RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2014, **4**, 32228

The preferred radical scavenging mechanisms of fisetin and baicalein towards oxygen-centred radicals in polar protic and polar aprotic solvents

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Received 24th March 2014
Accepted 20th June 2014

DOI: 10.1039/c4ra02577f

www.rsc.org/advances

Naturally occurring flavonoid molecules, *i.e.* fisetin (2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one) and baicalein (5,6,7-trihydroxy-2-phenyl-4*H*-chromen-4-one), have been investigated experimentally and theoretically for their ability to scavenge hydroxyl and superoxide anion radicals. The reaction enthalpies for the reaction of fisetin and baicalein with selected radical species, related to three mechanisms of free radical scavenging activity (HAT, SET-PT and SPLET), are calculated using the M05-2X/6-311+G(d,p) model. The calculated energy requirements indicated the preferred radical scavenging mechanisms in polar protic and aprotic solvents.

Introduction

Phenolic compounds are plant secondary metabolites commonly found in herbs and fruits. Flavonoids are one of the major groups among the phenolics with more than several thousand known compounds.[1]

Free radicals, oxygen-, nitrogen- or carbon-centred, which are constantly generated *in vivo*, are a part of metabolic processes. Among all radical species, oxygen-centred radicals are proposed to have a substantial role *in vivo*, since they can potentially damage almost all types of biologically important molecules like lipids (causing lipid peroxidation), amino acids, carbohydrates, and nucleic acids (causing mutations).[2]

The hydroxyl radical is considered the most reactive radical (with a half-life around 10^{-9} s) and the most damaging one by far. Hydrogen peroxide, in the presence of metal ions, is converted to a hydroxyl radical ($HO^{\bullet}$) and a hydroxide anion (HO^{-}). This reaction, called the Fenton reaction, is very important in biological systems, because most cells have some level of iron, copper, or other metals that can catalyze this reaction. The hydroxyl radical passes easily through membranes and cannot be kept out of cells. The uncontrolled action of hydroxyl radicals can have devastating effects within the body, since it reacts at diffusion rates with virtually any molecule found in its path, including macromolecules such as DNA, membrane lipids, proteins and carbohydrates.[2,3]

The initial step in most biological free radical reactions is the production of superoxide anion radical ($O_2^{\bullet -}$) (SOR), which is formed upon monovalent reduction of molecular oxygen. Superoxide can act both as an oxidant (by accepting electrons) or as a reductant (by donating electrons). Although it is not particularly reactive and thus does not cause much oxidative damage, it is biologically a very toxic agent with some bad implications. It acts as a precursor to other oxidizing agents like singlet oxygen, peroxynitrite and other highly reactive molecules. Superoxide anion also acts as a reducing agent of metal ions (Fe(III)) in the production of the highly reactive hydroxyl radical ($HO^{\bullet}$), which is converted from the hydrogen peroxide (H_2O_2). Furthermore, superoxide anion radical can react with the hydroxyl radical ($HO^{\bullet}$) to form singlet oxygen ($^1O_2$), which is not a radical form but reactive nonetheless. In reaction with nitric oxide ($NO^{\bullet}$), it produces peroxynitrate ($OONO^{-}$), another highly reactive oxidizing molecule. Because it is not particularly reactive, the chemistry of superoxide anion radical in living systems is most likely dominated by the hydroperoxyl, $^{\bullet}OOH$, radical, which is its protonated form. Hydroperoxyl radicals could also indicate the behaviour of peroxy $^{\bullet}OOR$ radicals, which are very common in living systems.[2–4]

If not counterbalanced by internal or external antioxidants, a high production of reactive oxygen species (ROS) consequently leads to oxidative stress, which has been proposed to play an important role in the pathogenesis of many, if not all, diseases. The antiradical properties of flavonoids are related to their ability to transfer their phenolic H-atoms to free radical forms. This transfer can be visualized through at least three

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mechanisms characteristic not only of flavonoids but phenolics in general: hydrogen atom transfer (HAT) (eqn (1)), sequential proton loss electron transfer (SPLET) (eqn (2)), and single electron transfer followed by proton transfer (SET-PT) (eqn (3)). All three mechanisms are competitive, and which mechanism will be predominant depends on the reaction conditions, indicating that the nature of present free radicals and the polarity of the solvent significantly influence the reaction pathway. In any case, the result of all three mechanisms is the same, as described in reactions (1)–(3).[5–7]

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

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

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

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

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

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

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

Baicalein is naturally occurring flavone found in the traditional Chinese medicinal herb Baikal skullcap. It is used in the treatment of many disease-related symptoms such as insomnia, fever and perspiration and also investigated with promising results in different areas such as anticancer, anti-inflammatory and antioxidant activities.[8–10] Fisetin is also a naturally occurring flavonol commonly found in strawberries and other fruits and vegetables. It is considered as a potent antioxidant capable of effective free radical scavenging *in vivo*. Its most striking beneficial medical effects are as follows: stimulating signalling pathways that enhance the long-term memory neuroprotective role, induction of neuronal differentiation, inhibition of the aggregation of the amyloid beta protein that may cause progressive neuronal loss in Alzheimer's disease and modulation of the expression of more than 20 genes at the transcription level.[11–13]

