



Comparative analysis of molecular properties and reactions with oxidants for quercetin, catechin, and naringenin

Artem G. Veiko¹ · Elena A. Lapshina¹ · Ilya B. Zavodnik¹

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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 evaluation of the electronic properties of some flavonoids belonging to different classes such as quercetin (flavonols), catechin (flavanols), and naringenin (flavanones) and their interactions with oxidants in 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 rate constants (s^{-1} , 20 °C) and the activation energies (kJ/mol) of DPPH reduction were 0.34 ± 0.06 and 23.0 ± 2.5 in the case of quercetin, 0.09 ± 0.02 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 had the highest value of HOMO energy, a planar structure and optimal electron orbital delocalization on all the phenolic rings due to the C2=C3 double bond in the C ring (absent in catechin and naringenin).

Keywords Quercetin · Catechin · Naringenin · Structure · Antioxidants · Chlorination

Introduction

Oxidative stress, a result of overproduction of reactive oxygen/nitrogen/chlorine species and/or impairment in the cellular antioxidative defense system, is considered as an important mechanism of cyto- and geno-toxicity. 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 belong to this group), are important natural antioxidants, free radical scavengers, and regulators of cellular redox balance and redox signaling

casades [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. According to their chemical structures, flavonoids are classified into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones.

The molecular structure and mechanisms of antioxidative, cytoprotective, and regulatory activities of flavonoids as well as their potential use (tested both ex vivo and in vivo) have been 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

✉ Ilya B. Zavodnik
zavodnik_il@mail.ru; zavodnik_ib@grsu.by

¹ Department of Biochemistry, Yanka Kupala State University of Grodno, Bulvar Leninskogo Komsomola, 50, 230030 Grodno, Belarus

function, modulation of inflammatory responses, and antimicrobial action [5, 6]. Flavonoids interact with many proteins and activate, inhibit, upregulate, or downregulate such cascades as protein kinases, AMPK, MAPK, NF- κ B, and the TGF β -2/PI3K/AKT pathways, p53-mediated apoptotic events, 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 of a hydrophobic nature, considerably prevent cellular reactive oxygen species (ROS) production by mitochondria ($IC_{50} \approx 1\text{--}2 \mu\text{M}$) and inhibit the redox enzymes, NAD(P)H oxidases, xanthine oxidases, monooxygenases, cyclooxygenases, and lipoxygenases [9].

Direct antiradical/antioxidant capacities of flavonoids are related to redox properties of their readily oxidized phenolic hydroxyl groups and conjugated rings. Bors et al. were the first to claim three 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]. In our experiments, the flavonoids considerably inhibited membrane lipid peroxidation (the IC_{50} values were equal to $9.7 \pm 0.8 \mu\text{M}$, $8.8 \pm 0.7 \mu\text{M}$, and $46.8 \pm 4.4 \mu\text{M}$ in the case of quercetin, catechin, and naringenin, respectively) and decreased glutathione oxidation in mitochondria and erythrocytes treated with *tert*-butyl hydroperoxide [14, 15].

We suggested that the biochemical effects of some flavonoids (quercetin, catechin, and 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]. The capacity of flavonoids (e.g., quercetin, baicalin, luteolin, hesperetin, gallic acid, gallic acid gallate, epigallocatechin gallate, and scutellarein) to inhibit key proteins involved in the coronavirus infective cycle has recently been reviewed [16].

The mechanisms of direct antioxidant activity of polyphenols can be clarified via (1) hydrogen atom transfer (HAT) and proton-coupled electron transfer (PCET) where the proton and electron are simultaneously transferred to the radical in one kinetic step; (2) single electron transfer followed by proton transfer (SET-PT), which is a two-step mechanism; (3) sequential proton loss electron transfer (SPLET), a

