	-7.742189	-9.055135
ΔE = E (HOMO) β – E (LUMO) β, eV	-9,893135	-8.14522	-9.150667
Lowest Level Energy α, eV	-42.063153	-42.440206	-42.205243
Lowest Level Energy β, eV	-42.057281	-42.444943	-42.165635
Highest Level Energy α, eV	6.299569	6.340062	6.296683
Highest Level Energy β, eV	6.299778	-8.997514	6.303410
Dipole Moment, D	1.602	0.986	2.107
QSAR properties			
Surface area (grid), $Å^2$	453.58	453.62	470.21
Volume, $Å^3$	736.63	753.52	764.33
Hydration energy, kcal/mol	-22.97	-32.53	-31.12
Ab initio UHF (6-31G)			

Parameters		Naringenin	Quercetin	(+)-Catechin
Total Energy, kcal/mol,		-595475.5297	-688649.2008	-643136.5287
Electronic Kinetic Energy, kcal/mol		595905.9009	689036.7450	643608.5332
eK, ee & eN Energy, kcal/mol		-1516527.37	-1770727.35	-1704258.65
Nuclear Repulsion Energy, kcal/mol		921051.8416	1082078.1516	1061122.1226
Torsion angles (C3-C2-C1'-C2')		86.4	180	117.9
The total net excess charge of the ring	A	0.437	0.54	0.351
	B	0.069	0.128	0.018
	C	-0.26	-0.352	-0.049

According to our *ab initio* calculations the net negative excess charges of the C rings (Table 1) of quercetin, catechin and naringenin make these parts of flavonoid molecules attractive for electrophilic attack. The flavonoid B rings possess positive but small excess charges and the A rings of the flavonoids demonstrate large positive excess charges. In the quercetin molecule, the calculated HOMO was delocalized mainly on the B ring and on the C2 = C3 double bond, and, partially, on the A and C rings. In catechin and naringenin molecules, electron orbitals were delocalized mainly on the B ring (Fig. 2).

The calculated highest net negative excess charges were found for hydroxyl groups at the 4'- position in the case of naringenin, at the 3'- and 7- positions in the case of quercetin and at the 5- and 7- positions in the case of catechin (Table 2). The lowest energies of hydrogen atom abstraction were calculated for the hydroxyl groups at the 4'- position in the case of naringenin, at the 3- and 3'- positions in the case of quercetin and at the 3'- and 4'- positions in the case of catechin (Table 2). We computed the O-H bond dissociation energies (BDE) as the energy evolution associated with the homolytic rupture of the OH bond [25].

Table 2
Total net excess charge and hydrogen atom removal energy (kJ/mol) of quercetin, catechin and naringenin hydroxyl groups

	Hydroxyl group	OH-3	OH-5	OH-7	OH-3'	OH-4'
Naringenin	Bond dissociation energy	----	-365,59	-352,19	----	-333,35
	Total net excess charge	----	-0.317	-0.355	----	-0.372
Quercetin	Bond dissociation energy	-309,15	-367,35	-355,63	-316,69	-539,42
	Total net excess charge	-0.344	-0.311	-0.353	-0.392	-0.338
Catechin	Bond dissociation energy	-435,34	-435,76	-334,85	-317,27	-313,93
	Total net excess charge	-0.33	-0.361	-0.37	-0.345	-0.346

3.2. Radical-flavonoid interactions

We evaluated parameters of DPPH-flavonoid interactions (stoichiometry, apparent rate constants, activation energy) using the dependences of the radical reduction rates on the concentrations of flavonoids and the temperature and the equations (1) and (2).

$v = k\,c^{n}$, (1)

were v is the apparent reduction rate, n is the order of the reaction and k is the apparent rate constant of DPPH reduction (Table 3). The apparent rates of the reduction reactions were estimated from the kinetic curves of DPPH reduction by varying concentrations of the flavonoids (2.5–20 μM). For calculations, we represented the Eq. (1) in logarithm form $\ln v = \ln k + n \ln c$ (Figs. 3). It should be noted that we registered the reaction rates under conditions of DPPH excess. The apparent reaction rate constant of the DPPH reduction by quercetin was much higher in comparison with catechin, and naringenin did not demonstrate antioxidant activity. The stoichiometry of the DPPH reduction was 1:1 (Table 3).

Table 3
Parameters of flavonoid – oxidant interactions: DPPH reduction, flavonoid autoxidation, inhibition of HOCl – induced haemolysis, and water solubility of flavonoids

Parameters	Quercetin	Catechin	Naringenin
Apparent reaction rate of DPPH reduction, $M^{-1}s^{-1}$ (20 ºC)	4000 ± 1000	1100 ± 200	-
Activation energy of reduction, kJ/mol (10 μM flavonoid)	23.0 ± 2.5	32.5 ± 2.5	-
Reduction stoichiometry	1.05 ± 0.05	0.99 ± 0.05	-
Activation energy of autoxidation, kJ/mol (50 μM flavonoid)	50.8 ± 6.1	58.1 ± 7.2	-
IC_{50} for haemolysis inhibition, μM	1.0 ± 0.2	1.1 ± 0.2	1.2 ± 0.2
Water solubility, mg/l	0.512 [26]	2260 [27]	4.38 [28]

Representing the dependence of the reaction rates on the temperature according to the Arrhenius Eq. (2), we calculated the activation energy of the electron transfer to the DPPH radical (Figs. 4, Table 3).

$v = A \exp(-\Delta Ea / RT)$, (2)

where v is the rate of the DPPH reduction at the temperature T (K), A is the pre-exponential factor, R is the Gas constant and ΔEa is the activation energy of the reduction.

3.3. Flavonoid autoxidation: effect of antioxidants

Flavonoid oxidations by air oxygen (autoxidations) resulted in their spectral changes in time (Figs. 5A – 7A). For quercetin autoxidation, we observed progressive decrease of absorbance maxima at 255 nm and 378 nm and appearance of a new peak at 329 nm (in ethanol / phosphate buffer mixture, 30 % / 70 %, pH 7.4) (Fig. 6A). Catechin autoxidation resulted in appearance of a new maximum at 435 nm without significant changes in the peak at 279 nm (Fig. 7A) and we did not observe spectral changes for naringenin (Fig. 6A). Figure 8 shows kinetic curves of quercetin autoxidation in the absence and in the presence of antioxidants: reduced glutathione, ascorbic acid and melatonin. From the time - dependences of the flavonoid absorbance changes (decrease in D_{378} in the case of quercetin, and increase in D_{435} in the case of catechin) we determined the apparent initial autoxidation rates at different temperatures (10–50 ºC) and calculated activation energies of the reactions, according to the Arrhenius Eq. (2) (Table 3).