The present paper aims to provide quantitative tools to thoroughly and comprehensively determine the antiradical mechanisms of fisetin and baicalein by calculating the energy requirements for the reactions of these molecules with hydroxyl and superoxide anion radicals in different media. Calculated energy requirements may indicate which radical scavenging mechanism is thermodynamically preferred and point out active sites for radical inactivation. Joint application of theoretical calculations and experimental measurements in determining the antiradical activity of fisetin and baicalein is aimed at proving the transferability of the results obtained by different methods.

Results and discussion

EPR measurements

The free radical scavenging activities of fisetin and baicalein towards two oxygen-centred free radicals, hydroxyl and superoxide anion, are estimated by EPR spectroscopy. Upon addition of flavones, it is observed that the signals are quenched to different extents, indicating different radical scavenging activities by the tested compounds. The antioxidant activity (AA) is calculated with respect to the relative heights of the hydroxyl and superoxide peaks marked with circles in Fig. 1 and 2.

The standard Fenton reaction system[14] generates $^{\bullet}$OH radicals to a high extent, which forms stable spin-adducts with the spin-trap DEPMPO and gives the characteristic EPR signal of the DEPMPO/OH adduct. It is observed that the addition of fisetin and baicalein to the Fenton reaction system decreases the amount of DEPMPO/OH adduct (Fig. 1). The antioxidant activity is calculated with respect to the relative height of the third peak in the EPR spectrum of the spin-adduct (marked with a circles in Fig. 1).

Fig. 2 shows characteristic EPR spectra of DEPMPO/OOH adduct generated in UV irradiated riboflavin/EDTA systems. The addition of fisetin (Fig. 2a) and baicalein (Fig. 2b) to the reaction systems notably decreases the amount of the formed DEPMPO/OOH adduct.

Table 1 shows that baicalein is a slightly stronger antioxidant compared to fisetin. Taking into account the structure of the molecule it is possible to assume that the influence of the position C5 is more dominant in comparison to the *ortho* hydroxyl groups in ring B. Compared to other structurally related flavone molecules (Table 1), it is also evident that the C5 position has an important role in the selectivity towards the hydroxyl radical since kaempferol (3,5,7,4′-tetrahyroxy flavone) shows the highest percentage of hydroxy radical reduction (kaempferol $\sim$ quercetin $\sim$ morin $\sim$ baicalein > fisetin). The obtained results are in accordance with the results of Wang[15] and Heijnen[16] who found that kaempferol was one of the strongest scavengers for the Fenton-generated hydroxyl radical (an IC_{50} of 0.5 μM).

Fisetin is found to be a more potent superoxide anion radical scavenger compared to baicalein (Table 1). Here we established

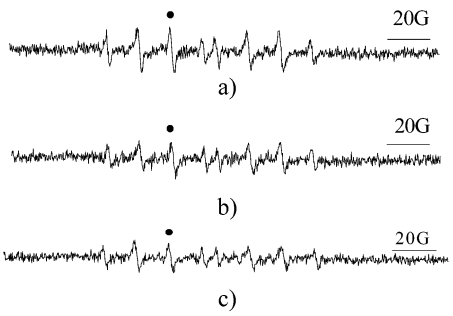

Fig. 1 Characteristic EPR spectra of DEPMPO/OH (a) and DEPMPO/OH adducts of fisetin (b) and baicalein (c) generated in a Fenton reaction system. Closed circles mark characteristic EPR peaks used for measuring oxidant scavenging activity of fisetin and baicalein.

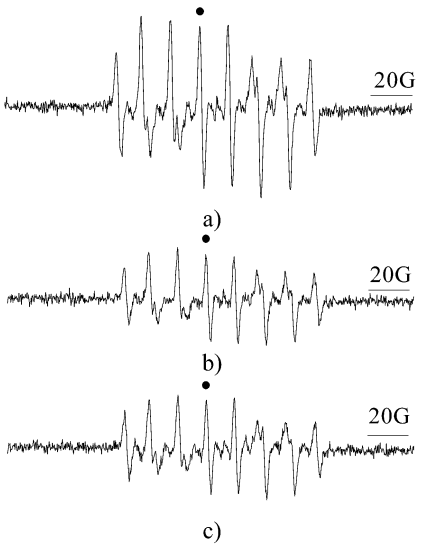

Fig. 2 Characteristic EPR spectra of DEPMPO/OOH (a) and DEPMPO/OOH adduct of fisetin (b) and baicalein (c) generated in the UV irradiated riboflavin/EDTA system. Closed circles mark characteristic EPR peaks used for measuring oxidant scavenging activity of fisetin and baicalein.

