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ORIGINAL RESEARCH

Energy requirements of the reactions of kaempferol and selected radical species in different media: towards the prediction of the possible radical scavenging mechanisms

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Abstract Kaempferol, one of the most bioactive plant flavonoids was experimentally and theoretically (at M05-2X/6-311G(d,p) level of theory) investigated for its ability to scavenge potentially, highly damaging hydroxyl and superoxide anion radicals. Relating the obtained hydroxyl radical activity sequence with kaempferol structural features, it could be assumed that C4′-OH functional most probably renders it as hydroxyl radical scavenger, while C5-OH group has more prominent role compared to *ortho*-hydroxy groups in B ring. However, kaempferol's activity toward superoxide anion radical implicates *ortho*-hydroxy groups in B ring as more relevant. Theoretical calculations point to HAT and SPLET mechanisms as operative for kaempferol in all solvents under investigations.

Keywords Kaempferol · Radical scavenging activity · Hyrdoxyl radical · Superoxide anion radical · HAT and SPLET mechanisms

Introduction

Flavonoids are natural polyphenolic compounds reported to exert a wide range of positive health effects mainly arising from their antioxidant ability. The protective role of flavonoids is manifested in their capability to "sacrifice" first in the oxidative processes transforming free radicals into stable, deprotonated, forms. The antioxidant activity of flavonoids generally has broader significance. The protection role of flavonoids against diet-related oxidative stress could have nutritional significance in the preservation of dietary lipids essential to cell functioning and the protection against the toxicity of potentially harmful lipid oxidation products. As antioxidants, flavonoids can (i) bind metal ions, (ii) inhibit enzymes involved in free radical production, and (iii) directly scavenge free radicals. According to their bioavailabilities and chemical properties, they may scavenge most of the oxygen species that are produced during oxidation stress i.e., singlet oxygen, carbon, and mainly oxygen-centered free radicals as hydroxyl, superoxide anion, peroxy, alkoxy as well as nitric oxide radicals [1–3].

In the radical scavenging mechanisms, the inactivation of reactive radical species is related to the flavonoids (ArOH) ability to transfer the phenolic H-atom ($1H^+/1e^-$) [4]. This transfer can be visualized through at least three mechanisms characteristic of not only flavonoids but also phenolics generally: hydrogen atom transfer (HAT) (eq. 1), sequential proton loss electron transfer (SPLET) (eq. 2), and single-electron transfer followed by proton transfer

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(SET-PT) (eq. 3) [5–8]. The net result of all the three mechanisms is the same—the production of the most stable flavonoid radical ArO^-. The calculated energy requirements for each mechanism, BDE (bond dissociation enthalpy) (HAT), IP (ionization potential) and PDE (proton dissociation enthalpy) (SET-PT), and PA (proton affinity) and

$$ArOH + R^\bullet \rightarrow ArO^\bullet + RH \quad (1)$$

$$ArOH \rightarrow ArO^- + H^+ \quad (2a)$$

$$ArO^- + R^\bullet \rightarrow ArO^\bullet + R^- \quad (2b)$$

$$R^- + H^+ \rightarrow RH \quad (2c)$$

$$ArOH + R^\bullet \rightarrow ArOH^{+\bullet} + R^- \quad (3a)$$

$$ArOH^{+\bullet} + R^- \rightarrow ArO^\bullet + RH \quad (3b)$$

ETE (electron transfer energy) (SPLET) may indicate which radical scavenging mechanism is thermodynamically preferred and point out the active site for radical inactivation.

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one) (Fig. 1), a dietary constituent also known as *indigo yellow,* is a natural flavonoid that can be found mainly in broccoli, tea, kale, ginko, cabbage, endive, leek, beans, tomato, strawberries, grapes, and many medical herbs used in the traditional medicine (*Bauhinia microstachya, Chromolaena odorata, Ardisia japonica, Aloe vera, Amburana cearensis, Cassia alata, Centella asiatica, Ammi majus, Angelica keiskei, Bunium persicum, Bauhinia forficata, Capparis spinosa)* [9]. It is known as a strong antioxidant which helps to prevent arteriosclerosis by inhibiting the oxidation of low-density lipoprotein and the formation of platelets in the blood [9]. It also reduces the risk of developing some types of cancer, induces apoptosis in glioma cells, and shows anti-viral activity against cytomegalovirus, influenza virus, herpes simplex virus, and human immunodeficiency virus (HIV) [9].

The present paper aims to provide quantitative tools to thoroughly and comprehensively determine the radical scavenging mechanisms of kaempferol by calculating the energy requirements of the reactions of kaempferol and selected radical species in different media. The calculated energy requirements may indicate which radical scavenging mechanism is thermodynamically preferred and point out the active sites for radical inactivation. Joint application of theoretical calculations and experimental measurements in assessment of radical scavenging activity of kaempferol is aimed to prove the transferability of the results obtained by the different approaches.

