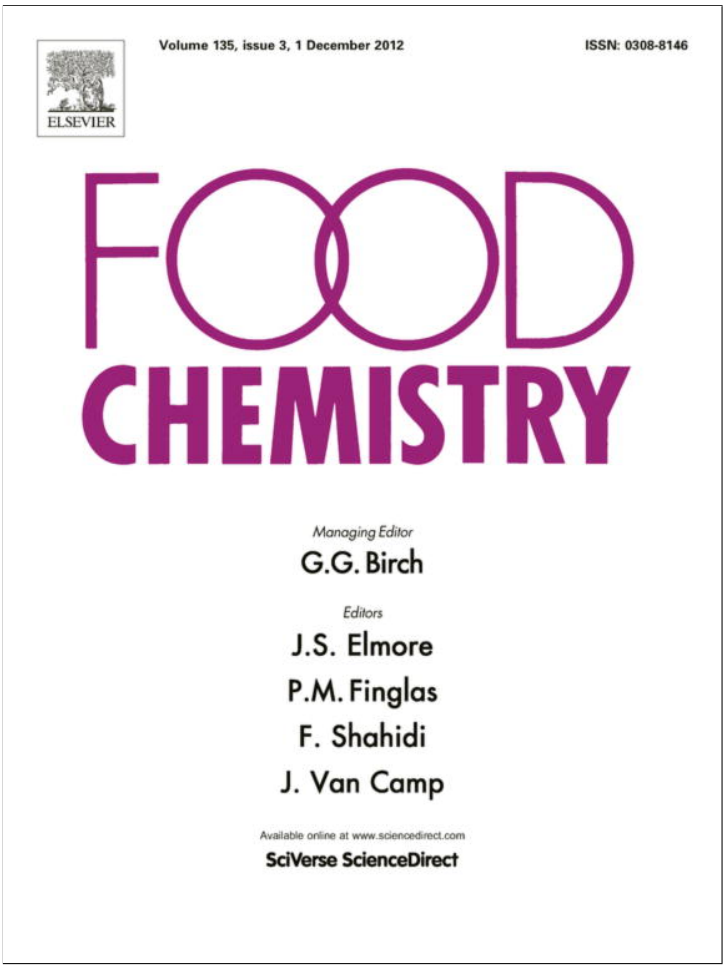

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Free radical scavenging activity of morin 2′-O$^-$ phenoxide anion

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ABSTRACT

Due to intramolecular H-atom transfer, deprotonation of the most acidic 3-OH group of morin yields 2′-O$^-$ phenoxide anion. The reaction enthalpies related to mechanisms of free radical scavenging activity of this dominant species at a physiological pH of 7.4 were calculated by PM6 and DFT methods in gas-phase, water, benzene and DMSO. Results indicate the 4′-OH group of 2′-O$^-$ phenoxide anion is the active site for radical inactivation. The thermodynamically favoured mechanism depends on the polarity of the reaction media: in polar solvents (water and DMSO), the sequential proton loss electron transfer (SPLET) mechanism is preferred while in non-polar benzene (and in gas-phase), the hydrogen atom transfer (HAT) mechanism is responsible for the free radical scavenging activity of the morin phenoxide anion. Results show that the fast, semiempirical PM6 method fairly mimics more accurate, though time-consuming DFT methodologies.