the following activity ranking for superoxide anion radicals compared with other flavone molecules: quercetin > fisetin > baicalein > morin > kaempferol. Regarding the obtained radical activity sequence with the structural features and substitution patterns of these flavone molecules, it could be assumed that *ortho*-hydroxy groups in the B ring (quercetin, fisetin) and a pyrogallol functional (in the A ring of baicalein) have more prominent roles in the activity towards superoxide anion radical. Also, although present in almost all molecular structures (except fisetin), the C5 group is not the one which determines the superoxide anion radical scavenging activity of the investigated molecules. The substitution patterns of morin and kaempferol also suggest that the *ortho* hydroxyl system in ring B is the one influencing the activity towards superoxide anion radicals. The obtained results are quite opposite to those obtained for hydroxyl radical scavenging by the same molecules[21] in which the C5–OH showed greater prominence compared to *ortho*-hydroxy groups in the B ring. The established differences could also be related to different scavenging mechanisms governing reduction of different oxygen species.

Electrochemical measurements

Electrochemical measurements concerning aqueous solutions of baicalein and fisetin indicate that both compounds display complex electrochemistry. Regarding baicalein, two oxidation peaks are discernible from the cyclic voltammograms recorded in aqueous solutions. Following reports in the recent literature,[17] the first anodic peak can be associated with the 2e process of the oxidation of two OH moieties located at the A ring. This oxidation product is further oxidized irreversibly as no corresponding cathodic peak is observed upon the reversal of potential sweep (Fig. 3, left).

In the case of fisetin (Fig. 3, right), three distinct anodic peaks were observed, in agreement with the available literature.[18] In contrast to baicalein, the first oxidation step involved in the electrochemical oxidation of fisetin relates to the oxidation of the catecholic group in the B ring, which involves the elimination of two electrons and two H^+ ions. The oxidation product formed in the first step undergoes fast intramolecular rearrangement and is oxidized further.[18] Regarding radical scavenging activity of baicalein and fisetin, cyclic voltammetry indicates potent radical scavengers.[19] Namely, Lindberg Mandsen *et al.*[19] have correlated the oxidation onset potential of different flavonoids with the rate of scavenging peroxyl and DPPH radicals, showing the existence of limiting oxidation onset potential above which flavonoid is no longer an efficient radical scavenger. Both baicalein and fisetin have low oxidation potentials indicating high radical scavenging activities.

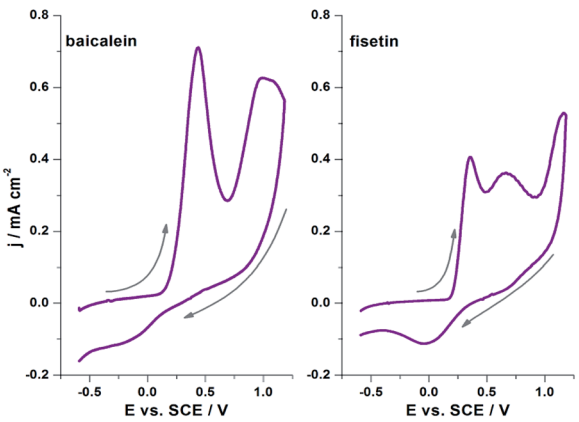

Fig. 3 Background-corrected cyclic voltammograms of baicalein (left) and fisetin (right; $c = 5 \times 10^{-4}$ mol dm^{-3}) in aqueous solution; pH = 7, potential sweep rate 500 mV s^{-1}, N_2-purged solutions.

Table 1 Radical scavenging activity of fisetin, baicalein and structurally related flavones

Oxidant scavenging (% of radical reduction)	fis.	baic.	quer.[21]	mor.[21]	kaem.[a]
$^{\bullet}$OH, EPR tested	30	35	37	36	43
$^{\bullet}O_2^-$, EPR tested	50	42	55	34	26
$^{\bullet}O_2^-$, electrochemically tested	74	54	67	34	42

[a] Unpublished result.

The radical scavenging activity of baicalein and fisetin toward electrochemically generated $^{\bullet}O_2^-$ was probed in DMSO solution containing 0.1 M Bu_4NPF_6, following an approach proposed by Rene *et al.*[20] and further elaborated by us.[21] Within this approach, relative reduction of the anodic peak related to the oxidation of the electrochemically formed $^{\bullet}O_2^-$ in the presence of 1 mM flavonoid (Fig. 4) is taken as the measure of $^{\bullet}O_2^-$ radical scavenging activity.[21] In both cases, a radical transfer mechanism dominates in the reaction between $^{\bullet}O_2^-$ radical and investigated flavonoids, as suggested by the appearance of the cathodic prepeak and negligible changes of the amplitude of cathodic peak[20] (Fig. 3). Comparing baicalein and fisetin $^{\bullet}O_2^-$ scavenging activities, fisetin displays somewhat higher radical scavenging rate with a 74% ± 4% signal reduction compared to 54% ± 3% signal reduction in the case of baicalein (Table 1). The established activity ranking for superoxide anion radicals compared to several other flavone molecules is: fisetin > quercetin > baicalein > kaempferol > morin (Table 1).