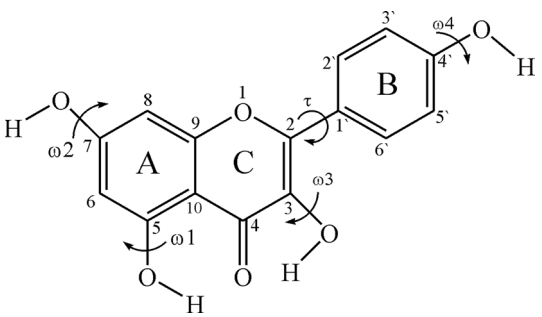

Fig. 1 Chemical structure of kaempferol

Experimental procedure

Chemicals

The following substances were used: kaempferol (Merck, USA), ethylenediaminetetraacetic acid (EDTA) (Merck, USA), sodium hydroxide (Merck, USA), ethanol (Uvasol, Merck, USA), ferrous sulfate (Merck, USA), dimethylformamide (DMF) (Merck, USA), 2-deoxyribose (Sigma, USA), hydrogen peroxide (Sigma, USA), thiobarbituric acid (TBA) (Sigma, USA), 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) (ENZO Life Sciences, Inc.), riboflavin (Merck, USA), tetrabutylammonium hexafluorophosphate (Bu_4NPF_6) (Merck, USA), dimethyl sulfoxide (DMSO) (Merck, USA), and lithium perchlorate ($LiClO_4$) (Merck, USA).

2-Deoxyribose degradation test

The procedure of 2-deoxyribose oxidative degradation assay was taken from Lopes et al., 1999 [10]. Upon the initiated Fenton reaction, 2-deoxyribose is attacked by the $^\bullet$OH radicals. In this reaction, the malonaldehyde is produced. On heating with thiobarbituric acid (TBA) at low pH, malondialdehyde (MDA) gives colored thiobarbituric acid/malondialdehyde adduct (TBA-MDA) which strongly absorbs at 532 nm. The reaction was monitored through two steps; the first one without an iron(II) chelator (kaempferol and EDTA in this case) and the second one with an iron chelator. The iron(II) (prepared in 0.1 M HCl, concentration range 2.5–35 μM), 2-deoxyribose (final concentration 50 mM), and 50 μl of hydrogen peroxide (4 mM) were added to the pH 7.2 buffer (final volume of 1 ml). The reactions were carried out first for 10 min at room temperature and then stopped by the addition of 1 ml 4 % phosphoric acid (v/v) followed by 1 ml 1 % TBA (w/v, in 50 mM NaOH). After boiling the mixture for 15 min, the visible absorbance of the formed 2-deoxyribose degradation product was measured at 532 nm. The second step of the reaction was carried out using the same procedure but with the addition of iron(II) chelators (kaempferol and EDTA) (0.01–0.2 mM) after the addition of hydrogen peroxide.

EPR spectra

The EPR spin-trapping experiment was carried out in the following manner: (a) the selected reactive oxygen species (ROS) ($^{\bullet}OH$ and $^{\bullet}O_2^-$) were produced by a pure chemical radical generating systems and their amounts were determined by the amplitude of the selected EPR signals which originated from the spin adducts formed by the particular trapping radicals; (b) the same experiment was repeated after the addition of kaempferol, which should lead to the decreased intensity of EPR signal, since a certain amount of the produced radicals is removed. The ability of kaempferol to remove free radicals was evaluated by the difference between the relative amplitudes of the EPR signals of spin adducts in radical generating systems, with and without the addition of kaempferol. Results were presented as oxidant scavenging (% of radical reduction), which represents the relative decrease of radical production: *% of radical reduction* $= 100 \times (I_0 - I_a)/I_0$: where I_0—relative height of the third low-field EPR peak of the spin adduct of the control system and I_a—relative height of the same EPR peak in the spectrum of the sample containing kaempferol.

Hydroxyl radical scavenging

The ability of kaempferol to scavenge $^{\bullet}OH$ radical was tested using the Fenton reaction as the $^{\bullet}OH$ producing system and DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) as the spin-trapping agent. The Fenton reaction system contained 0.5 mM H_2O_2 and 0.075 mM $FeSO_4$. The spin-trap DEPMPO was purified and tested for hydroxylamine impurities by a previously established procedure [11]. The final concentration of DEPMPO was 50 mM. The final concentration of DMF solution of kaempferol in the investigated system was 0.1 mM. The sample with no kaempferol served as a control. Deionized 18 MΩ H_2O was used in all the experiments. EPR spectra were recorded at room temperature using Varian E104-A EPR spectrometer operating at X-band (9.51 GHz) with the following settings: modulation amplitude 2 G, modulation frequency 100 kHz, microwave power 10 mW, time constant 0.032 s, field center 3410 G, and scan range 200 G. The spectra were recorded using EW software (Scientific Software, Bloomington, IL, USA). The samples were drawn into 10 cm long gas-permeable Teflon tubes (wall thickness 0.025 mm and internal diameter 0.6 mm; Zeus industries, Raritan, USA). The measurements were performed using quartz capillaries in which the Teflon tubes were placed.