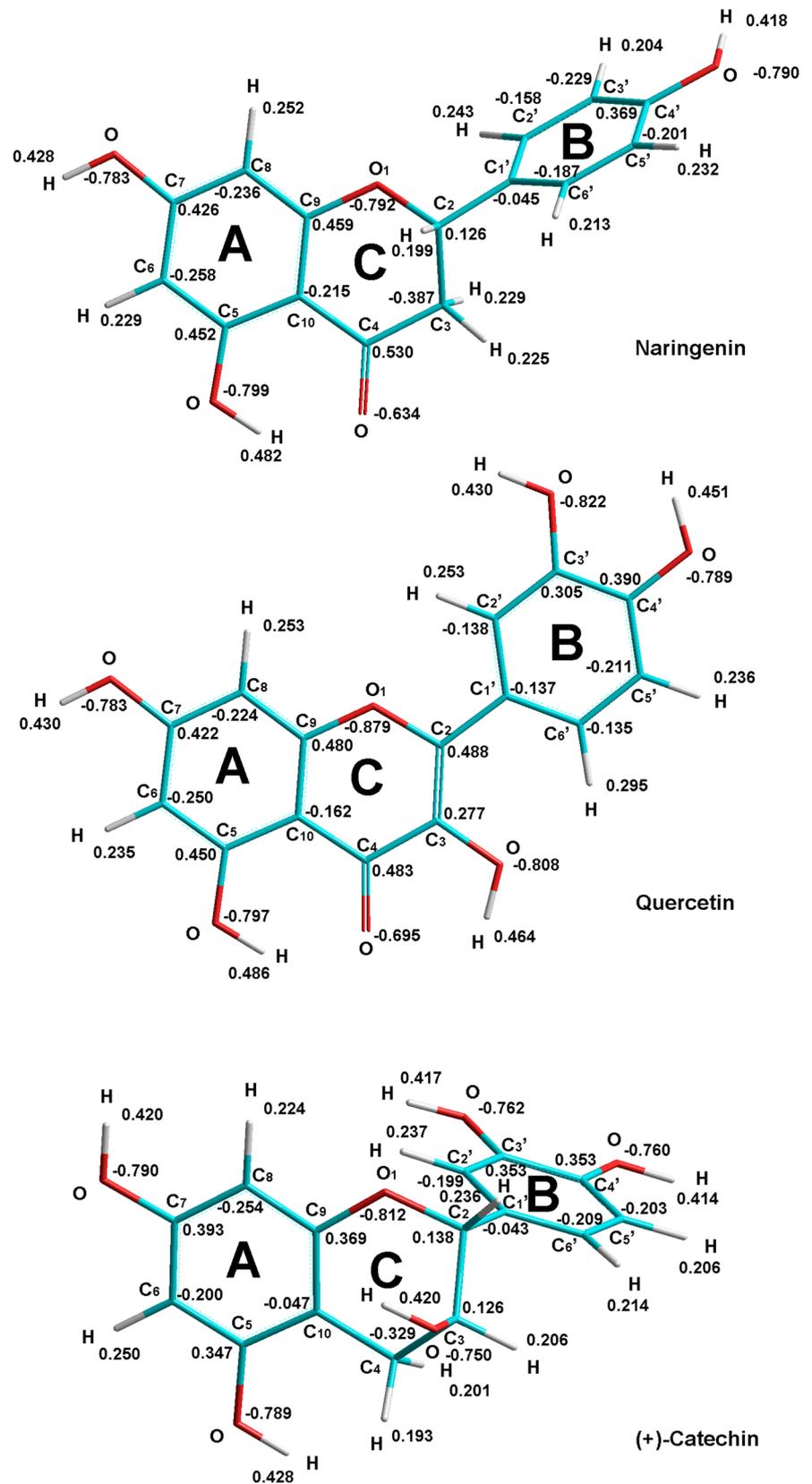
reverse mechanism with respect to SET-PT which represents relatively stable polyphenol anion formation after the proton loss as the first step of this mechanism; and (4) formation of stable adducts with radicals [17, 18]. In nonpolar environments (e.g., lipid bilayer), PCET is the only active process of free radical scavenging by flavonoids. Under nonacidic conditions, SPLET is the major mechanism and both the mechanisms take place in reactions against peroxy or DPPH radicals [11, 19]. Simultaneously, flavonoids display prooxidant 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 glutathione [4].

Since electronic and molecular parameters are crucial for biochemical activity, calculations of the structure and thermochemical parameters of flavonoids were widely performed earlier using methods of quantum chemistry [8, 17, 20–22]. Relationships between molecular structures of flavonoids and their biological activities are still not fully understood. The goal of the present study was a comparative analysis of the chemical structures of quercetin, catechin, and naringenin belonging to various flavonoid subclasses (optimal molecular geometry, electronic properties, reactive sites) and their antioxidative and anti-inflammatory activities. Knowledge about flavonoid structures and interactions with different targets seems to be useful to predict biological effects of phytopharmaceuticals.

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 in 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.

In our study, we used methods of theoretical semi- and non-empirical quantum chemical calculations for evaluating the charge distribution in the flavonoid molecules, dipole moments, bond dissociation energies (BDE), and their electronic and molecular parameters that determine the nature of their reactions with radicals. The antioxidative activities of flavonoids were assessed in the model reactions of stable DPPH radical reductions and flavonoid autoxidations

Fig. 1 Optimized molecular structures of quercetin, catechin, and naringenin (the optimization was performed by the semi-empirical AM1 method), calculated excess charges of atoms of flavonoid molecules (using the non-empirical ab initio method with 6-31G basis and UHF method with the Polak–Ribière gradient algorithm)



and by a spectroscopy method to measure the reaction rate constants, activation energies, and stoichiometry, using a spectroscopy method. The anti-inflammatory potential of the flavonoids was established as ability to prevent hypochlorous acid-induced red blood cell hemolysis due to flavonoid chlorinations by hypochlorous acid, a dominant mediator of inflammation.