All the flavonoids studied effectively interacted with HOCl, one of the powerful biological oxidants, these interactions (flavonoid chlorination) resulted in considerable changes in the flavonoid absorption spectra (Figs. 5B – 7B). Spectral changes were similar in the case of quercetin and catechin autoxidation and chlorination, naringenin chlorination (and not autoxdation) induced decrease in the peak at 282 nm and appearance of a new maximum at 370–375 nm (Figs. 5). Ascorbic acid, reduced glutathione, and not melatonin, prevented flavonoid autoxidation (Fig. 8).

3.4. Prevention of hypochlorite-induced haemolysis by flavonoids

The interaction of HOCl with erythrocytes resulted in effective hemolysis [29]. We evaluated the effect of flavonoids on the susceptibility of rat erythrocytes to hypochlorous acid-induced damage. We measured the rates of hemolysis from the kinetic curves of HOCl – induced hemolysis. Flavonoids considerably prevented erythrocyte lysis, as was determined from the dependences of the hemolysis rates on the flavonoid concentrations (Fig. 9). The calculated apparent IC_{50} concentrations of the flavonoids (Fig. 9, Table 3) for inhibition of the hemolysis were considerably lower than the HOCl hemolytic concentrations and demonstrated high protective effects of all the flavonoids studied.

4. Discussion

The reductive-oxidative reactions of quercetin, catechin, naringenin, their transient and stable oxidation products, the relationship between free radical scavenging activity of flavonoids and their electronic structure have been widely studied for several decades [1, 9, 14–18, 22]. The ability of non-glycosylated flavonoids to functioning as ROS scavengers is related to the number of hydroxyl groups and connected with their capacity for hydrogen radical donation, with their capability to form stable phenoxyl radicals (a property that is shared by aromatic compounds containing *o*-dihydroxyl groups or close hydroxyl and carbonyl groups [9, 20, 30]. Flavones and flavan-3-ols are most effective against reactive oxygen species [30].

It has been recently found 48 stable conformers of quercetin, due to rotation of their five hydroxyl groups and AC and B rings, among which are 24 conformers have a planar structure and the dipole moment of the conformers varies within the range of 0.35–9.87 D [31]. Earlier we suggested that the C2 = C3 bond in the C-ring determined the planar geometry of quercetin as well as its semiquinone radicals and quinones as optimal forms in vacuum [14]. Similarly, Erkos et al. described stable quercetin conformation as a planar one [22]. The quercetin planar structure (according to our AM1 geometry optimization) is optimal for delocalization of AB and C rings electron orbitals (Fig. 2). The catechin and naringenin molecules without the double C2=C3 bond in the C ring did not have any planar structures (Fig. 1, Table 1). On the opposite, it was suggested that in catechin molecule, an intramolecular hydrogen bond placed the B, A and C rings in the same plane [2]. The dipole moment defines molecule orientation during interaction and interaction rate [22]. According to our calculations, the dipole moments of the highly polarizable AC ring and the B ring have opposite directions in the quercetin molecule (data not shown), thus quercetin has the lowest dipole moment in comparison with the other two flavonoids, the molecule is non-polar and possesses low water solubility (Table 1, 2). Earlier Rasulev et al. calculated the dipole moment for quercetin as μ = 2.41 D [20], whereas according Erkos et al., this parameter is 3.013 D [22].

The negative excess charge on the OH groups as well as on the rings of quercetin and its radicals may play a dominant role in their reactions with radicals and biological activities [22]. The calculated excess negative charges in our experiments allowed to suggest OH group being in positions 3’- and 7- in quercetin, 4’- in naringenin and 5- and 7- in catechin as the most probable sites for hydrogen atom abstraction in radical reduction. Zhu et al. suggested that antioxidative (radical scavenging) activity of flavonoids was directly correlated with the calculated energy of hydrogen abstraction of the phenolic hydroxyl groups (bond dissociation enthalpy, BDE) and the order of free radical scavenging activities of a number of flavonoids was quercetin > catechin > luteolin > taxifolin > kaempferol > apigenin [1]. The authors showed that the 3'-OH and 4'-OH groups possessed the lowest BDEs in the case of quercetin. Similarly, the spin density of 4'-OH group of quercetin was closely related to its antioxidant activity [1]. The lowest BDEs and the highest excess negative charges of the 3’-OH group of quercetin and the 4’-OH group of naringenin allow to suggest these groups as the most reactive sites of these flavonoid molecules. It was suggested that the removal of the H-atom on the hydroxyl group on the C-4’ position of the quercetin B ring made the most reactive radical, H-atom transfer dominated over single electron transfer [22].

Our calculation shows that the Heat of formation absolute values for the flavonoids increase in the order of naringenin < catechin < quercetin according to molecule stability. It was shown previously that the Heat of formation value was negatively correlated well with the antioxidant activity of polyphenols [32]. The LUMO energy of the flavonoids decreased in the order of catechin > naringenin > quercetin and the HOMO energy redused in the order of quercetin > catechin > naringenin (Table 1). Quercetin has the highest value of HOMO energy, thus, quercetin is the best electron donor among the flavonoids studied (possesses the highest reductive potential). Similarly, quercetin has the lowest value of LUMO energy, and it is the best electron acceptor. The regions of the highest density of the HOMO map (Fig. 2) are preferable for electrophilic attack. The generation of electron density maps in quercetin (and in the quercetin radical)

showed that the C2 = C3 double bond in the C ring of quercetin (absent in catechin and naringenin) provides planarity and optimal electron orbital delocalization (HOMO) on all phenolic rings in the quercetin (and not in catechin and naringenin) molecule (Fig. 2). The system of the conjugated bonds in polyphenol molecules should delocalize electron density and stabilize the transient quercetin semiquinone radicals formed.