Superoxide anion radical scavenging

The ability of kaempferol to scavenge $^{\bullet}O_2^-$ radicals was tested by using the UV-irradiated riboflavin/EDTA generating system. The reaction mixture which contained 0.3 mM riboflavin, 5 mM EDTA, and 50 mM DEPMPO was irradiated by a Xe lamp (500 W) at room temperature for 30 s. The final concentration of ethanolic solutions of kaempferol in system was 0.1 mM. Experimental conditions were the same as for the hydroxyl radical measurements.

Cyclic voltammetry measurements

Electrochemical behavior of kaempferol (0.5 mM) was investigated using cyclic voltammetry (CV) in aqueous and ethanol solutions in conventional one-compartment three-electrode electrochemical cell with working glassy carbon disk electrode (base surface area 0.196 cm^2). Large Pt foil and saturated calomel electrode (SCE) served as the counter and reference electrode, respectively. Aqueous solutions were supported by 0.1 M K_2SO_4, while the measurements were performed at pH 3, 7, and 9. Ethanolic solution was supported by 0.1 M $LiClO_4$. During measurements, the dissolved O_2 was removed by purging solutions with high-purity N_2 (5 N). Between the measurements, GC surface was renewed by polishing with diamond paste after which it was thoroughly washed with acetone and deionized water.

Superoxide radical scavenging activity was probed electrochemically using cyclic voltammetry for the analysis of kaempferol reactivity toward electrochemically generated $^{\bullet}O_2^-$. All the experiments were performed in 0.1 M solution of tetrabutylammonium hexafluorophosphate (Bu_4NPF_6) in dimethyl sulfoxide (DMSO) stored over the molecular sieve (3 Å). Prior to the experiments, GC electrode was polished with diamond paste and thoroughly washed with acetone and deionized water. All the experiments were performed at laboratory temperature using Gamry PCI-4/750 potentiostat/galvanostat at a scan rate 100 mV s^{-1} in the potential window between −0.3 and −0.95 V versus SCE in O_2-saturated solutions (5 N) with increasing amounts of kaempferol.

DFT calculations

Conformational analysis

The conformations of different kaempferol forms (neutral, radical, radical cation, and anion) are fully optimized by the new local density functional method (M05-2X), developed by the Truhlar group [12] and 6-311G(d,p) basis set [13] implemented in the Gaussian 09 package [14]. This functional also yields satisfactory overall performance for the main group thermochemistry and thermochemical kinetics, as well as organic, organometallic, biological, and

noncovalent interactions. The M05-2X functional has been successfully applied by several authors [15–21]. It should be also noted that the M05-2X functional nicely reproduces the nonplanarity of the dihedral angle between the rings B and C in morin and quercetin [15, 19–21].

The structures obtained on this way were verified by the normal mode analysis to be minimum on the potential energy surface. No imaginary frequencies were obtained. The influence of water, dimethylsulfoxide (DMSO), ethanol, and dimethylformamide (DMF) as solvents was approximated by a solute electron density (SMD) solvation model [22], the continuum solvation model based on the quantum mechanical charge density of a solute molecule interacting with a continuum description of the solvent. "D" in the model name stands for "density" and denotes that full solute electron density is used without defining the partial atomic charges. It should be noted that all the species under investigation were fully optimized in all solvents, including frequency calculations. The vibrational frequencies were obtained from diagonalization of the corresponding M05-2X Hessian matrices. The nature of the stationary points is determined by analyzing the number of imaginary frequencies: 0 for minimum and 1 for transition state.

Mutual position of rings B and C was obtained by rotating around torsion angle τ (O1–C2–C1′–C2′ atoms, Fig. 1). The torsion angle τ was scanned in steps of 10° without constrains on all other geometrical parameters. The effects of the torsion angles ω_1(H–O5–C5–C6), ω_2(H–O7–C7–C8), ω_3(H–O3–C3–C2), and ω_4(H–O4′–C4′–C3′) were analyzed in order to determine the preferred relative positions of the rings B and C. Obtained structures were further optimized without any constrain around each potential minimum.

Thermodynamic parameters

Free radical scavenging activity of the antioxidants is highly influenced by the properties of the scavenged radical species [23, 24], and the reaction enthalpy is a thermodynamic quantity which can help in determining the preferred antiradical mechanism. In an exothermic reaction ($\Delta H < 0$), the newly formed radical is more stable than the starting one implying the reaction path as possible. Otherwise, in an endothermic reaction ($\Delta H > 0$) the reaction path is not favored, since the newly formed radical is less stable than the starting one.