Materials and methods

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.

Calculation of flavonoid molecular geometry and electronic properties

Flavonoid molecules were considered theoretically by performing both the semi-empirical molecular orbital theory and ab initio calculations. The Austin Model 1 (AM1) semi-empirical method within unrestricted Hartree–Fock (UHF) formalism in the self-consistent field approximation and the Polak–Ribiere algorithm were considered to optimize fully the geometry of the flavonoid molecules. The electronic structure of the systems was calculated according to an ab initio method [22, 23]. We performed all the calculations by 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 corresponding radical formed after removal of hydrogen atom of each hydroxyl group.

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 DPPH reduction by flavonoids. Briefly, 10 μl of flavonoids at different concentrations (ethanol solutions)

was added to 1990 μl of a DPPH solution (100 μM in ethanol) at 10–40 °C. The reduction of DPPH was monitored spectrophotometrically as its absorbance change at 520 nm using a Jasco V-650 UV–VIS Spectrophotometer (Japan). We estimated parameters of DPPH–flavonoid interactions (stoichiometry, apparent rate constants, activation energy) using Eqs. (1) and (2). The DPPH reduction rate equation can be presented as follows:

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

where v is the apparent reduction rate, n is the order of the reaction, and k is the apparent rate constant of DPPH reduction. The apparent rates of the reduction reactions were estimated from the time dependences of DPPH reduction by varying concentrations of the flavonoids (2.5–20 μM). For calculations of the apparent rate constant and the order of the reaction, we represented Eq. (1) in the logarithm form $\ln v = \ln k + n \ln c$. 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:

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

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 ΔE_a is the activation energy of the reduction.

Flavonoid autoxidation and chlorination

Flavonoid autoxidation was monitored spectrophotometrically 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 10–50 °C under gentle stirring. We used phosphate-buffered saline (PBS: 0.15 mol l⁻¹ NaCl, 1.9 mmol l⁻¹ KH₂PO₄, 8.1 mmol l⁻¹ K₂HPO₄, pH 7.4). Prevention of flavonoid autoxidation by antioxidants was assessed under incubation in the presence of 100 μM reduced glutathione, 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.

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 of Belarus (Protocol No. 22/15 of 01.10.2015) and were followed by the National Institute 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) contained a mixture of HOCl and NaOCl at a ratio of approximately 1:1 and was 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 varying concentrations of HOCl (5–75 μM) in PBS, pH 7.4, at 25 °C, and the kinetic curves of hemolysis were determined spectrophotometrically from the changes in optical density of the suspension at 670 nm and 25 °C in the presence or absence of flavonoids. Erythrocyte suspensions had been preincubated with flavonoids for 5 min before HOCl was introduced.

Statistical analysis

The results of the experiments were expressed as the means of four or five replicates \pm SEM. The differences between the values for the parameters measured in groups were analyzed using the Student's *t* test or the nonparametric Mann–Whitney test depending on the normality of distribution values in the groups. The normality of distribution was determined by the 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 the *p* value was less than 0.05.

Results

For better understanding of the biochemical reactivity of the flavonoids (quercetin, catechin, and naringenin) and their radical-scavenging sites, we compared their structures, properties, and reactions with oxidants.

Molecular properties and geometry of flavonoids

We calculated the optimized geometry (Fig. 1), molecular, and electronic parameters of quercetin, catechin, and naringenin molecules: heat of formation, dipole moment, surface area, etc. using the semi-empirical molecular orbital theory (Table 1) and excess charges of the atoms of the molecules (Fig. 1) by an ab initio method. 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' \approx 180° due to the double C2=C3 bond in the C ring of the quercetin structure. Based on the AM1 calculations, the dipole moment of flavonoid molecules increased in the order: quercetin (0.99 D) < naringenin (1.6 D) < catechin (2.1 D), which correlates with polarity and water solubility of the flavonoids (Table 1).

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 as the energy evolution associated with the homolytic rupture of the OH bond [25]. Our present findings are in line with the previous studies. The 4'- and 3'-OH groups (the catechol group) of the ring B and the 3-OH group of the ring C were found to be more reactive sites in the quercetin molecule [17]. 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 to be 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 promoted deactivating other radicals via proton and/or electron transfer [18].