In our experiments, flavonol quercetin was a more powerful antioxidant in comparison with flavan-3-ol (+)-catechin and flavanon naringenin. Quercetin demonstrated much higher apparent reaction rate and lower activation energy of DPPH reduction in comparison with catechin, and naringenin did not react with DPPH radical. One flavonoid molecule was consumed for reduction of one DPPH radical under our experimental conditions (Table 2). It was shown that at excess of flavonoids under the reduction of DPPH, the stoichiometry for flavonoids with one hydroxyl group was 1:1, that for flavonoids with two hydroxyl groups in the B-ring was 1:2 [33]. The IC_{50} values for flavonoid interactions with DPPH were determined, for catechin – 5.06 ± 0.08 μM, for quercetin – 4.36 ± 0.10 μM [1].

The activation energy of the DPPH reduction is higher in comparison with activation energy of diffusion, consequently, not every collision between the reactants would result in DPPH reduction.

Flavonoid autoxidation is one of the main reactions of ROS generation by flavonoids. It was suggested previously the monoexponential first-order kinetics ($k = 6.45 \times 10^{-2}\ M^{-1}\ s^{-1}$) of quercetin autoxidation, indicating one-electron oxidation of quercetin anion was followed by two fast steps of radical disproportionation and solvent addition to the resulting quinone. The main products of quercetin autoxidation (as well as enzymatic, chemical and electrochemical oxidations) are depside, phenolic acids, and quercetin-solvent adducts [34]. The activation energies of quercetin and catechin autoxidation were higher in comparison with that of the flavonoid interaction with DPPH. In our experiments, naringenin neither reduced DPPH, no was autoxidized, but effectively interacted with hypochlorous acid. The low radical-scavenging activity of naringenin was, probably, connected with the absence of two close hydroxyl groups or the hydroxyl and carbonyl groups in the molecule. Previously we observed a significant inhibition of membranous lipid peroxidation by naringenin, but its effectiveness was much lower in comparison with quercetin or catechin [14]. The flavonoid autoxidation and chlorination resulted in similar spectral changes, in spite of the products of these reactions being different (Figs. 5–7).

Hypochlorous acid (HOCl) generated from activated neutrophils could be associated with several pathological processes such as atherosclerosis. Earlier we evaluated the sequence of cellular events after red blood cell exposure to HOCl: rapid cell morphological transformations, membrane structural transitions, oxidation of cellular constituents, enzyme modifications, and colloid-osmotic hemolysis [29]. The flavonoids dose-dependently prevented HOCl-induced hemolysis and protective effects of three flavonoids were similar. We suggested a direct interaction of flavonoids with hypochlorous acid – flavonoid chlorinations and a decrease of effective HOCl concentration in erythrocyte suspensions. It is known that the reaction of HOCl with phenolic compounds occurs through an electrophilic attack on an aromatic carbon at the ortho- and para- positions to yield chlorine-substituted products [35, 36]. Our

theoretical calculation demonstrates possible sites for electrophilic attack in the flavonoid molecules (negatively charged C atoms, Fig. 1). Similar reactions of 3-chlorotyrosine and 3,5-dichlorotyrosine formations from the reaction of HOCl with tyrosine (free or peptide-bound) have been studied and chlorotyrosines have been used as a marker of involvement of HOCl in the processes [37].

Conclusions

The calculated planar conformation on the of quercetin is optimal for delocalization of electron orbitals (HOMO) on the AB and C rings. The catechin and naringenin molecules without the double C2=C3 bond in the C ring have switched conformations. The net negative excess charges of the C rings of quercetin, catechin and naringenin, the small positive excess charges of the B rings make these parts of the flavonoid molecules attractive for electrophilic attack. The calculated highest net excess charges in the hydroxy groups at the 4'- position in the case of naringenin, at the 3'- and 7- positions in the case of quercetin, and at the 5- and 7- positions in the case of catechin allowed us to suggest these groups to be the most probable sites for hydrogen atom abstraction in the radical reduction reactions. The 3'-OH group of the B-ring of quercetin has the highest excess negative charge and the lowest energy of hydrogen atom abstraction for the flavonoids studied and should be suggested as the most reactive reductive site. The stoichiometry of the DPPH reduction by flavonoids (quercetin, catechin), is 1:1. The activation energies of flavonoid autoxidation were higher in comparison with that of the DPPH radical reduction. Naringenin did not reduce DPPH and did not autoxidized. Thus, quercetin and catechin and not naringenin were effectively oxidized by the DPPH radical and air oxygen (autoxidation) and the flavonoids studied effectively prevented HOCl-induced haemolysis due to direct scavenging of hypochlorous acid (flavonoid chlorination). The best antioxidant quercetin has the highest value of HOMO energy and the planar structure and the optimal HOMO delocalization due to C2=C3 double bond in the C ring.

Declarations

Data availability

All data generated or analyzed during this study are included in this article.

Ethics declarations

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

The animal experiments were conducted in accordance with the ethical guidelines of the Ethics Committee of the Institute of Biochemistry of Biologically Active Compounds, National Academy of

Sciences, Belarus (Protocol No. 22/15 of 01.10.2015) and have been followed by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Consent to participate

All authors meet the qualifications for authorship and had an opportunity to read and comment the manuscript.

Consent for publication

All authors support publication of the manuscript in Molecular and Cellular Biochemistry.

Authors' contributions

Artem G. Veiko: Data curation, Visualization, Investigation, Software.

Elena A. Lapshina: Methodology, Conceptualization, Writing- Original draft preparation.

Ilya B. Zavodnik: Conceptualization, Supervision, Validation, Writing- Reviewing and Editing