The high $^{\bullet}O_2^-$ radical scavenging activities of baicalein and fisetin, observed here, should be related to previous reports where superoxide radical scavenging activity was tested in systems where $^{\bullet}O_2^-$ was produced by the action of the enzyme xanthine oxidase. In the latter case, the attenuation of the specific signal can be ascribed to both the superoxide scavenging and inhibitory action on xanthine oxidase.[22] Cos *et al.*[22] classified flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. The authors determined IC_{50} values for the reduction of superoxide levels, reporting a lower value for fisetin, compared to baicalein, in agreement with the greater radical scavenging activity observed here. However, according to the classification of the same authors, baicalein acts only as a xanthine oxidase inhibitor and not as a superoxide scavenger, while in the case of fisetin, both effects are operative. However, the experiments described here unambiguously confirm that both baicalein and fisetin act as $^{\bullet}O_2^-$ radical scavengers. Similar to the basic electrochemistry of baicalein and fisetin, it can be assumed that the reactive sites for the reaction with electrochemically generated $^{\bullet}O_2^-$ radicals are OH substitutions in the A ring of baicalein and the catecholic moiety in the B ring of fisetin.

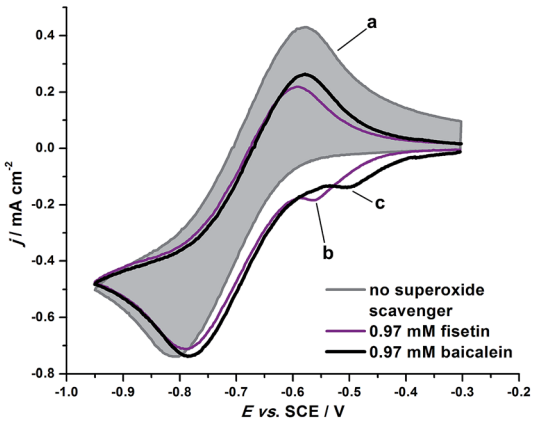

Fig. 4 Cyclic voltammetry of GC electrode in O_2-saturated DMSO solution supported by 0.1 M Bu_4NPF_6 with no superoxide radical scavenger added (curve a) and upon the addition of fisetin (curve b, c = 0.97 mM) or baicalein (curve c, c = 0.97 mM).

Antioxidative mechanisms of fisetin and baicalein with different free radicals

To be able to examine the influence of different radicals on an antioxidative mechanism, the reactive particle RO$^{\bullet}$ is introduced. In the present paper, this particle represents hydroxyl and superoxide anion radicals, which react with the most stable structures of fisetin and baicalein (Fig. 5). The scavenging properties of fisetin and baicalein are related to their ability to transfer a H atom to a free radical. The newly formed radicals (*e.g.* phenoxy radicals of fisetin and baicalein, FO$^{\bullet}$, BO$^{\bullet}$) are less reactive and more stable than the previous ones. The following reactions describe this H atom transfer:

$$FO\text{–}H + RO^{\bullet} \rightarrow F\text{–}O^{\bullet} + ROH \quad (4)$$

$$BO\text{–}H + RO^{\bullet} \rightarrow B\text{–}O^{\bullet} + ROH \quad (5)$$

Reaction enthalpy is a quantity that can successfully contribute to the understanding of different mechanisms operating in antiradical activity. If a reaction is exothermic, the newly formed intermediate or radical is more stable than the initial one, indicating that the reaction path is favourable. Otherwise, if the reaction is endothermic, the reaction path is not favoured.[23]

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

$$Ar\text{–}OH + RO^{\bullet} \rightarrow Ar\text{–}O^{\bullet} + ROH \quad (6)$$

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

$$\Delta H_{BDE} = H(ArO^{\bullet}) + H(ROH) - H(Ar\text{–}OH) - H(RO^{\bullet}) \quad (7)$$

where $H(ArO^{\bullet})$, $H(ROH)$, $H(Ar\text{–}OH)$, and $H(RO^{\bullet})$ are the enthalpies of the flavonoid radical, protonated radical, starting flavonoid compound, and reactive radical species, respectively.