1. Introduction

Morin (3,5,7,2′,4′-pentahydroxyflavone) belongs to the flavonoid group of polyphenolic compounds found ubiquitously within the plant kingdom. It can be found in the wood of old fustic, in osage orange, white mulberry, fig, almond and sweet chestnut, and many other herbs and fruits used as herbal medicines (Basile et al., 2000; Wijeratne, Abou-Zaid, & Shahidi, 2006). Morin has been the subject of a number of theoretical (Cody & Luft, 1994; Mendoza-Wilson, Santacruz-Ortega, & Balandran-Quintana, 2011; Payan-Gomez, Flores-Holguin, Perez-Hernandez, Pinon-Miramontes, & Glossman-Mitnik, 2011; Zielinska, Paradowska, Jakowski, & Wawer, 2008) and experimental studies dealing with its pharmacological activities, such as antioxidant properties (McPhail, Hartley, Gardner, & Duthie, 2003), inhibition of lipid peroxidation (Lian, Wang, Lo, Huang, & Wu, 2008), anticancer activity (Manna, Aggarwal, Sethi, Aggarwal, & Ramesh, 2007), anti-inflammatory activity (Galvez et al., 2001) and cardiovascular protection (Wu et al., 1995). In some cases morin may also have pro-oxidant action (Makris & Rossiter, 2001).

Despite the enormous interest in flavonoids as potential protective agents against the development of human diseases, the real contributions of such compounds to health maintenance remain unclear. The mechanisms explaining their biological activities are poorly understood and largely unknown, but it is possible that several different types of biochemical events are involved (Fraga, Galleano, Verstraeten, & Oteiza, 2010). Antioxidant activity could be a result of direct scavenging of free radicals, the sequestration of potential oxidants, altering the expression of the multiple genes encoding the enzymes with antioxidant function, and modulation of cell signalling (Leopoldini, Russo, & Toscano, 2011; Prochazkova, Boušova, & Wilhelmova, 2011). Scavenging of free radicals seems to play a notable part in the antioxidant activity of flavonoid compounds. An important influence on the free radical scavenging activity of flavonoids is the pH of the surrounding medium (Estevez, Otero, & Mosquera, 2010; Ji, Zhang, & Shen, 2006; Musialik, Kuzmicz, Pawlowski, & Litwinienko, 2009; Muzolf, Szymusiak, Gliszczynska-Swiglo, Rietjens, & Tyrakowska, 2008). Depending on the acidity, flavonoids may be partially or fully ionised, meaning that not only the neutral molecules but also the ions could be involved in the antioxidant actions.

Morin is an amphipathic molecule and due to its lipophilicity, is only sparingly soluble in aqueous media. As a pentahydroxy flavone, morin is more acidic than other flavonoids containing a

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7-OH group (Herrero-Martinez, Repolles, Bosch, Roses, & Rafols, 2008; Herrero-Martinez, Sanmartin, Roses, Bosch, & Rafols, 2005; Jovanovic, Steenken, Tosic, Marjanovic, & Simic, 1994; Musialik et al., 2009). Usually, the 7-OH group is the most acidic site in flavonoids, and the exception of morin is ascribed to the strong acidity of the cinnamoyl part of the molecule (Xie, Long, Liu, Qin, & Wang, 2006). Somewhat controversial are the reports regarding the first deprotonable OH group. Musialik et al. (2009) have designated the 2′-OH group of morin as the most acidic, with an experimentally determined $pK_{a1} = 5.2$, and the second dissociable hydroxyl group with a $pK_{a2} = 8.2$. These authors have explained the exceptionally strong acidity and unusual first deprotonation site of morin by the hydrogen bond stabilisation of 2′-O^- moiety with 3-OH group. Very recently, Payan-Gomez et al. (2011) have calculated pK_a values of morin using the PM6 method and have found that the most acidic hydrogen atom belongs to the 2′-OH group. However, these calculations were performed on the morin rotamer, which is not the most stable conformation. Other authors have proposed the 3-OH group as the most acidic (Mendoza-Wilson et al., 2011; Panhwar, Memon, & Bhanger, 2010). Previously, Jovanovic et al. (1994) published values of $pK_{a1} = 3.46$ and $pK_{a2} = 8.1$. The pK_{a1} value is much lower than that of other flavonols, such as quercetin ($pK_{a1} = 8.45$), naringenin ($pK_{a1} = 7.5$) and galangin ($pK_{a1} = 7.6$) (Musialik et al., 2009). To ascertain which OH group of morin could be easiest to deprotonate, in our previous work we calculated PA values using both the DFT and PM6 methods (Marković et al., 2012). DFT results indicated that in gas-phase, water, DMSO and benzene, the 3-OH group is the most acidic because of the lowest PA value. An interesting feature of deprotonation of the 3-OH group is that the resulting monoanion is the 2′-O^- phenoxide anion. On the basis of MP2 calculations in gas-phase, Binbuga, Hasty, Gwaltney, Henry, and Schultz (2007) reported that deprotonation at the 3-OH and 2′-OH groups of sulfonated morin lead to the same structure of resulting phenoxide anion. Undoubtedly, at a physiological pH of 7.4, the predominant form of morin is the phenoxide anion.