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Figures

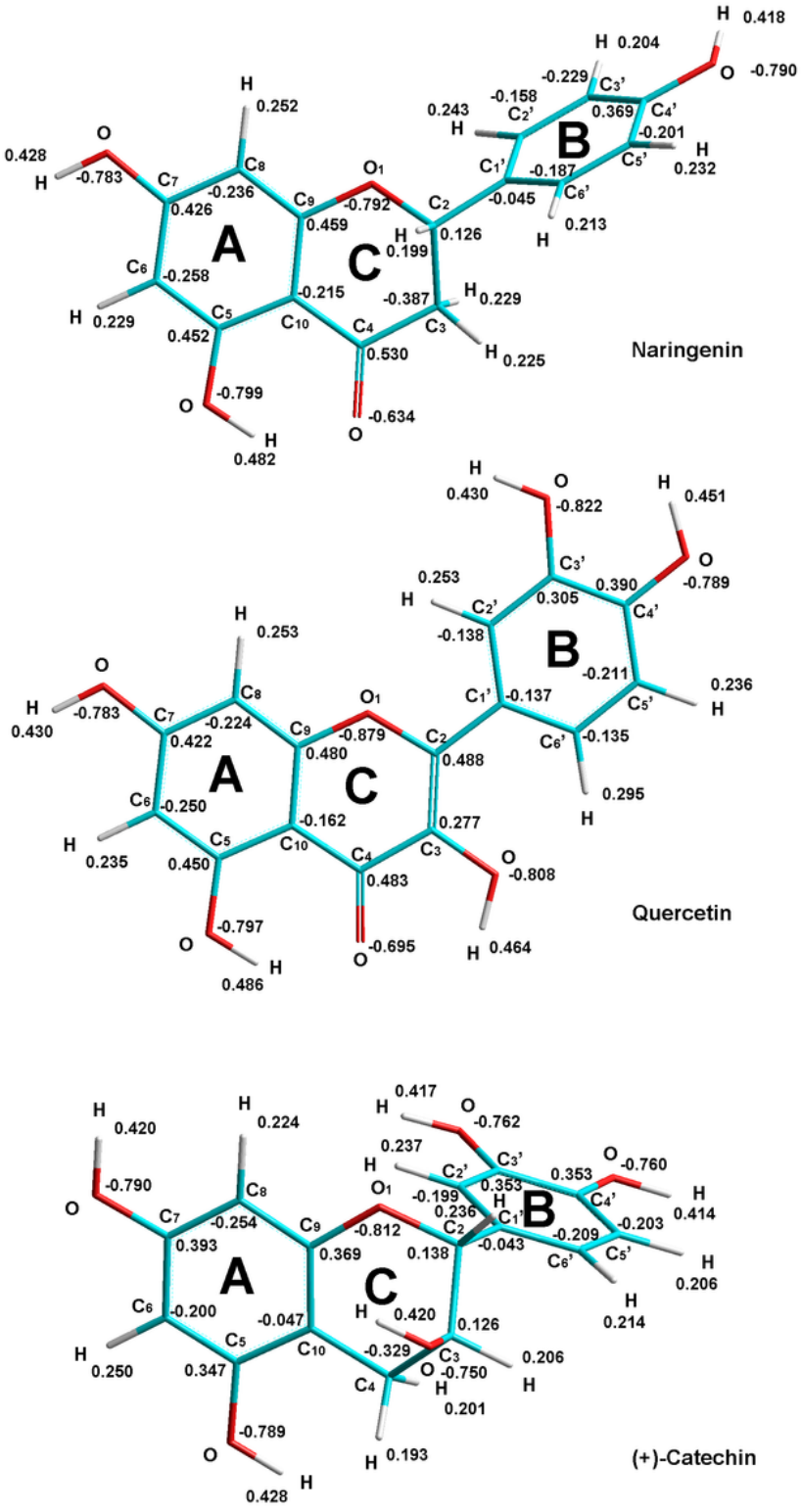

Figure 1

Optimized molecular structure of quercetin, catechin and naringenin (the optimization was performed by semi-empirical AM1 method), calculated excess charges of the atoms of flavonoid molecules (using the non-empirical Ab initio method with 6-31G basis and UHF method with Polak–Ribière gradient algorithm)

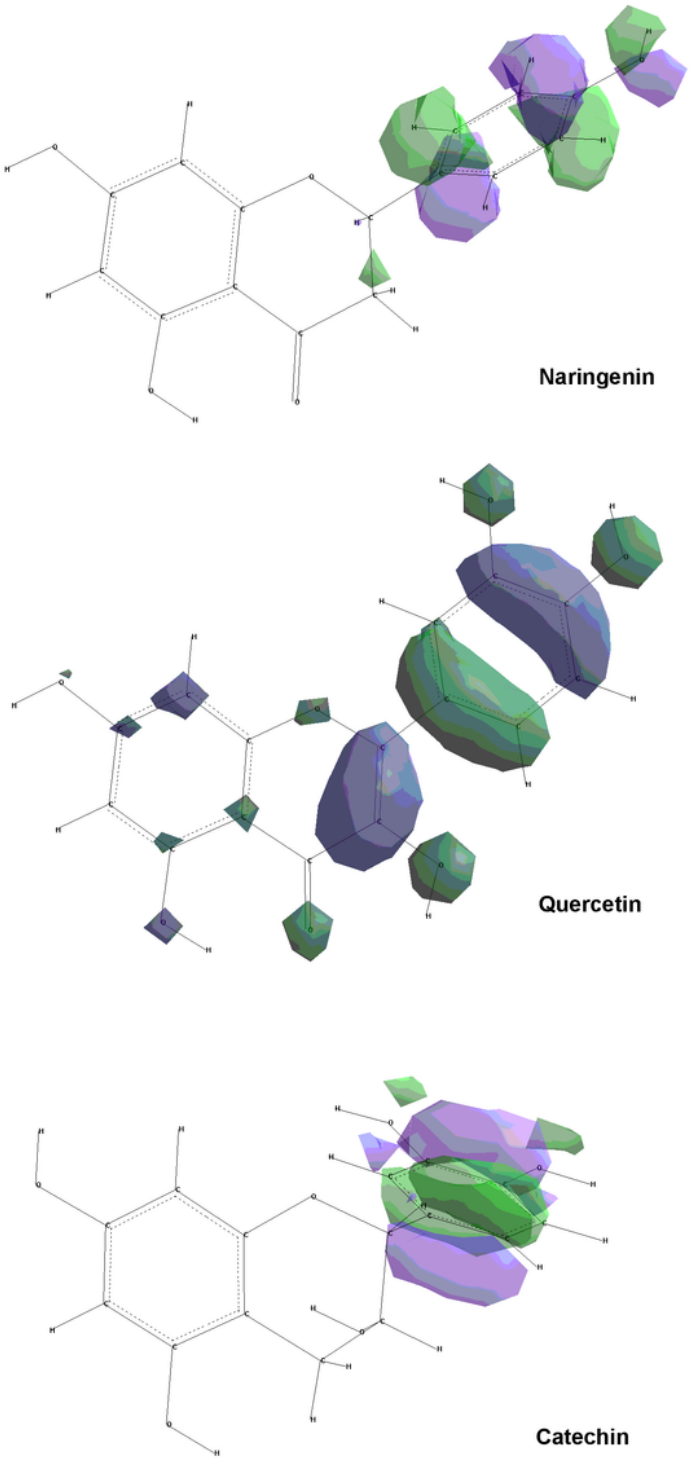

Figure 2

Calculated HOMO delocalization maps of quercetin, catechin and naringenin molecules (using non-empirical Ab initio method with 6-31G basis and UHF method with Polak–Ribière gradient algorithm)