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

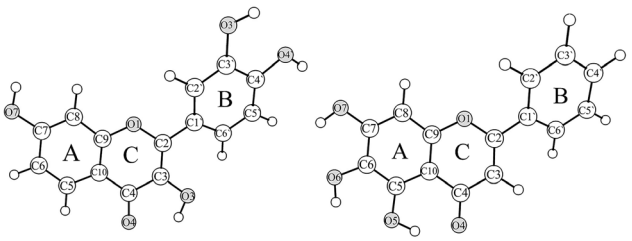

Fig. 5 Optimized and most stable structures of fisetin (left) and baicalein (right) in water.

$$Ar–OH + RO^{\bullet} \rightarrow Ar–OH^{\bullet+} + RO^{-} \quad (8)$$

ΔH_{IP} can be calculated as follows:

$$\Delta H_{IP} = H(Ar–OH^{\bullet+}) + H(RO^{-}) - H(Ar–OH) - H(RO^{\bullet}) \quad (9)$$

where the $H(Ar–OH^{\bullet+})$ and $H(RO^{-})$ are the enthalpies of the radical cation of the initial flavonoid and corresponding initial anion radical.

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

$$Ar–OH^{\bullet+} + RO^{-} \rightarrow Ar–O^{\bullet} + ROH \quad (10)$$

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

$$\Delta H_{PDE} = H(Ar–O^{\bullet}) + H(ROH) - H(Ar–OH^{\bullet+}) - H(RO^{-}) \quad (11)$$

The first step in the SPLET mechanism is deprotonation of the flavonoid by RO^{-}. The outcome of this reaction is the formation of the flavonoid anion $Ar–O^{-}$:

$$Ar–OH + RO^{-} \rightarrow Ar–O^{-} + ROH \quad (12)$$

ΔH_{PA} can be calculated as follows:

$$\Delta H_{PA} = H(Ar–O^{-}) + H(ROH) - H(Ar–OH) - H(RO^{-}) \quad (13)$$

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

$$Ar–O^{-} + RO^{\bullet} \rightarrow Ar–O^{\bullet} + RO^{-} \quad (14)$$

ΔH_{ETE} can be determined by the equation:

$$\Delta H_{ETE} = H(Ar–O^{\bullet}) + H(RO^{-}) - H(Ar–O^{-}) - H(RO^{\bullet}) \quad (15)$$

The species necessary to perform these calculations were generated from the most stable conformations of fisetin and baicalein. Calculations were performed in the aqueous phase, DMSO, ethanol and DMF (Tables 2 and 3).

The reaction enthalpies of fisetin and baicalein with hydroxyl, superoxide anion and peroxy radicals are related to three mechanisms of free radical scavenging activity (HAT, SET-

Table 2 Calculated reaction enthalpies (kJ mol^{-1}) for the reactions of fisetin with hydroxyl, superoxide anion and peroxyl radicals

M05-2X/6-311+G(d,p)

	Water $\varepsilon = 78.35$					DMSO $\varepsilon = 46.83$				
	HAT	SET-PT		SPLET		HAT	SET-PT		SPLET	
Fisetin	ΔH_{BDE}	ΔH_{IP}	ΔH_{PDE}	ΔH_{PA}	ΔH_{ETE}	ΔH_{BDE}	ΔH_{IP}	ΔH_{PDE}	ΔH_{PA}	ΔH_{ETE}
		91					95			
FOH-3 + $^{\bullet}OH$	−145		−236	−90	−55	−145		−240	−91	−54
FOH-3′ + $^{\bullet}OH$	−152		−243	−114	−38	−152		−247	−115	−37
FOH-4′ + $^{\bullet}OH$	−160		−251	−125	−35	−160		−255	−126	−34
FOH-7 + $^{\bullet}OH$	−108		−199	−120	11	−108		−204	−121	13
		409					418			
FOH-3 + $^{\bullet}OO^{-}$	77		−332	39	41	78		−341	39	39
FOH-3′ + $^{\bullet}OO^{-}$	70		−339	15	58	70		−348	14	56
FOH-4′ + $^{\bullet}OO^{-}$	62		−348	4	61	61		−356	3	59
FOH-7 + $^{\bullet}OO^{-}$	114		−296	9	108	113		−305	8	105
		184					188			
FOH-3 + $^{\bullet}OOH$	−9		−193	−47	38	−9		−197	−48	39
FOH-3′ + $^{\bullet}OOH$	−16		−200	−71	55	−16		−204	−72	56
FOH-4′ + $^{\bullet}OOH$	−24		−208	−82	58	−24		−212	−83	59
FOH-7 + $^{\bullet}OOH$	28		−156	−77	105	27		−161	−78	105
	Ethanol $\varepsilon = 24.85$					**DMF $\varepsilon = 37.22$**				
		104					98			
FOH-3 + $^{\bullet}OH$	−144		−248	−92	−52	−144		−242	−91	−53
FOH-3′ + $^{\bullet}OH$	−152		−256	−118	−34	−152		−250	−116	−36
FOH-4′ + $^{\bullet}OH$	−160		−265	−129	−31	−160		−258	−127	−33
FOH-7 + $^{\bullet}OH$	−108		−213	−124	15	−108		−206	−122	14
		438					424			
FOH-3 + $^{\bullet}OO^{-}$	79		−359	38	41	78		−346	39	39
FOH-3′ + $^{\bullet}OO^{-}$	71		−367	12	58	70		−354	14	57
FOH-4′ + $^{\bullet}OO^{-}$	63		−375	1	61	62		−362	3	60
FOH-7 + $^{\bullet}OO^{-}$	114		−323	7	108	114		−310	8	106
		197					190			
FOH-3 + $^{\bullet}OOH$	−8		−205	−49	41	−9		−199	−48	39
FOH-3′ + $^{\bullet}OOH$	−16		−213	−74	58	−16		−207	−73	57
FOH-4′ + $^{\bullet}OOH$	−24		−221	−86	61	−24		−215	−84	60
FOH-7 + $^{\bullet}OOH$	28		−169	−80	108	28		−163	−79	106