Antiradical properties of the morin phenoxide anion ($Ar(OH)_4O^-$) are related to the ability to transfer a phenolic H-atom to a free radical (e.g., alkoxyl radical, RO·). The formal H-atom abstraction from morin phenoxide anion is described by:

$$Ar(OH)_4O^- + RO^\cdot \rightarrow Ar(OH)_3O^-O^\cdot + ROH \quad (1)$$

and may involve complex processes. It has been recognised that such a reaction proceeds via at least three different mechanisms: hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SET-PT) and sequential proton loss electron transfer (SPLET). All three of these mechanisms may take place in parallel, but at different rates (Klein, Lukeš, & Ilčin, 2007; Litwinienko & Ingold, 2007; Wright, Johnson, & DiLabio, 2001).

In the HAT mechanism, the hydrogen atom is transferred from one of the OH groups of the morin phenoxide anion ($Ar(OH)_4O^-$) to the free radical:

$$Ar(OH)_4O^- \rightarrow Ar(OH)_3O^-O^\cdot + H^\cdot \quad (2)$$

The product of this reaction is the morin radical anion ($Ar(OH)_3O^-O^\cdot$). The HAT mechanism is characterised by the homolytic bond dissociation enthalpy (BDE) of the OH group. The O–H BDE is calculated by the following equation:

$$BDE = H(Ar(OH)_3O^-O^\cdot) + H(H) - H(Ar(OH)_4O^-) \quad (3)$$

$H(Ar(OH)_3O^-O^\cdot)$ is the enthalpy of the morin radical anion generated after H· abstraction, $H(H)$ is the enthalpy of the hydrogen atom, and $H(Ar(OH)_4O^-)$ is the enthalpy of the parent morin phenoxide anion. A lower BDE value is usually attributed to a greater ability of the hydroxyl group to donate a hydrogen atom which results in an easier free radical scavenging reaction. The HAT mechanism is favoured for radicals with high H-atom affinity and is preferred in non-polar solvents because it does not involve charge separation.

In the SET-PT mechanism the first step is the transfer of an electron from morin phenoxide anion:

$$Ar(OH)_4O^- \rightarrow Ar(OH)_4O^\cdot + e^- \quad (4)$$

This step can be characterised by the ionisation potential (IP) which can be calculated as follows:

$$IP = H(Ar(OH)_4O^\cdot) + H(e^-) - H(Ar(OH)_4O^-) \quad (5)$$

$H(Ar(OH)_4O^\cdot)$ is the enthalpy of the transient morin radical generated after electron abstraction and $H(e^-)$ is the enthalpy of an electron. The second step is the deprotonation of $Ar(OH)_4O^\cdot$:

$$Ar(OH)_4O^\cdot \rightarrow Ar(OH)_3O^-O^\cdot + H^+ \quad (6)$$

It can be described by O–H proton dissociation enthalpy (PDE) which can be calculated by the equation:

$$PDE = H(Ar(OH)_3O^-O^\cdot) + H(H^+) - H(Ar(OH)_4O^\cdot) \quad (7)$$

where $H(H^+)$ is the enthalpy of the proton. The net result of the SET-PT mechanism is the same as in the HAT mechanism.