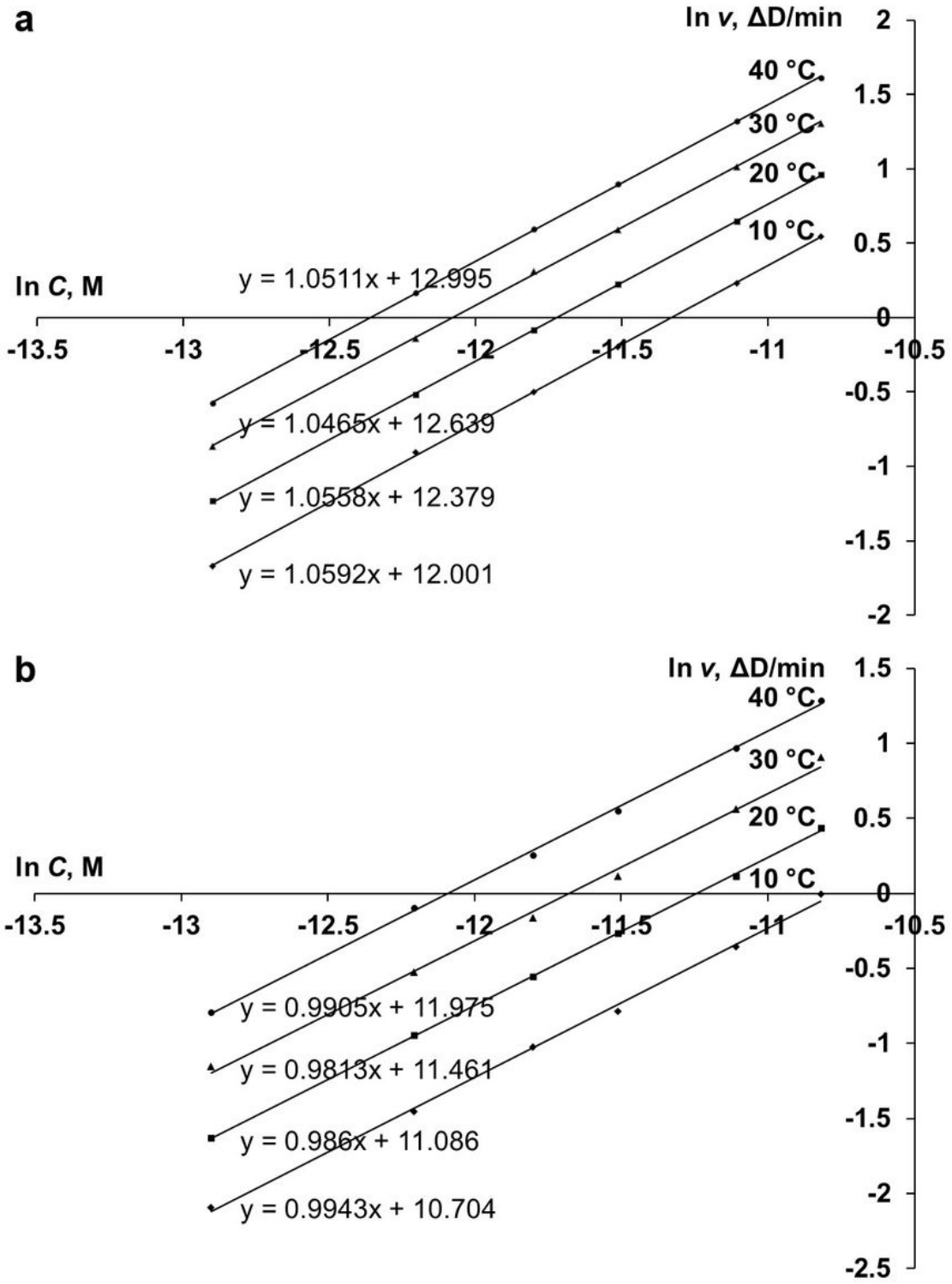

Figure 3

Concentration dependence of DPPH (100 μM) reduction by quercetin (a) and catechin (b) at different temperatures (10 - 40 °C) in ethanol, represented as logarithm form