Table 3 Calculated reaction enthalpies (kJ mol^{-1}) for the reactions of baicalein with hydroxyl, superoxide anion and peroxy radicals

M05-2X/6-311+G(d,p)

	Water $\varepsilon = 78.35$					DMSO $\varepsilon = 46.83$				
	HAT	SET-PT		SPLET		HAT	SET-PT		SPLET	
Baicalein	ΔH_{BDE}	ΔH_{IP}	ΔH_{PDE}	ΔH_{PA}	ΔH_{ETE}	ΔH_{BDE}	ΔH_{IP}	ΔH_{PDE}	ΔH_{PA}	ΔH_{ETE}
		78					82			
BOH-5 + $^{\bullet}$OH	−121		−199	−96	−25	−121		−203	−97	−23
BOH-6 + $^{\bullet}$OH	−165		−243	−112	−53	−165		−247	−113	−52
BOH-7 + $^{\bullet}$OH	−119		−197	−118	−2	−119		−201	−119	0
		397					406			
BOH-5 + $^{\bullet}$OO^{-}	101		−296	32	68	101		−304	32	69
BOH-6 + $^{\bullet}$OO^{-}	57		−339	17	40	57		−348	17	41
BOH-7 + $^{\bullet}$OO^{-}	103		−294	11	91	103		−302	11	92
		171					175			
BOH-5 + $^{\bullet}$OOH	15		−156	−54	68	15		−160	−54	69
BOH-6 + $^{\bullet}$OOH	−29		−200	−69	40	−29		−204	−70	41
BOH-7 + $^{\bullet}$OOH	17		−154	−75	91	17		−158	−76	92
	Ethanol $\varepsilon = 24.85$					**DMF $\varepsilon = 37.22$**				
		92					85			
BOH-5 + $^{\bullet}$OH	−120		−212	−99	−21	−121		−206	−98	−23
BOH-6 + $^{\bullet}$OH	−164		−256	−115	−50	−165		−250	−113	−51
BOH-7 + $^{\bullet}$OH	−119		−210	−121	2	−119		−204	−119	0
		425					411			
BOH-5 + $^{\bullet}$OO^{-}	103		−322	31	72	102		−309	32	70
BOH-6 + $^{\bullet}$OO^{-}	58		−367	16	43	58		−353	16	42
BOH-7 + $^{\bullet}$OO^{-}	104		−321	10	94	104		−308	11	93
		184					178			
BOH-5 + $^{\bullet}$OOH	16		−168	−56	72	15		−162	−55	70
BOH-6 + $^{\bullet}$OOH	−28		−212	−71	43	−29		−206	−70	42
BOH-7 + $^{\bullet}$OOH	17		−167	−77	94	17		−161	−76	93

PT and SPLET) and are calculated by the DFT method. The reaction enthalpies are presented in Tables 2 and 3.

The preferred mechanisms of antiradical activity of fisetin and baicalein are estimated from the ΔH_{BDE}, ΔH_{IP}, and ΔH_{PA} values. Namely, the lowest of these values indicates which mechanism is favourable. The preferred site of antiradical action can be estimated from the sum of the enthalpies involved in a particular free radical scavenging mechanism (BDE for HAT; IP and PDE for SET-PT and PA and ETE for SPLET).

The enthalpies for the reactions of hydroxyl radicals with fisetin and baicalein show that these reactions are exothermic in all solvents. As can be seen from Table 2, the C4′–OH group of fisetin has the lowest ΔH_{BDE} values in all solvents, representing the first site that can donate its H-atom, followed by C3′ < C3 < C7. The obtained order is in agreement with previously obtained results.[18] The lowest ΔH_{BDE} value in baicalein has the C6 position, followed by C5 < C7 (Table 3). On the other hand, ΔH_{PA} values of all present OH groups, for the reactions of hydroxyl radical with fisetin, give the following sequence: C4′ < C7 < C3′ < C3, indicating proton transfer from the C4′ group is easier compared to other OH groups. The activity ranking sequence for baicalein, set according to the obtained ΔH_{PA} values for the reactions with hydroxyl radical is: C7 < C6 < C5, indicating that proton transfer from the C7 hydroxyl group is favoured. ΔH_{PA} values calculated for different solvents, polar protic (water and ethanol) and polar aprotic (DMSO and DMF), for both molecules are comparable with the ΔH_{BDE} values, indicating that the HAT and SPLET mechanisms are competitive under these conditions.