In order to examine the influence of different radicals to an antiradical mechanism of the most stable kaempferol conformer (conformer I), the reactive particle $RO^{\bullet}$ was introduced. In the present paper, this particle represents superoxide anion and hydroxyl radicals.

In HAT mechanism, the hydrogen atom is transferred from phenolic compound to the free radical $RO^{\bullet}$:

$$K-OH \; + \; RO^{\bullet} \rightarrow \; K-O^{\bullet} + \; ROH \tag{4}$$

ΔH_{BDE} for the HAT mechanism can be calculated using the following equation:

$$\Delta H_{BDE} = H(KO^{\bullet}) + H(ROH) - H(K-OH) - H(RO^{\bullet}), \tag{5}$$

where the $H(KO^{\bullet})$, $H(ROH)$, H(K–OH), and $H(RO^{\bullet})$ are the enthalpies of the phenolic radical, molecule obtained after hydrogen atom abstraction from the phenolic compound, starting phenolic compound, and reactive radical species, respectively. Lower ΔH_{BDE} values can be attributed to a greater ability of phenolic compound to donate a hydrogen atom to $RO^{\bullet}$ species.

The first step in the SET-PT mechanism is the transfer of an electron from phenolic compound to free radical species, yielding the phenolic radical cation $Ph–OH^{\bullet+}$ and the corresponding anion.

$$K-OH \; + \; RO^{\bullet} \rightarrow \; K-OH^{\bullet+} + \; RO^{-} \tag{6}$$

ΔH_{IP} for the first step of the SET-PT mechanism can be calculated as follows:

$$\Delta H_{IP} = H(K-OH^{\bullet+}) + H(RO^{-}) - H(K-OH) - H(RO^{\bullet}), \tag{7}$$

where the H(K–$OH^{\bullet+}$) and $H(RO^{-})$ are the enthalpies of the radical cation of initial phenolic compound and anion generated from the corresponding initial radical.

The second step of this mechanism is the deprotonation of K–$OH^{\bullet+}$ by RO^{-}:

$$K-OH^{\bullet+} + \; RO^{-} \rightarrow \; K-O^{\bullet} + \; ROH \tag{8}$$

ΔH_{PDE} can be calculated using the following equation:

$$\Delta H_{PDE} = H(K-O^{\bullet}) + H(ROH) - H(K-OH^{\bullet+}) - H(RO^{-}) \tag{9}$$

The first step in the SPLET mechanism is the deprotonation of phenolic compound by RO^{-} or other bases. The outcome of this reaction is the formation of the phenoxide anion K–O^{-}:

$$K-OH \; + \; RO^{-} \rightarrow K-O^{-} + \; ROH \tag{10}$$

ΔH_{PA} can be calculated as follows:

$$\Delta H_{PA} = H(K-O^{-}) + H(ROH) - H(K-OH) - H(RO^{-}) \tag{11}$$

In the next step, electron transfer from K–O^{-} to $RO^{\bullet}$ takes place:

$$K-O^{-} + \; RO^{\bullet} \rightarrow \; K-O^{\bullet} + \; RO^{-} \tag{12}$$

ΔH_{ETE} can be determined by the following equation:

$$\Delta H_{\mathrm{ETE}} = H(\mathrm{K-O^{\bullet}}) + H(\mathrm{RO^{-}}) - H(\mathrm{K-O^{-}}) - H(\mathrm{RO^{\bullet}}) \quad (13)$$

The potential antiradical activity of kaempferol, for each reactive site (OH group), is simulated in the reactions with hydroxyl ($^{\bullet}$OH) and superoxide anion ($^{\bullet}$OO^{-}) radicals, the radical species among the most important in biological and food chemistry. Reaction enthalpies for the reaction of kaempferol with selected radicals, related to three mechanisms of free radical scavenging activity (HAT, SET-PT, and SPLET), are calculated using M05-2X/6-311G (d, p) model.

Results and discussion

Antiradical activity of kaempferol toward hydroxyl and superoxide anion radicals: spectroscopic measurements

Radical scavenging activity of kaempferol toward hydroxyl radical is evaluated using the Fenton reaction in 2-deoxyribose degradation test and hydroxyl radical producing system with DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) as the spin-trapping agent. Its activity toward superoxide anion radical is evaluated by using the UV-irradiated riboflavin/EDTA generating system and by cyclic voltammetry.