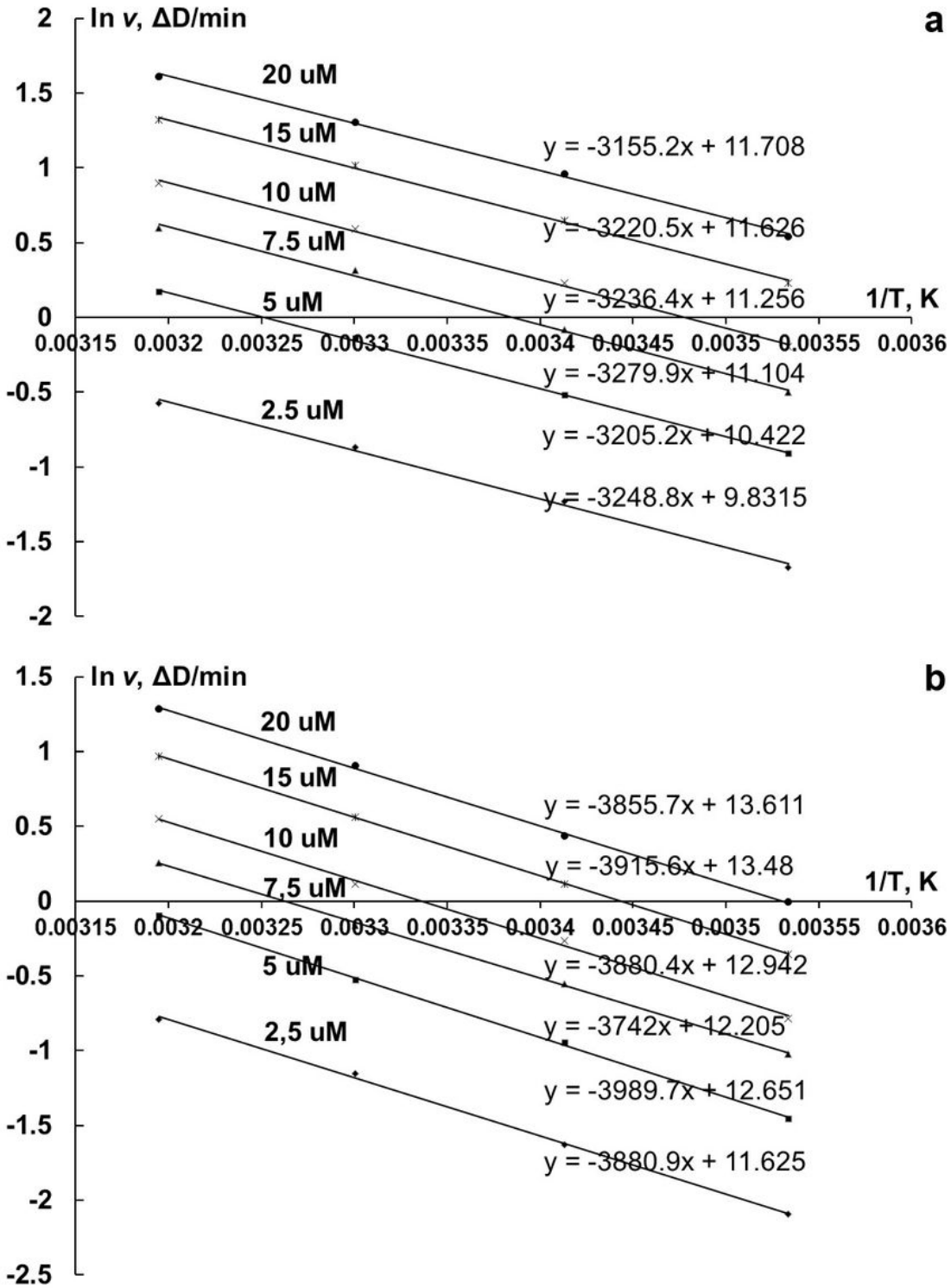

Figure 4

Arrhenius plots of DPPH (100 μM) reduction by quercetin (a) and catechin (b) (2.5 μM – 20 μM) in ethanol

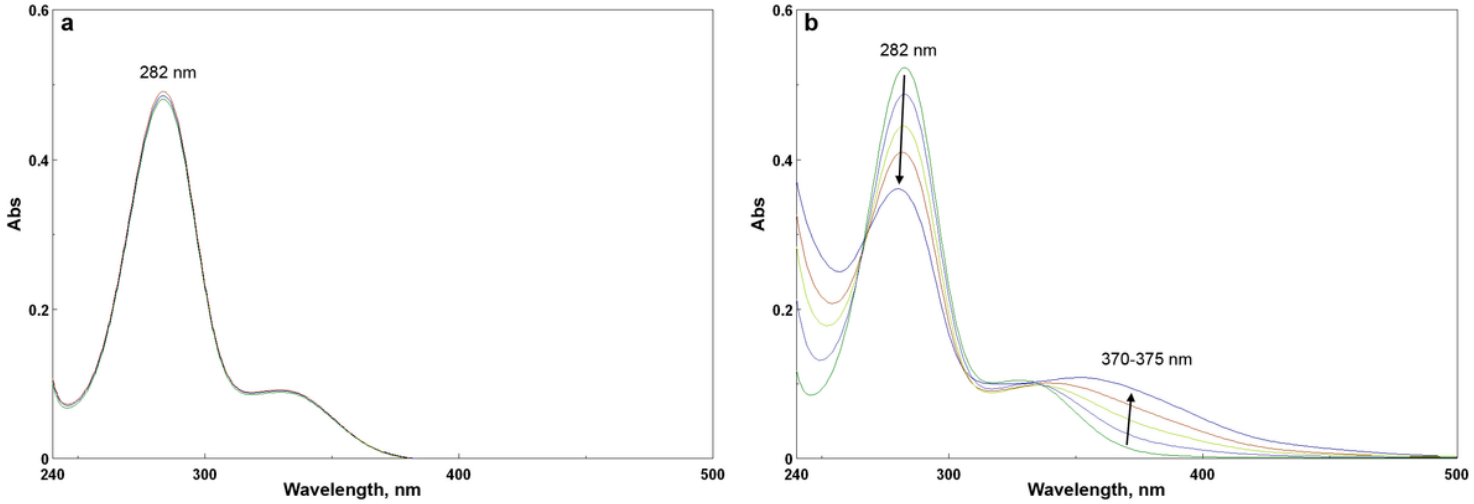

Figure 5

UV-VIS spectra of naringenin under autoxidation (A) and chlorination (B) (in the presence of 0, 25, 50, 100, and 150 μM HOCl) in ethanol / PBS mixture (30 % / 70 %), pH 7.4, flavonoid concentration was 50 μM, the temperature of autoxidation was 50 ºC, 1-hour interval, the temperature of chlorination was 25 ºC, 1 min of incubation with HOCl