In the case of the superoxide anion radical, the reactions representing all three mechanisms are endothermic in all solvents (Tables 2 and 3). Thus, the newly formed radical is less stable than the initial one, indicating that the polar solvents are not suitable media for the reactions of fisetin and baicalein with superoxide anion radicals. For this reason, the opposite reaction for electron transfer is investigated:[24]

$$\text{Ar–OH} + \text{OO}^{\bullet -} \rightarrow \text{Ar–OH}^{\bullet -} + \text{O}_2 \quad (16)$$

ΔH_{IPr} can be calculated as follows:

$$\Delta H_{IPr} = H(\text{Ar–OH}^{\bullet -}) + H(\text{O}_2) - H(\text{Ar–OH}) - H(\text{OO}^{\bullet -}) \quad (17)$$

where $H(\text{Ar–OH}^{\bullet -})$, $H(\text{O}_2)$, and $H(\text{OO}^{\bullet -})$ are the enthalpies of the radical anion of the initial flavonoid, the corresponding initial anion radical molecule of oxygen, and the superoxide anion radical, respectively. The following results for ΔH_{IPr} were obtained: 243, 242, 240, and 241 for fisetin and 221, 220, 217,

and 219 kJ mol^{-1} for baicalein in water, DMSO, ethanol, and DMF, respectively. It is obvious that this reaction step is endothermic. Despite the fact that the IP values are almost two times lower than those for the forward reaction, this reaction step is still significantly endothermic.

The obtained results are not so surprising taking into account the fact that the superoxide anion radical is a small and polar species, which could be surrounded by more solvent molecules and thus additionally stabilized. For this reason, the reactions in all solvents are more or less endothermic, indicating that the superoxide anion radical is not very reactive under these conditions. Since peroxyl radical is the protonated form of the superoxide anion radical,[4] it is possible to expect its reaction with fisetin and baicalein instead of a superoxide anion radical. Therefore, the enthalpies of the reactions of peroxyl radical with fisetin and baicalein are also given in Tables 2 and 3. The obtained ΔH_{PA} values in all media are significantly less than the corresponding ΔH_{BDE} values, indicating that SPLET is the prevailing mechanism in all solvents. Since the C4′–OH group of fisetin has the lowest ΔH_{PA} values in all solvents (Table 2), it represents the most reactive site for abstraction of a H-atom, followed by C7 < C3′ < C3. In the case of baicalein, the C7–OH group has a somewhat lower ΔH_{PA} value compared to the C6 and C5 positions. These results are good agreement with the BDE, IP, PDE, PA, and ETE values for fisetin and baicalein.[18,36]

Experimental

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 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 particular trapping radicals; (b) the same experiment was repeated after addition of fisetin and baicalein, which should lead to the decreased intensity of the EPR signal since a certain amount of produced radicals is removed. The ability of fisetin and baicalein 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 flavone molecules. The results are presented as oxidant scavenging (% of radical reduction), which represents the relative decrease in radical production: % of radical reduction $= 100 \times (I_0 - I_a)/I_0$, where I_0 is the relative height of the third low-field EPR peak of the spin-adduct of the control system and I_a is the relative height of the same EPR peak in the spectrum of the sample containing flavones.

Generation of $^{\bullet}OH$ radical

The ability of fisetin and baicalein to scavenge $^{\bullet}OH$ radicals was tested using the Fenton reaction as an "$^{\bullet}OH$ producing" system. 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.[14] The final concentration of DEPMPO was 50 mM. The final concentration of all samples was 0.01 mM. The sample with no antioxidants served as a control. Deionized 18 MΩ H_2O was used in all experiments. EPR spectra were recorded at room temperature using a 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 centre, 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 Teflon tubes were placed.

Generation of $^{\bullet}O_2^-$ radicals

The superoxide radical ion was produced by a pure chemical radical generating system, the UV (Xe lamp of 500 W) irradiated riboflavin/EDTA generating system containing 0.3 mM riboflavin, 5 mM EDTA and 50 mM DEPMPO. The irradiation was performed at room temperature, and the final concentrations of ethanolic solutions of fisetin and baicalein were 0.1 mM.

The ability of fisetin and baicalein to remove superoxide anion radical was evaluated by the difference between the relative amplitudes of the EPR signals of spin-adducts in radical generating system with and without the addition of fisetin and baicalein. Results were, as in the case with hydroxyl radical, presented as oxidant scavenging activity (% of radical reduction).