Radical–flavonoid interactions

Figures 3 and 4 represent the dependences of radical reduction rates on the concentrations of flavonoids and the temperature. It should be noted that we recorded the reaction rates under conditions of DPPH excess. Parameters of DPPH–flavonoid interactions (stoichiometry, apparent rate constants, and activation energy) are listed in Table 3. As is seen, 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

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	−85,033.7341	−99,164.7732	−93,070.1819
Binding energy, kcal/mol	−3640.1191	−3717.6003	−3861.3785
Isolated atomic energy, kcal/mol	−81,393.6150	−95,447.1729	−89,208.8034
Electronic energy, kcal/mol	−513,116.2131	−601,021.9335	−597,927.1805
Core–core interaction kcal/mol	428,082.4789	501,857.1603	504,856.9985
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
$\Delta E = E$ (HOMO) $\alpha - E$ (LUMO) α , eV	−9.902148	−7.742189	−9.055135
$\Delta E = E$ (HOMO) $\beta - E$ (LUMO) β , eV	−9.893,135	−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)			
Total energy, kcal/mol	−595,475.5297	−688,649.2008	−643,136.5287
Electronic kinetic energy, kcal/mol	595,905.9009	689,036.7450	643,608.5332
eK, ee & eN energy, kcal/mol	−1,516,527.37	−1,770,727.35	−1,704,258.65
Nuclear repulsion energy, kcal/mol	921,051.8416	1,082,078.1516	1,061,122.1226
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

activation energy of DPPH reduction by quercetin was much lower than that of catechin. It means that quercetin is a more preferable reductant of the DPPH molecule than catechin. The stoichiometry of the DPPH reduction was 1:1 (Table 3).

Flavonoid autoxidation: effect of antioxidants

Flavonoid oxidations by air oxygen (autoxidations) resulted in their spectral changes in time (Figs. 5a, 6, 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. 5a). 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 °C) and calculated activation energies of the reactions according to the Arrhenius Eq. (2) (Table 3).

Fig. 2 Calculated HOMO delocalization maps of quercetin, catechin, and naringenin molecules (using the non-empirical ab initio method with 6-31G basis and UHF method with the Polak–Ribière gradient algorithm)