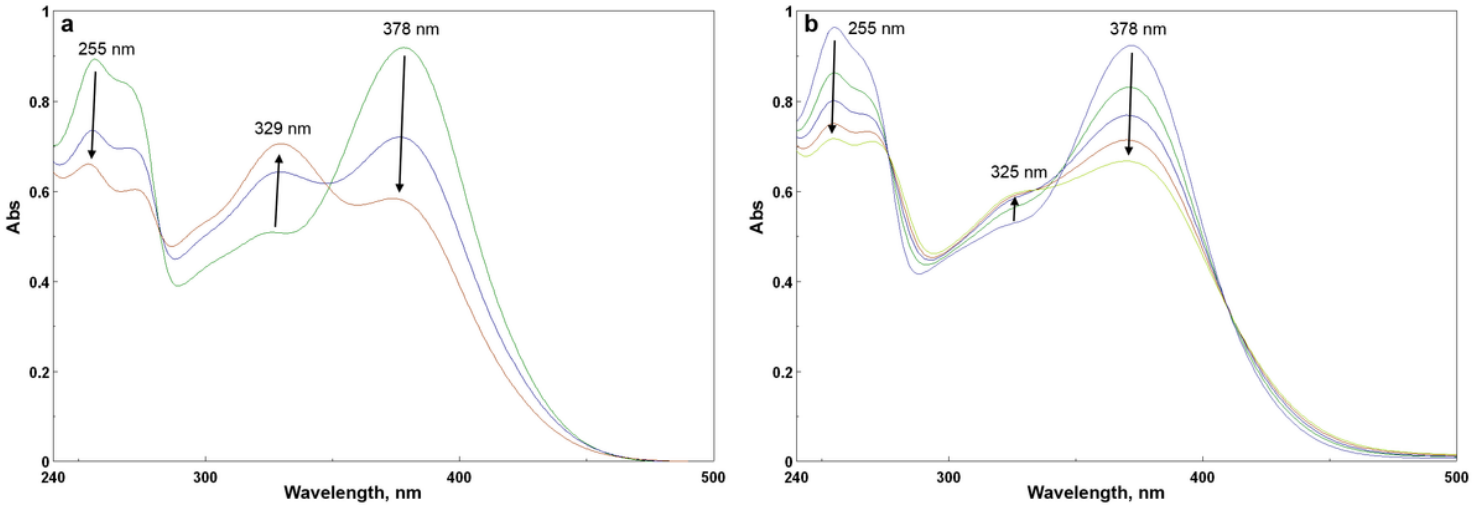

Figure 6

UV-VIS spectra of quercetin under autoxidation (A) and chlorination (B) (in the presence of 0, 25, 50, 100, and 150 μM HOCl) in ethanol / PBS mixture (30 % / 70 %), pH 7.4, flavonoid concentration was 50 μM, temperature of autoxidation was 50 ºC, 1-hour interval, temperature of chlorination was 25 ºC, 1 min of incubation with HOCl

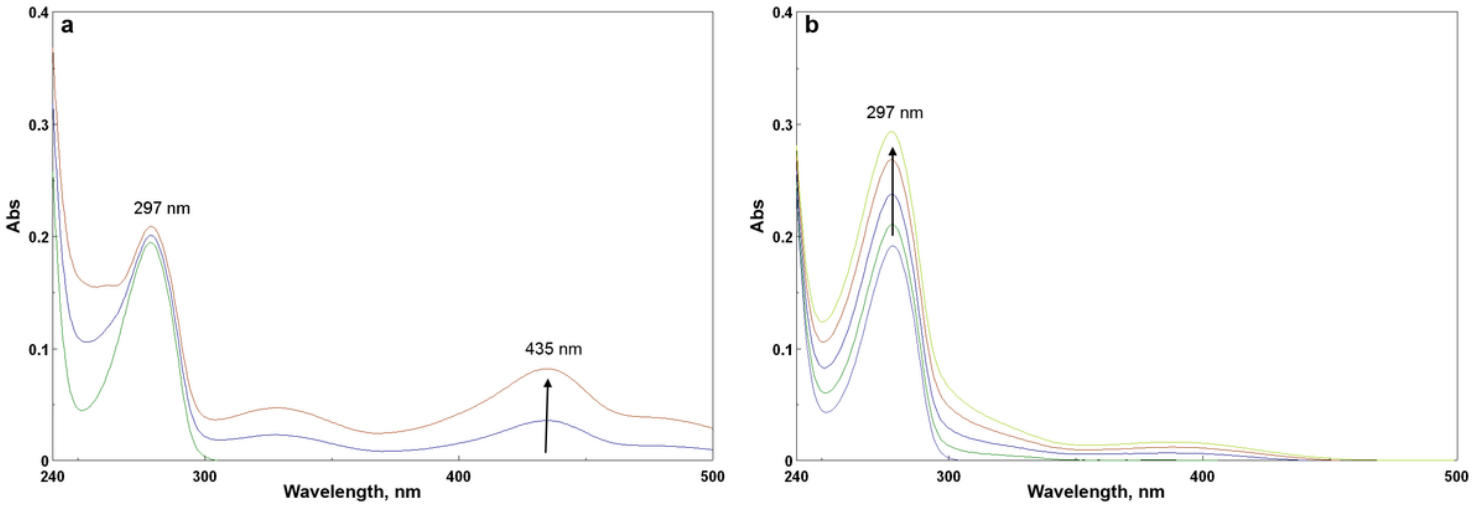

Figure 7

UV-VIS spectra of catechin under autoxidation (A) and chlorination (B) (in the presence of 0, 25, 50, 100, and 150 μM HOCl) in ethanol / PBS mixture (30 % / 70 %), pH 7.4, flavonoid concentration was 50 μM, temperature of autoxidation was 50 ºC, 1-hour interval, temperature of chlorination was 25 ºC, 1 min of incubation with HOCl