Deprotonation of the morin phenoxide anion, which results in the formation of morin phenoxide dianion ($Ar(OH)_3(O^-)_2$), is the first step in the SPLET mechanism (Foti, Daquino, & Geraci, 2004; Litwinienko & Ingold, 2007):

$$Ar(OH)_4O^- \rightarrow Ar(OH)_3(O^-)_2 + H^+ \quad (8)$$

This step corresponds to the proton affinity (PA) of morin phenoxide dianion $Ar(OH)_3(O^-)_2$. The PA can be calculated by the equation:

$$PA = H(Ar(OH)_3(O^-)_2) + H(H^+) - H(Ar(OH)_4O^-) \quad (9)$$

where $H(Ar(OH)_3(O^-)_2)$ is the enthalpy of the morin phenoxide dianion generated after the proton abstraction. In the second step, electron transfer from $Ar(OH)_3(O^-)_2$ takes place:

$$Ar(OH)_3(O^-)_2 \rightarrow Ar(OH)_3O^-O^\cdot + e^- \quad (10)$$

It is related to the electron transfer enthalpy (ETE) which can be determined by the equation:

$$ETE = H(Ar(OH)_3O^-O^\cdot) + H(e^-) - H(Ar(OH)_3(O^-)_2) \quad (11)$$

The net result of SPLET is again the same as in HAT and SET-PT; the formation of corresponding radical anion species $Ar(OH)_3O^-O^\cdot$. SET-PT and SPLET mechanisms are favoured in polar media because of the charge separation. They are preferred for radicals with high electron affinity.

As a part of ongoing investigations (Marković et al., 2012) this paper extends PM6 and DFT calculations to the mechanisms of the radical scavenging activity of morin phenoxide anion. The energetics related to the radical scavenging activity of phenolic compounds is usually calculated for neutral molecules in gas-phase (Leopoldini et al., 2011), excluding the important influence of the pH of the surrounding medium on the free radical scavenging activity of these molecules (Estevez et al., 2010; Ji et al., 2006; Musialik et al., 2009; Muzolf et al., 2008). Morin is an unusually acidic flavonoid which is mostly present as the monoanion at a physiological pH of 7.4. The energy requirements, enthalpies of morin phenoxide anion species, in gas-phase, polar and non-polar solvents, are calculated with the aim of indicating the thermodynamically favourable free radical scavenging mechanism and the preferred site of radical inactivation. Computations were performed in water and benzene which may represent biological liquids and membranes, respectively. Despite the fact that the existence of morin phenoxide anions in benzene is doubtful, such

species may be present in emulsions consisting of oil and water phases where may express interfacial antioxidant action (Frankel, 2007, chap. 3).

2. Computational details

DFT calculations were performed using the Gaussian 09 package (Frisch et al., 2009) and PM6 calculations using MOPAC2009™ programme package (MOPAC 2009).

2.1. DFT calculations

The conformations of phenoxide anion, radical anions, and dianions are fully optimised with the new local density functional method (M05-2X), recently developed by the Truhlar group (Zhao, Schultz, & Truhlar, 2006), using the 6-311++G(d,p) basis set as implemented in the Gaussian 09 package (Frisch et al., 2009). This new hybrid meta exchange–correlation functional is parameterised so that it includes both nonmetallic and metallic compounds. This function also yields satisfactory overall performance for the main-group thermochemistry and thermochemical kinetics, as well as organic, organometallic, biological and non-covalent interactions. To calculate the thermodynamic properties in the solvent environment (water, benzene, and DMSO), calculation with SMD (Marenich, Cramer, & Truhlar, 2009), as implemented in Gaussian 09, was used in combination with DFT calculation at the M05-2X/6-311++G(d,p) level. The vibrational frequencies are obtained from diagonalisation of the corresponding M05-2X Hessian matrices. The nature of the stationary points is determined by analysing the number of imaginary frequencies: 0 for the minimum and 1 for the transition state. The relative enthalpies were calculated at 298 K.