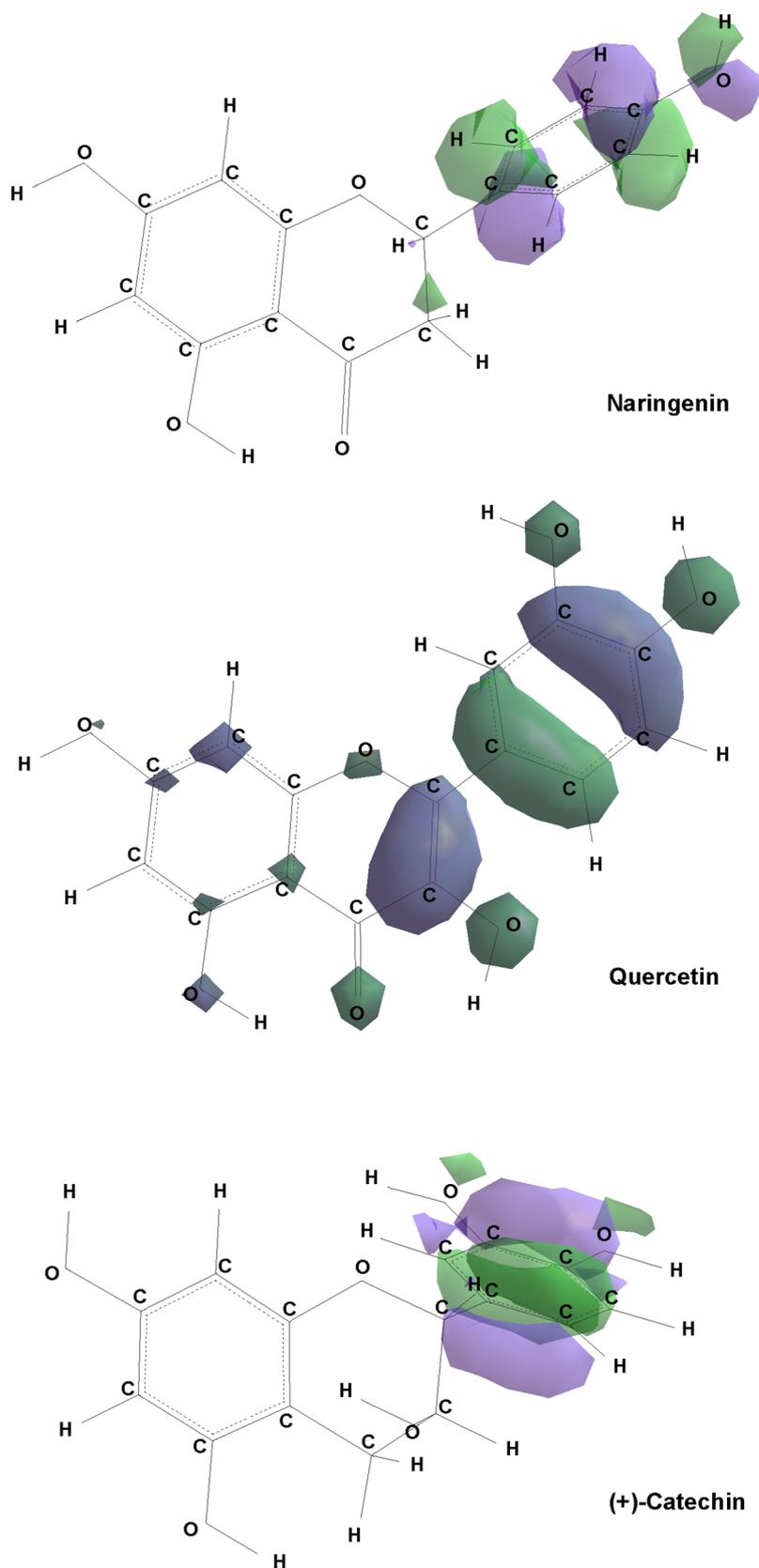
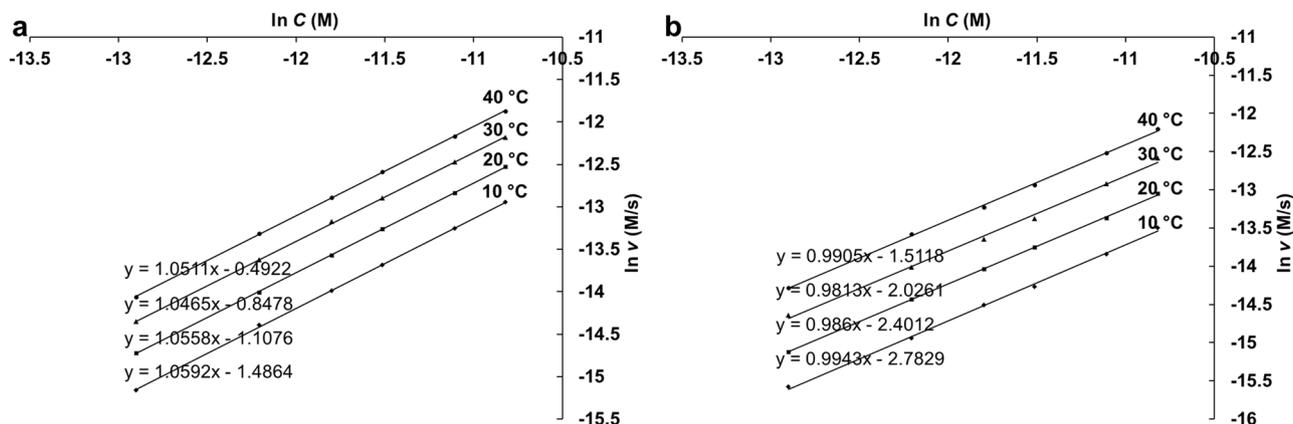
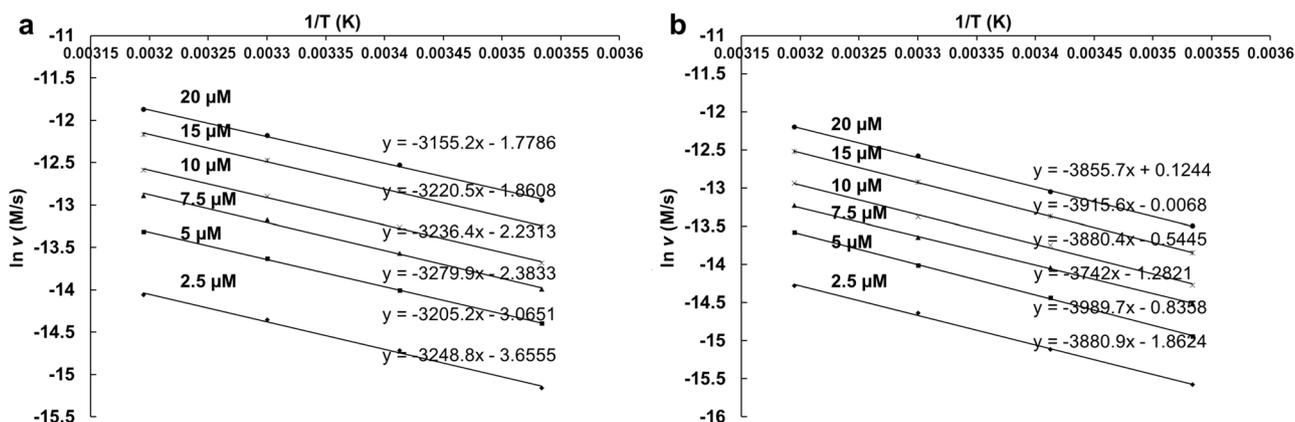


Table 2 Total net excess charge and hydrogen atom removal energy (kJ/mol) of quercetin, catechin, and naringenin hydroxyl groups