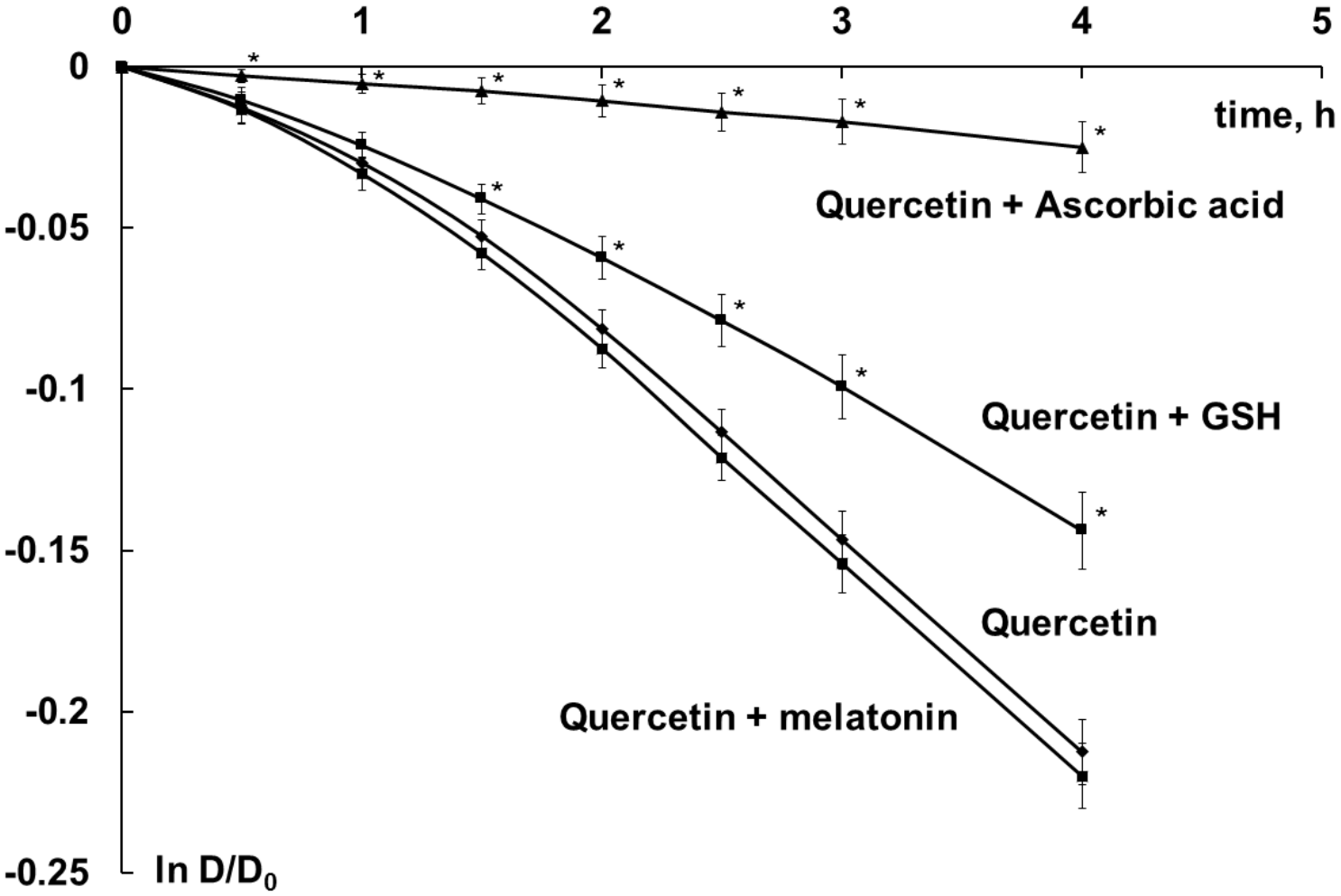

Figure 8

Time - dependences of quercetin absorbance changes (D380) in the absence of antioxidants, and in the presence of melatonin, reduced glutathione, ascorbic acid in ethanol / PBS mixture (30 % / 70 %), pH 7.4, at 25 ºC, flavonoid concentration was 50 μM, antioxidant concentrations were 100 μM; * - p <0.05 in comparison with quercetin alone

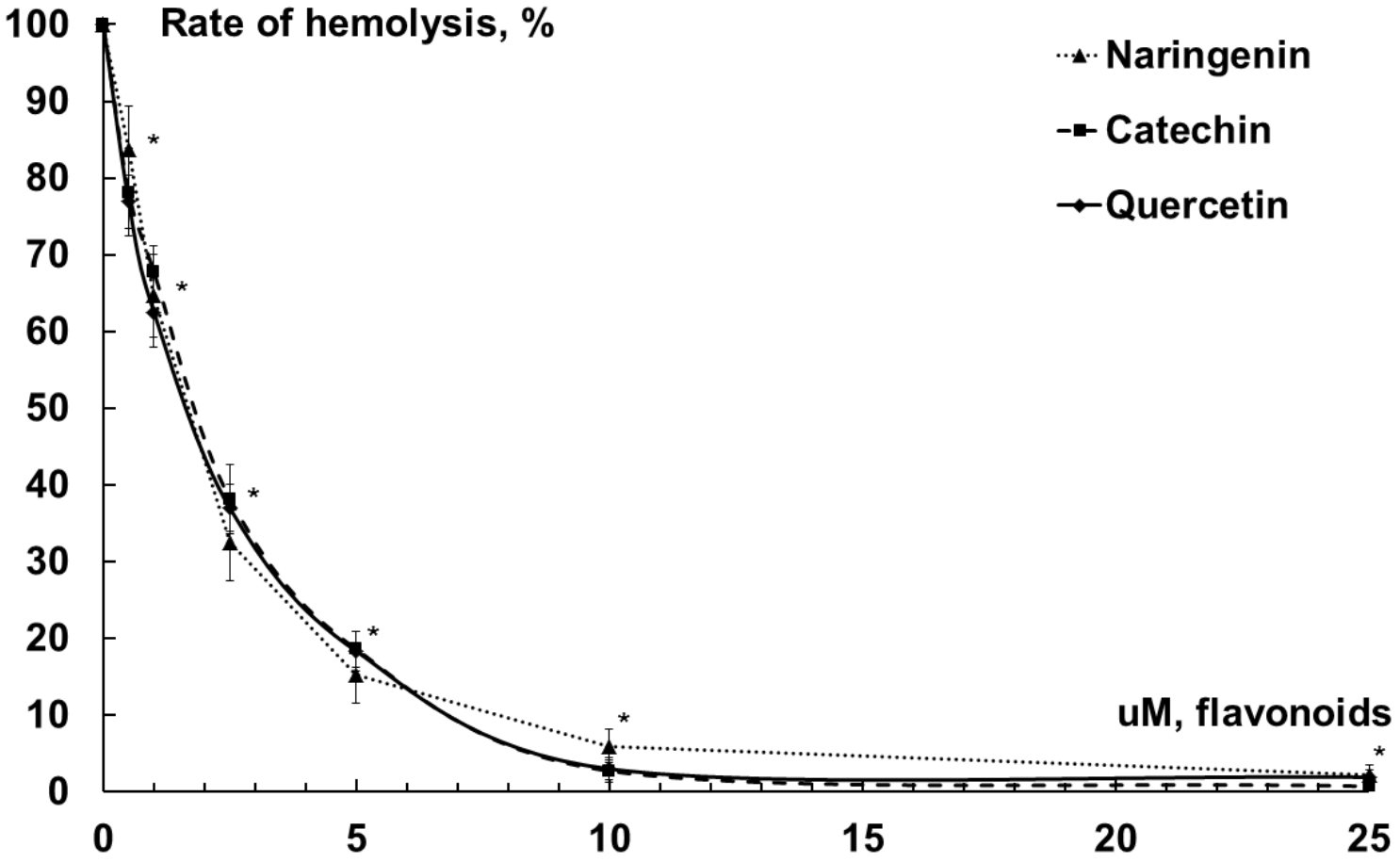

Figure 9

Inhibition by flavonoids (quercetin, catechin, narungenin) of rat erythrocyte hemolysis induced by HOCl. Erythrocyte suspension (hematocrit 0.05%) was mixed with HOCl (75 μM) in the absence or the presence of varying concentrations of flavonoids in PBS, pH 7.4, at 25 °C; * - p <0.05 compared with hemolysis in the absence of flavonoids