The potential energy surfaces are obtained in relation to the torsion angle τ between the rings B and C, defined by the 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 following torsion angles rotations were also studied: ω1 (H–O5–C5–C6), ω2 (H–O7–C7–C8), ω3 (H–O3–C3–C2), and ω4 (H–O4′–C4′–C3′). Afterwards, the structures were further optimised without any constrain around each potential minimum. Particular attention was devoted to the interpretation of the reactivity of OH groups.

The rate constants were calculated according to the equation:

$$k(T) = \frac{kT}{h} exp(-\Delta^{\ddagger}G^{o}/RT)$$

where $k(T)$, k, h, R, and $\Delta^{\ddagger}G^{o}$ denote rate constant at temperature T, Boltzmann constant, Planck constant, gas constant, and free activation energy, respectively (Ochterski, 2000).

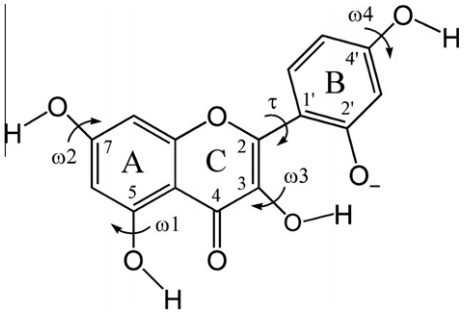

Fig. 1. Chemical structure of morin 2′-O⁻ phenoxide anion.

2.2. MOPAC calculations

In MOPAC calculations, the geometries of phenoxide anion, radical anions and dianions were optimised using the PM6 method. The eigenvector following (EF) optimisation procedure was carried out with a final gradient norm under 0.01 kcal mol^{-1} Å^{-1}. The solvent contribution to the enthalpies of formation of morin species was computed employing COSMO (Conductor-like Screening Model) calculations implemented in MOPAC 2009™. This approach was used for all structures. The solvation enthalpies of hydrogen atom (H·), proton (H^+) and electron (e^-), for solvents dealt with in this report, are taken from literature (Klein et al., 2007; Rimarčik, Lukeš, Klein, & Ilčin, 2010).

3. Results and discussion

In the morin molecule there are five possible sites for deprotonation associated with the hydroxyl groups. As presented in the Introduction, either the 3-OH or 2′-OH group could be deprotonated first. The DFT calculations in gas-phase, DMSO and benzene indicate that the deprotonation of the most acidic 3-OH group as well as of the 2′-OH group of morin, results in the same structure, i.e., 2′-O⁻ phenoxide anion (Supplementary material Fig. S1). If the 3-OH group is deprotonated first, then the intramolecular H-atom transfer from 2′-OH group to 3-O⁻ site occurs, resulting in the formation of 2′-O⁻ phenoxide anion. This occurrence is probably the consequence of the negative charge delocalisation over the cinnamoyl part of molecule, which stabilises the 2′-O⁻ phenoxide anion. The DFT calculations in water using the SMD solvation model suggest that the deprotonation of 3-OH group results in the formation of 3-O⁻ phenoxide anion, implying that both anions can exist in aqueous solution. The coexistence of the two anions in the aqueous solution can be attributed to the polarity of the solvent, i.e. to the intermolecular hydrogen bonds which prevent spontaneous proton transfer from 2′-O to 3-O. The proton transfer from 2′-O to 3-O requires an energy barrier of 4.96 kJ mol^{-1}, whereas the reverse reaction requires activation energy of 6.07 kJ mol^{-1}. The low activation barriers indicate that the interconversions between the two anions are very fast (the rate constants for forward and reverse reactions amount to 6.4×10^{11} and 3.9×10^{11} s^{-1}). According to the PM6 calculations obtained for all studied media, i.e., gas-phase, water, DMSO and benzene, 2′-O⁻ phenoxide anion is the result of 3-OH group (as well as 2′-OH group) deprotonation (see Fig. S2). As can be seen from the Figs. S1 and S2, regarding the deprotonation of 3-OH group, the PM6 calculations fairly reproduce the DFT results. In the case of 2′-OH group deprotonation in water and DMSO, the DFT and PM6 results are identical. The discrepancy, which arises in the gas-phase and benzene, are rationalised in the PM6 calculations by hydrogen bond formation between 3-OH and 2′-O⁻ groups, unlike the DFT calculations which suggest a preserved hydrogen bond between 3-OH and 4-C=O groups.