	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

**Fig. 3** Concentration dependence of DPPH (100 μM) reduction by quercetin (**a**) and catechin (**b**) at different temperatures (10–40 °C) in ethanol, represented as the logarithm form**Fig. 4** Arrhenius plots of DPPH (100 μM) reduction by quercetin (**a**) and catechin (**b**) (2.5–20 μM) in ethanol**Table 3** Parameters of flavonoid–oxidant interactions: DPPH reduction, flavonoid autoxidation, inhibition of HOCl-induced hemolysis, and water solubility of flavonoids

Parameters	Quercetin	Catechin	Naringenin
Apparent reaction rate of DPPH reduction, s^{-1} (20 °C)	0.34 ± 0.06	0.09 ± 0.02	–
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 hemolysis 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]

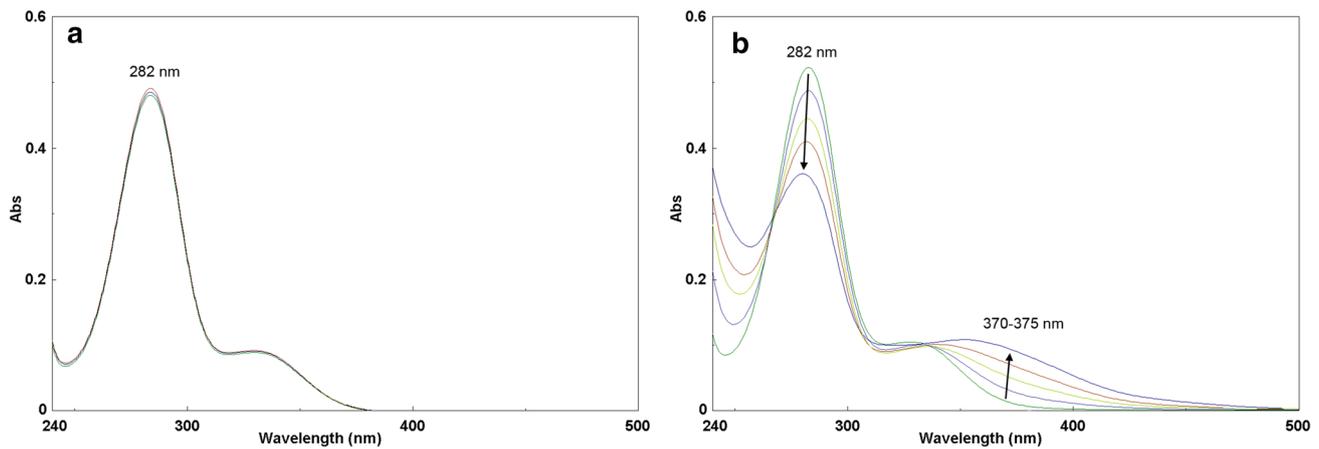


Fig. 5 UV–VIS spectra of naringenin under autoxidation (a) and chlorination (b) (in the presence of 0, 25, 50, 100, and 150 μM HOCl) in ethanol/PBS mixture (30%/70%), pH 7.4, the flavonoid con-

centration was 50 μM , the temperature of autoxidation was 50 $^{\circ}\text{C}$, 1-h interval, the temperature of chlorination was 25 $^{\circ}\text{C}$, 1 min of incubation with HOCl

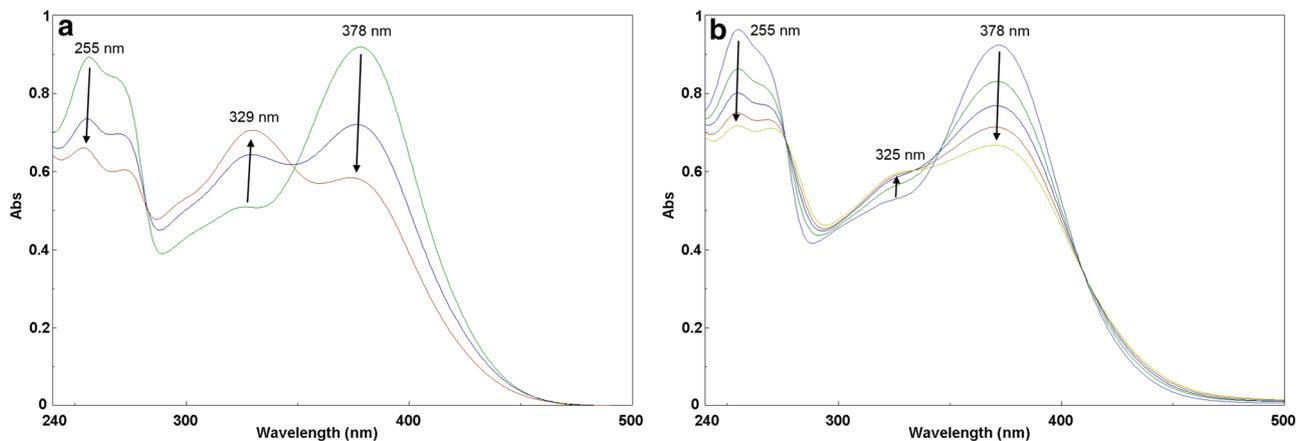


Fig. 6 UV–VIS spectra of quercetin under autoxidation (a) and chlorination (b) (in the presence of 0, 25, 50, 100, and 150 μM HOCl) in ethanol/PBS mixture (30%/70%), pH 7.4, the flavonoid concentration

was 50 μM , the temperature of autoxidation was 50 $^{\circ}\text{C}$, 1-h interval, the temperature of chlorination was 25 $^{\circ}\text{C}$, 1 min of incubation with HOCl