The oxidative degradation of 2-deoxyribose induced by Fenton reagents is presented in Fig. S1. When only kaempferol is present in the system (Fig. S1, trace o), it acts as an iron chelator that inhibits hydroxyl radical production up to 73 % at concentrations of 0.15 mM and maximum of iron(II) concentration of 35 μM. The same efficiency shows quercetin but at concentration three times smaller than kaempferol's [25]. The mechanism of iron(II)-promoted Fenton reaction attenuation is tested by EDTA which is a known iron chelator. Upon gradual addition of the equimolar EDTA to the reaction system, the Fenton reaction is almost completely attenuated (up to 88 %) (Fig. S2, trace▲). This fact implicates iron(II)-EDTA complexation as the key factor in eliminating the available iron [25, 26]. In the absence of EDTA, kempferol inhibits 2-deoxyribose degradation up to 73 % (Fig. S1, trace o) but at a concentration two orders of magnitude higher than EDTA. This result suggests that the inhibition of the Fenton reaction proceeds by iron chelation rather than the other possible paths. At fixed EDTA concentration (5 μM) (Fig. S1, trace ■), a hydroxyl radical is generated although not at the same rate as when only iron(II) is present (Fig. S1, trace ●). In the presence of EDTA (Fig. S1, trace ▲), kaempferol inhibits the reaction more effectively than when only EDTA is present (Fig. S1, trace ■). This result suggests that kaempferol, like fisetin and morin [26, 27], besides the ability to act as chelation agent is also able to act as a radical scavenging agent in the presence of the strong chelator like EDTA. This fact is supported by the competition experiments (Fig. S3) showing partial kaempferol recovery from iron to kaempferol complex induced by different EDTA concentrations.

Figure 2 shows the characteristic EPR spectra of DEPMPO/OH (Fig. 2a) and DEPMPO/OOH (Fig. 2b) adducts generated in the Fenton and UV-irradiated riboflavin/EDTA systems. The addition of kaempferol to the reaction systems notably decreases the amount of the formed DEPMPO/OH and DEPMPO/OOH adducts. Scavenging activity of kaempferol toward hydroxyl radical is calculated with respect to the relative height of the third peak in the EPR spectrum of the spin adduct (marked with closed circle in Fig. 2a) while toward superoxide radical with respect to the relative height of the fourth low-field peak of the EPR spectrum of the spin adduct (marked with open circle in Fig. 2b; Table 1).

As Table 1 shows, kaempferol has been found to be more potent hydroxyl than the superoxide radical scavenger compared to quercetin, morin [25, 26], fisetin, and baicalein. The same results are obtained by Wang [28] and Heijnen [29], who evaluated the radical scavenging activity of several flavonoids and found that kaempferol was one of the strongest scavengers for the Fenton-generated hydroxyl radical, with an IC_{50} of 0.5 μM, and also very strong peroxynitrite scavenger, with an IC_{50} of 0.35 μM. Here, the established activity ranking for hydroxyl radical is kaempferol > quercetin ≈ morin ≈ baicalein > fisetin. Relating the obtained radical activity sequence with structural features and substitution patterns of these flavone molecules, it could be assumed that along with C4′-OH functional, which most probably renders these molecules as hydroxyl radical scavengers, C5-OH group has more prominent role in scavenging hydroxyl radical compared to *ortho*-hydroxy groups in B ring. However, quite opposite sequence for kaempferol activity toward superoxide anion radical (quercetin > fisetin > baicalein > morin > kaempferol) implicate *ortho*-hydroxy groups in B ring and pyrogallol functional (in A ring of baicalein) as more relevant. It should be noted that C4′-OH functional also renders these molecules as superoxide anion radical scavengers. The established differences could also be related to different scavenging mechanisms governing reduction of different oxygen species.

The pronounced ability of kaempferol to decrease hydroxyl radical concentration may play a key role in its radical scavenging activity in vivo. It would be possible to assume that scavenging activity of kaempferol toward superoxide radical may also be of relevance in biological systems, since the superoxide radical formation indirectly

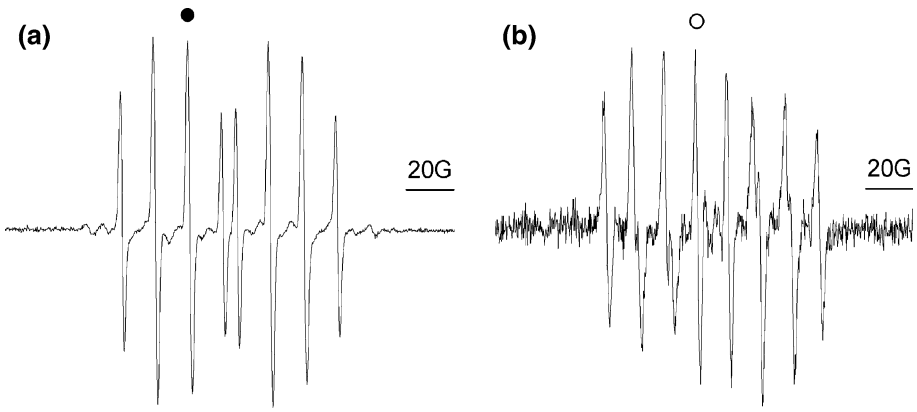

Fig. 2 Characteristic EPR spectra of **a** DEPMPO/OH adduct generated in the Fenton reaction system, and **b** DEPMPO/OOH adduct generated in the UV-irradiated riboflavin/EDTA system. *Closed* and *open circles* mark characteristic EPR peaks used for measuring oxidant scavenging activity of kaempferol