3.1. Conformational analysis of morin 2′-O⁻ phenoxide anion

According to published experimental results on pK_a determinations (Herrero-Martinez et al., 2005; Herrero-Martinez et al., 2008; Jovanovic et al., 1994; Musialik et al., 2009) and our results of theoretical calculations presented in the previous section, it is obvious that at the physiological condition of pH 7.4 morin mainly exists as a monoanionic, 2′-O⁻ phenoxide anion, specie. To determine the preferred relative positions of the rings B and C, the conformational space of 2′-O⁻ phenoxide anion structure (Fig. 2) is investigated as a function of the torsional angle τ (O1–C2–C1′–C2′) between those rings. The minimisation procedure for the phenoxide anion structure, according to the general rules indicated in the Computational

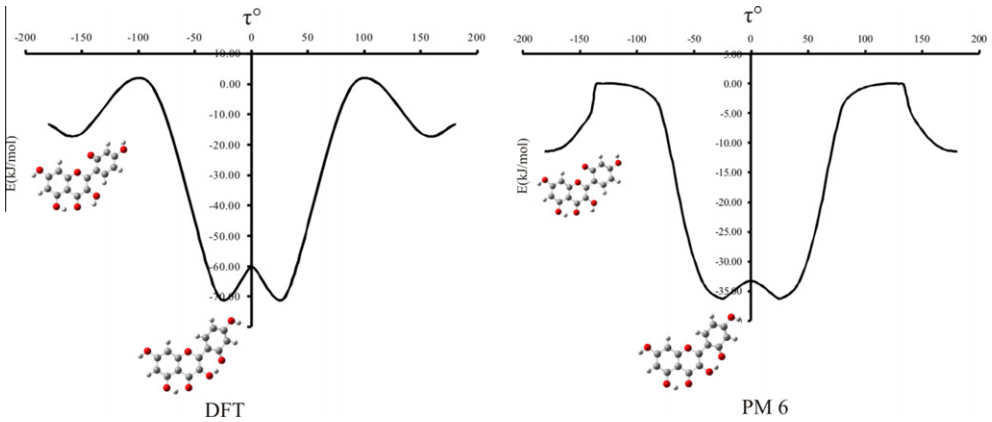

Fig. 2. DFT and PM6 energy profiles for rotation around C2–C1′ bond of morin 2′-O⁻ phenoxide anion in the gas-phase.

details, performed at the M05-2X level with the 6-311++G** basis set and PM6 method, yields a non-planar conformation as the more stable one.

The analysis of the geometries of morin rotamers shows that all of them were nonplanar, implying that the dihedral angle between the ring C and the ring B is different from zero. Since the high scavenging activity of the molecule (due to the increased conjugation) correlates with the planarity (as measured by the torsion angle) of the ring B, compared to the rest of the molecule (AC bicycle), the conformational space is analysed as a function of the torsional angle τ.

The PM6 calculations show that by removing any constraint on the torsional angle, the conformational absolute minimum for a more stable structure occurs at $\tau = -27.2°$ followed by a relative minimum at $\tau = -180°$ with energy difference of 24.84 kJ mol^{-1}. The potential energy maximum lies at $\tau = -120°$, and the interconversion barrier between the two minima is 36.32 kJ mol^{-1}. The similar results of conformational analyses are obtained by the DFT method. The absolute minimum is at $-30.5°$ while the relative one is $-152.5°$ with the energy difference between the two minima being 53.14 kJ mol^{-1}. The potential energy maximum lies at $\tau = -90.5°$, and the interconversion barrier between the two minima is about 69.94 kJ mol^{-1}, which is significantly higher than the corresponding value for morin (Marković et al., 2012).