All the flavonoids studied effectively interacted with HOCl, a powerful biological oxidant. These interactions (flavonoid chlorination) resulted in considerable changes in the flavonoid absorption spectra (Figs. 5b, 6b, 7b). Spectral changes were similar in the case of quercetin and catechin autoxidation and chlorination, while naringenin chlorination (and not autoxidation) induced a decrease in the peak at 282 nm and appearance of a new maximum at 370–375 nm (Fig. 5). Ascorbic acid, reduced glutathione, and not melatonin, prevented flavonoid autoxidation (Fig. 8).

Prevention of hypochlorite-induced hemolysis by flavonoids

The interaction of HOCl with erythrocytes resulted in effective hemolysis [29]. We assessed the effect of flavonoids on the susceptibility of rat erythrocytes to hypochlorous acid-induced damage, measuring 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.

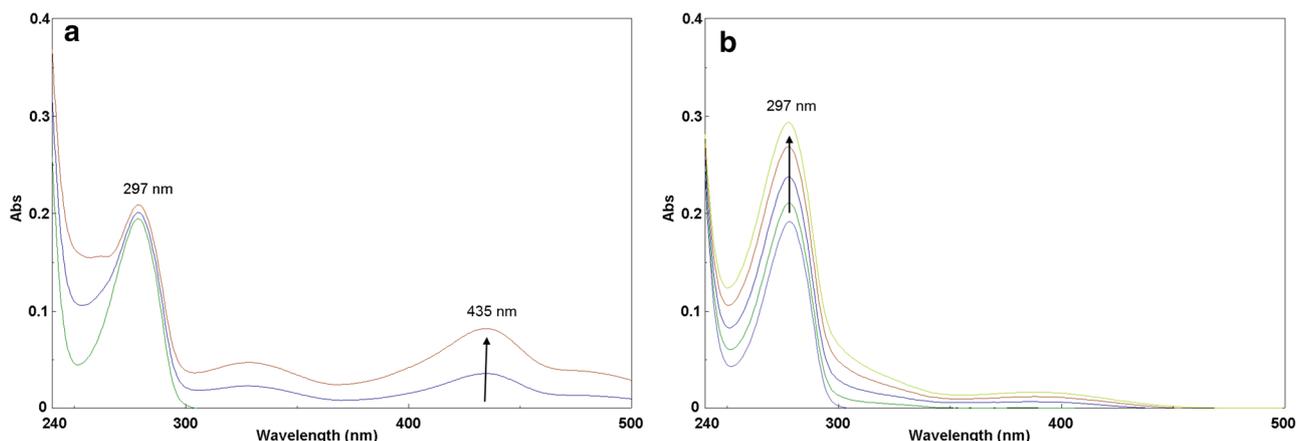


Fig. 7 UV–VIS spectra of catechin under autoxidation (a) and chlorination (b) (in the presence of 0, 25, 50, 100, and 150 μM HOCl) in ethanol/PBS mixture (30%/70%), pH 7.4, the flavonoid concentration

was 50 μM, the temperature of autoxidation was 50 °C, 1-h interval, the temperature of chlorination was 25 °C, 1 min of incubation with HOCl

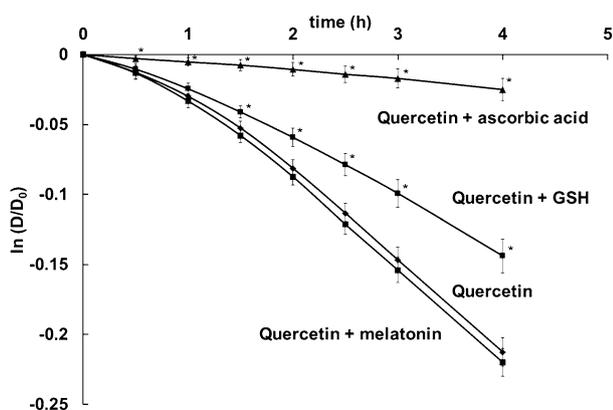


Fig. 8 Time dependences of quercetin absorbance changes (D_{380}) in the absence of antioxidants, and in the presence of melatonin, reduced glutathione, ascorbic acid in ethanol/PBS mixture (30%/70%), pH 7.4, at 25 °C, the flavonoid concentration was 50 μM, the antioxidant concentrations were 100 μM; * $p < 0.05$ in comparison with quercetin alone