Cyclic voltammetry

The electrochemical behaviour of fisetin and baicalein was investigated using cyclic voltammetry in aqueous and ethanol solutions employing a conventional three-electrode electrochemical cell with a working glassy carbon (GC) disk electrode (base surface area 0.196 cm^2). Pt foil and a saturated calomel electrode (SCE) served as the counter and reference electrodes, respectively. Measurements were performed at room temperature using a Gamry PCI-4/750 potentiostat/galvanostat. Aqueous solutions were supplemented with 0.1 M K_2SO_4, and pH was adjusted to 7. Ethanol solutions were supplemented with 0.1 M $LiClO_4$. During the measurements, dissolved O_2 was removed by purging solutions with high-purity N_2 (5 N). Between each measurement, the GC surface was renewed by polishing with diamond paste after which it was thoroughly washed with ethanol and deionized water.

Superoxide radical scavenging activity was probed electrochemically using cyclic voltammetry. All the experiments were performed in a 0.1 M solution of tetrabutylammonium hexafluorophosphate (Bu_4NPF_6) in dimethyl sulfoxide (DMSO) stored over a molecular sieve (3 Å). Prior to the experiments, the GC electrode was polished with diamond paste and thoroughly washed with ethanol and deionized water. The experiments were performed at a scan rate of 100 mV s^{-1} in the potential window between −0.3 and −0.95 V *vs.* SCE in O_2-saturated solutions with increasing amounts of fisetin or baicalein.

Computational method

The conformations of species of baicalein and fisetin involved in radical scavenging mechanisms are fully optimized by the new local density functional method (M05-2X), developed by the Truhlar group[25–27] by using the 6-311+G(d,p) basis set implemented in the Gaussian 09 package.[28] The M05-2X functional has been recommended for kinetic, thermochemistry calculations, by their developers,[26] and it has been also successfully used by other authors.[29–35] The M05-2X functional is also among the best performing functionals for calculating reaction energies involving free radical species.[24] Moreover, it satisfactorily reproduces nonplanarity in the molecules of some naturally occurring molecules like flavonids.[33,36,37]

To calculate the thermodynamic properties in the solvent environment: water, dimethylsulfoxide (DMSO), ethanol and dimethylformamide (DMF), the SMD[38] solvation model was used with M05-2X/6-311+G(d,p) model.

The nature of the stationary points is determined by analysing the number of imaginary frequencies: 0 for minimum and 1 for transition state. Therefore, the obtained structures were verified by normal mode analysis. Note that no imaginary frequencies were obtained.

Conclusions

EPR measurements prove fisetin and baicalein are hydroxyl and superoxide anion radical scavengers. Regarding the obtained radical scavenging sequence with the structural features of fisetin, baicalein and a few other structurally related flavones, it could be assumed that along with the C4′–OH functional group, which most likely renders these molecules as hydroxyl radical scavengers, a C5–OH group has a more prominent role in scavenging hydroxyl radicals compared to the *ortho*-hydroxy groups in the B ring, while C3′–OH modifies the activity. However, the superoxide anion radical scavenging sequence indicates that the *ortho*-hydroxy groups in the B ring and the pyrogallol functional group (in A ring of baicalein) as more relevant. It should be noted that the C4′–OH functional group also renders these molecules as superoxide anion radical scavengers. The established differences could also be related to different scavenging mechanisms governing the reduction of different oxygen species. The results of CV measurements confirm the good superoxide scavenging activity of fisetin and baicalein. The difference in superoxide anion radical activity, obtained by EPR and CV measurements, could be rationalized in terms of different modes of superoxide anion radical production.

The reaction enthalpies for the reactions of fisetin and baicelin with hydroxyl radicals are exothermic in all solvents. The calculated energy requirements for the reactions of the investigated molecules and hydroxyl radicals point to HAT and SPLET as the operative radical scavenging mechanisms in all solvents under investigation. It should be also noted that the C4′–OH group of fisetin is the most favoured site for homolytic and heterolytic O–H breakage in all solvents and by both mechanisms. The most favoured site for homolytic and heterolytic O–H breakage in baicalein are the C6–OH (HAT) and C7–OH (SPLET) positions in all solvents.

The obtained results also show that there is no mechanism suitable for the reaction of fisetin and baicalein with superoxide anion radicals in all solvents. The main reason for this behaviour probably lies in the fact that the negatively charged superoxide radical anion is additionally stabilized in the polar solvents, which results in a considerably reduced reactivity with fisetin and baicalein. Regarding the reactivity of fisetin and baicalein as a peroxyl radical scavenger, it is found that the SPLET mechanism prevails over HAT H-abstraction as the thermodynamically more feasible reaction channel in polar protic as well as aprotic polar solvents.

Acknowledgements

The authors gratefully acknowledge financial support from the Ministry of Science of Republic of Serbia (Projects no. 172015 and 174028) and the Ministry of Science, Education and Sports of the Republic of Croatia (Projects no.: 079-0000000-3211 and 098-0982464-2511).

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