Table 1 Oxidant scavenging activity of kaempferol and structurally related flavones

Oxidant scavenging (% of radical reduction)	kaempferol	Quercetin [25]	morin [27]	fisetin[a]	baicalein[a]
DEPMPO-OH (hydroxyl radical)	43	37	36	30	35
DEPMPO-OOH (superoxide)	26	55	34	50	42

[a] unpublished results

leads to hydroxyl radical formation. It is known that the oxidative stress involves the most reactive oxygen and nitrogen species for which the formation of superoxide anion is required. Namely, superoxide dismutase enzyme converts the superoxide anion into hydrogen peroxide which further can be, in the presence of reduced transition metals, like Fe(II) or Cu(I) ions, converted into highly reactive hydroxyl radical known to cause damage to highly important biological molecules like DNA, lipids, proteins, etc.

Electrochemical behavior of kaempferol

CV measurements (Fig. S4) indicate complex pH-dependent electrochemistry of kaempferol. Oxidation onset potential is observed to shift to lower values with decreasing pH, as previously demonstrated for the case of compounds with similar structure such as quercetin and fisetin [25, 30]. This indicates the participation of protons in the electrochemical steps, i.e., that the deprotonation accompanies and facilitates the deelectronation [31]. While at pH 3, only one oxidation peak is observed in investigated potential window, at pH 7 and 9 clearly discernible oxidation peaks are observed, while cathodic peaks are not as noticeable. Similarly to fisetin [30], the first oxidation peak can be ascribed to the oxidation of hydroxyl moiety at B ring, while second peak can be ascribed to oxidation of hydroxyl group located at C ring. Earlier reports suggest that the first charge transfer is followed by fast chemical reaction which involves structural reorganization of the first oxidation product. Comparing the electrochemical

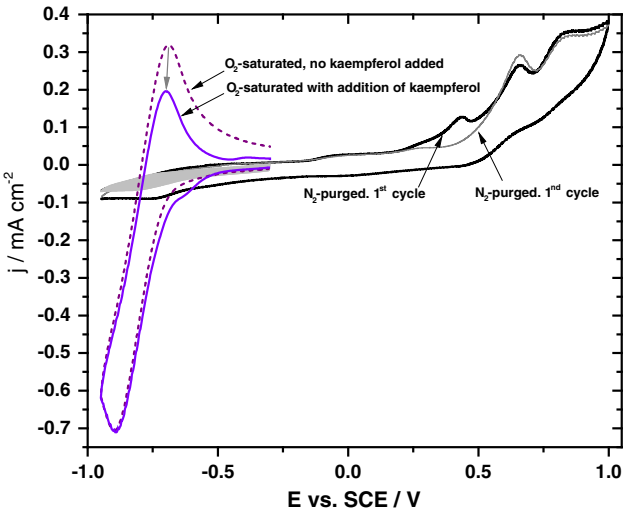

Fig. 3 Cyclic voltammograms of O_2-saturated 0.1 M Bu_4NPF_6 solution in DMSO in the potential window between −0.3 and −0.95 V versus SCE with (full line) and without kaempferol (*dashed line*). Cyclic voltammograms of 1.5 mM solution of kaempferol in the same supporting electrolyte solution are enclosed

behavior of kaempferol and quercetin in neutral methanol solution, Yang [32] has observed that the first oxidation product of quercetin, formed upon oxidation of C3′,C4′-dihydroxyl moiety at B ring, is more stable than the corresponding compound obtained by hydroxyl group oxidation at B ring of kaempferol.

Superoxide radical scavenging activity of kaempferol is probed electrochemically by analyzing cyclic voltammograms of O_2-saturated DMSO solutions upon successive

additions of kaempferol. $^{\bullet}O_2^-$ scavenging activity is diagnosed as decrease of reverse anodic peak corresponding to superoxide radical oxidation upon addition of kaempferol (Fig. 3). Scavenging activity is quantified in a same manner as previously described [26], through the relative change of charge under anodic peak in the presence of 1 mM polyphenolic compound. In the potential window where $^{\bullet}O_2^-$ is formed and is reacting with present kaempferol, there are no faradic processes corresponding to oxidation/reduction of kaempferol itself (Fig. 3), guarantying the correctness of analysis. In addition, repetitive cyclic voltammograms indicate that kaempferol oxidation products remain adsorbed on electrode surface. Similarly to previously analyzed quercetin and morin [26], presence of cathodic pre-peak and retained amplitude of cathodic peak (Fig. 3) indicate predominant radical transfer mechanism [33] with the first two reaction steps:

$$O_2 + e^- \longrightarrow k_s {}^{\bullet}O_2^- \quad (14)$$

$${}^{\bullet}O_2^- + AH \longrightarrow k_f HO_2^- + A^{\bullet} \quad (15)$$

and the overall reaction is as follows:

$$O_2 + e^- + 2AH \rightarrow H_2O_2 + A^- + nonradicalproducts \quad (16)$$

Obtained results have two important implications. First of all, electrochemical test undoubtedly confirms that kaempferol is superoxide anion radical scavenger. This is to be related to reports where superoxide radical scavenging activity is tested in the system where $^{\bullet}O_2^-$ is produced by the action of the enzyme xanthine oxidase, and the attenuation of the specific signal could be ascribed to both superoxide scavenging and inhibitory action on xanthine oxidase [1]. While some of these reports point out kaempferol as excellent superoxide scavenger [28], the other reports classify kaempferol exclusively as xanthine oxidase inhibitor [1]. It is possible to suppose that both effects are operative and cannot be investigated separately on xanthine oxidase $^{\bullet}O_2^-$ generating system.

Here, the obtained results indicate kaempferol as rather good superoxide scavenger (% of signal reduction is 42 ± 2 in the presence of 1 mM kaempferol). The percentage of the signal reduction in the presence of quercetin, investigated in the same electrochemical system, is 67 ± 5 [26]. Nevertheless, certain discrepancies between electrochemical and EPR evaluation of superoxide scavenging activity may arise due to different modes of $^{\bullet}O_2^-$ production. As hypothesized by Rene [33], complications concerning the nature and modifications of the heterogeneous electron-transfer kinetics may occur, influencing the final result. The authors proposed this assumption due to the difficulties in fully explaining a shift of cathodic prepeak for a series of flavonoids, observable in the case of kaempferol, too (Fig. 3). In spite this all, conclusion regarding very good superoxide scavenging activity of kaempferol is unambiguous.

As kaempferol and quercetin have similar structure with the only difference of C3′-OH moiety in the case of quercetin, it appears that the proposition made by Wang et al. [28], regarding the role of OH substitutions in B ring, is plausible. Originally, the authors proposed that substitution at C4′ position may be sufficient to render a flavonol as a scavenger of superoxide and/or an inhibitor of xanthine oxidase.

According to our results on high activity of kaempferol toward $^{\bullet}O_2^-$, it appears that more specific assumption regarding C4′-OH moiety can be made, leading to a conclusion that the presence of this group is a prerequisite for high superoxide scavenging, while the presence of other OH substituents in B ring modifies this activity. This conclusion can be supported by a single example of galangin, flavonol with the same substitution pattern of A and C rings as kaempferol but with no substituent in B ring, which was found to be xanthine oxidase inhibitor with an additional pro-oxidant effect on the production of superoxide [1].

DFT calculations

The preferred mechanism of antiradical activity of kaempferol can be estimated from ΔH_{BDE}, ΔH_{IP}, and ΔH_{PA} values. Namely, the lowest of these values indicates which mechanism is favorable, and the preferred site of antiradical action can be estimated from the sum of enthalpies involved in a particular free radical scavenging mechanism (BDE for HAT; IP and PDE for SET-PT; and PA and ETE for SPLET). In Tables 2 and S1, the reaction enthalpies and the corresponding free energies are listed. Since the entropy factor is small, positive, and nearly constant (0.54–0.55 kJ K^{-1} mol^{-1}), the sign of enthalpy change implies the same sign of free Gibbs energy change, no matter what the temperature value is. On the basis of thermodynamical values in Table 2, it is clear that only HAT and SPLET are the operative radical scavenging mechanisms of kaempferol in all solvents under investigations. The hydrogen atom is abstracted mainly from 3-OH group on the C ring when the reaction takes place via HAT mechanism which confirms the role of the C3-OH group in antiradical mechanism of kaempferol. Also this result is in agreement with BDE values obtained by Rong [34]. The 7-OH group in the ring A is the dominant position for reaction via SPLET mechanism in all solvents.