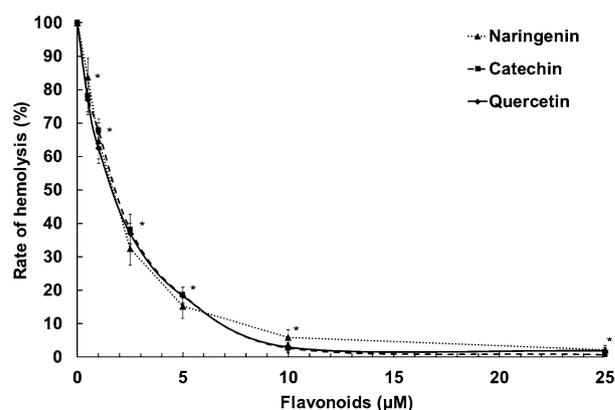


Fig. 9 Inhibition by flavonoids (quercetin, catechin, naringenin) of rat erythrocyte hemolysis induced by HOCl. Erythrocyte suspension (hematocrit 0.05%) was mixed with HOCl (75 μM) in the absence or the presence of varying concentrations of flavonoids in PBS, pH 7.4, at 25 °C; * $p < 0.05$ compared with hemolysis in the absence of flavonoids

Discussion

The reductive–oxidative reactions of quercetin, catechin, naringenin, their transient and stable oxidation products, as well as 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 function as ROS scavengers is related to the number of hydroxyl groups due to their capacity for hydrogen radical donation and 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].

Forty-eight stable conformers of quercetin have recently been found due to rotation of their five hydroxyl groups and AC and B rings 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 the catechin molecule, an intramolecular hydrogen bond placed the B, A, and C rings in the same plane [2]. The dipole moment governs 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); therefore, 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, 3). Earlier, Rasulev et al. calculated the dipole moment for quercetin as $\mu = 2.41$ D [20], whereas according to 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 that the OH groups, located in positions 3'- and 7-in quercetin, in position 4'-in naringenin, and in positions 5- and 7- in catechin, were 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 some 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 the 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 produced the most reactive radical [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. On the contrary, it was shown previously that the heat of formation value negatively correlated 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 was reduced 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 the 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, the flavonol quercetin was a more powerful antioxidant in comparison with flavan-3-ol (+)-catechin and flavanon naringenin. Quercetin demonstrated much higher apparent reaction rate constant and lower activation energy of DPPH reduction in comparison with catechin, and naringenin did not react with the DPPH radical. One flavonoid molecule was consumed for reduction of one DPPH radical under our experimental conditions (Table 3). It was shown that at excess of flavonoids under the reduction of DPPH, the stoichiometry for flavonoids with one hydroxyl group was 1:1 and that for flavonoids with two hydroxyl groups in the B ring was 1:2 [33]. The IC₅₀ values for flavonoid interactions with DPPH were determined. They were 5.06 ± 0.08 μM for catechin and 4.36 ± 0.10 μM for quercetin [1]. The activation energy of the DPPH reduction is higher in comparison with the 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 that the monoexponential first-order kinetics ($k = 6.45 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$) 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 did not reduce DPPH and it was not 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 although the products of these reactions were different (Figs. 5, 6, 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 the protective effects of the three flavonoids were similar. We suggested a direct interaction of the 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].

Conclusion

The best antioxidant quercetin has the highest value of HOMO energy as well as the planar structure and the optimal HOMO delocalization due to the C2=C3 double bond in the C ring. 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 and 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 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 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) was 1:1. The activation energies of flavonoid autoxidation were higher in comparison with that of the DPPH radical reduction. Naringenin was not autoxidized and it did not reduce DPPH. The flavonoids quercetin (despite low solubility) and catechin (with high water solubility) exerted efficient protective effect against free radicals and oxidants. All the flavonoids studied effectively prevented HOCl-induced hemolysis due to direct

scavenging of hypochlorous acid (flavonoid chlorination). In this way, the high antioxidative and anti-inflammatory potentials of the polyphenolic compounds could contribute to numerous health benefits.

Author contributions AGV: Data curation, Visualization, Investigation, Software. EAL: Methodology, Conceptualization, Writing—Original draft preparation. IBZ: Conceptualization, Supervision, Validation, Writing—Reviewing and Editing.

Data availability All the data generated or analyzed during this study have been included in this article.

Declarations

Conflict of interest The authors declare 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 of Belarus (Protocol No. 22/15 of 01.10.2015) and were followed by the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Informed consent All the authors meet the qualifications for authorship and had an opportunity to read and comment the manuscript. All authors support publication of the manuscript in *Molecular and Cellular Biochemistry*.

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