Examination of the reaction enthalpies for the reactions of kaempferol with hydroxyl radical shows that they are exothermic in all solvents. On the basis of the values in

Table 2 Calculated reaction enthalpies (kJ/mol) for the reactions of kaempferol with hydroxyl radical, superoxide radical anion, and peroxyl radical

M05-2X/6-311G(d,p)										
Kaempferol	**Water (ε = 78.35)**					**DMSO (ε = 46.83)**				
	HAT	SET-PT		SPLET		HAT	SET-PT		SPLET	
	ΔH_{BDE}	ΔH_{IP}	ΔH_{PDE}	ΔH_{PA}	ΔH_{ETE}	ΔH_{BDE}	ΔH_{IP}	ΔH_{PDE}	ΔH_{PA}	ΔH_{ETE}
		99					242			
KOH-3 $+^{\cdot}OH$	−148		−247	−127	−21	−139		−380	−219	80
KOH-4′ $+^{\cdot}OH$	−125		−223	−127	3	−119		−361	−230	111
KOH-5 $+^{\cdot}OH$	−95		−194	−123	28	−76		−317	−207	131
KOH-7 $+^{\cdot}OH$	−95		−194	−141	46	−91		−333	−245	153
		373					692			
KOH-3 $+^{\cdot}OO^{-}$	66		−307	19	46	103		−588	−24	128
KOH-4′ $+^{\cdot}OO^{-}$	89		−284	19	70	123		−569	−36	158
KOH-5 $+^{\cdot}OO^{-}$	118		−254	23	96	166		−526	−12	179
KOH-7 $+^{\cdot}OO^{-}$	118		−254	5	114	151		−541	−50	201
	Ethanol (ε = 24.85)					**DMF (ε = 37.22)**				
		135					244			
KOH-3 $+^{\cdot}OH$	−149		−284	−149	−1	−138		−383	−222	83
KOH-4′ $+^{\cdot}OH$	−128		−263	−151	23	−119		−363	−231	112
KOH-5 $+^{\cdot}OH$	−95		−230	−144	49	−76		−320	−207	131
KOH-7 $+^{\cdot}OH$	−97		−232	−165	68	−91		−335	−245	154
		454					697			
KOH-3 $+^{\cdot}OO^{-}$	74		−379	12	62	103		−594	−28	131
KOH-4′ $+^{\cdot}OO^{-}$	96		−358	10	86	122		−575	−37	159
KOH-5 $+^{\cdot}OO^{-}$	129		−325	17	112	166		−531	−13	179
KOH-7 $+^{\cdot}OO^{-}$	127		−327	−5	131	150		−547	−51	201

Table 2, it is clear that kaempferol reacts with hydroxyl radical via both HAT and SPLET mechanisms in all solvents. For protic polar solvents (water and ethanol), HAT and SPLET are competitive mechanisms, while in other two aprotic polar solvents (DMSO and DMF), SPLET is the prevailing mechanism.

In the reaction with superoxide anion radical, kaempferol (Table 2) undergoes SPLET mechanism which is dominant in all solvents, while the reaction by HAT mechanism is endothermic in all solvents. Moreover, the aprotic polar solvents (DMF and DMSO) are more suitable medium for reactions of kaempferol with superoxide anion radical.

Conclusion

Kaempferol shows radical scavenging activity toward hydroxyl and superoxide anion radicals. The results of 2-deoxyribose degradation test suggest that the anti-Fenton activity of kaempferol may originate both from iron chelation and radical scavenging. Activity ranking, established by EPR measurements, for hydroxyl radical is kaempferol > quercetin ≈ morin ≈ baicalein > fisetin and for superoxide anion radical: quercetin > fisetin > baicalein > morin > kaempferol. The obtained differences could be rationalized in terms of structural features generally governing the antioxidant behavior of the flavonoids, predominately the substitution pattern of B ring, and characteristics of radical species itself. Namely, it may be concluded that C4′-OH functional generally renders flavone molecules as hydroxyl and superoxide anion radical scavengers, while C3′-OH modifies the activity. Additionally, it seems that C5-OH group has more important role in scavenging activity toward hydroxyl radical compared to *ortho*-hydroxy groups in B ring. In activity toward superoxide anion radical, *ortho*-hydroxy groups in B ring and pyrogallol functionals (in A ring of baicalein) are more relevant. Results of CV measurements confirm very good superoxide scavenging activity of kaempferol.

Reaction enthalpies and free energies, calculated using the M052X/6-311G(d,p) level of theory, of the individual steps of three possible radical scavenging mechanisms (HAT, SET-PT, and SPLET) offer insight into the influence of the medium on the thermodynamically preferred mechanism as well as the preferred sites of the antiradical

action. In protic polar solvents (water and ethanol), kaempferol reacts with hydroxyl radical via competitive HAT and SPLET mechanisms, while in aprotic polar solvents (DMSO and DMF) SPLET prevails. Preferred sites of action are C3-OH (via HAT) and C7-OH (via SPLET) functionals. Aprotic polar solvents (DMF and DMSO) are more favorable for the interaction with superoxide anion radical. In those solvents kaempferol undergoes SPLET mechanism.

As theoretical calculations imply, the difference in superoxide anion radical activity, obtained by EPR and CV measurements, could be rationalized not only in terms of different modes of superoxide anion radical production but also in terms of the impact of the medium used in those measurements (ethanol in EPR and DMSO in CV).

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