It is worth mentioning that in going from $\tau = 0°$ to $\pm 40°$, the potential curve is not flat, and has a maximum at $\tau = 0°$. The energy barrier for this rotation is much lower than in previous case, and amounts 2.93 and 9.51 kJ mol^{-1} for PM6 and DFT calculations, respectively. This means that the rotation on this side requires a negligible amount of energy. The non-planarity of the most stable structure can be caused by the steric repulsion between 3-OH and 2′-O⁻ groups.

3.2. Free radical scavenging mechanisms of morin 2′-O⁻ phenoxide anion

The reaction enthalpies related to the three mechanisms of free radical scavenging activity (HAT, SET-PT and SPLET) of 2′-O⁻ phenoxide anion are calculated by the DFT method. The PM6 calculations are also performed and compared to DFT results. The species necessary to perform these calculations are generated from the most stable conformation of 2′-O⁻ phenoxide anion. The BDE of the phenolic OH group represents the reaction enthalpy of HAT. The IP is related to the first step of SET-PT mechanism while the PA is related to the first step of SPLET – abstraction of a proton from one of the phenoxide anion OH groups. Therefore, BDE, IP and PA may be used to determine the thermodynamically preferred reaction pathway (Klein et al., 2007; Rimarčik et al., 2010). The calculations were performed in the gas-phase, water, benzene and DMSO. Water and benzene were chosen as typical polar and non-polar solvents, respectively. Water is the main constituent of all physiological liquids and at a physiological pH of 7.4, flavonoids could display complex solution behaviour, since there can be an equilibrium pool of both neutral and anionic forms of the compound (Webb & Ebeler, 2004). In plasma, flavonoids can act as protein-bound conjugates. The poor solubility in water and the amphipathic nature of morin allows its partition into lipid bilayers and by increasing its local concentration can express the antiradical activity. The possible place of action is also at water/lipid interfaces (Oteiza, Erlejman, Verstraeten, Keen, & Fraga, 2005).

The DFT and PM6 reaction enthalpies related to the three mechanisms of antiradical activity of 2′-O⁻ phenoxide anion in different media are presented in Table 1.

As can be seen from Table 1, the preferred mechanism of action in the gas-phase and non-polar benzene is HAT because the BDE value of 4′-OH group of the 2′-O⁻ phenoxide anion is lower than the corresponding IP and PA values. In water, the BDE and IP values are considerably higher than the PA values. According to the DFT results, the SPLET mechanism is a thermodynamically more favourable reaction pathway in water. On the basis of the PM6 results, the PA is significantly lower than the BDE and IP values, while the ETE (7-OH) is slightly lower than the IP value. This indicates that there is competition between the SPLET and SET-PT mechanisms, the SPLET mechanism is again the thermodynamically more likely reaction pathway in water. As for the reaction in the polar aprotic solvent DMSO, the HAT mechanism could be competitive to the SPLET, because the second step of the SPLET mechanism, i.e., ETE, in spite the low values of PA, has higher energy value than the corresponding BDE. It should be noted, that these differences are much less pronounced in the results obtained by the M05-2X method. Fig. 3 depicts the simplified presentation of the HAT, SET-PT and SPLET mechanisms of morin 2′-O⁻ phenoxide anion.

The PA values are significantly lower than gas-phase values, mainly due to the large H^+ solvation enthalpy values in all solvents studied. In benzene, the calculated PAs are noticeably higher than they are in water and DMSO. On the other hand, there is no such pronounced difference between BDEs in the environments studied. In all media, the most abstractable hydrogen atom of 2′-O⁻ phenoxide anion is that from the 4′-OH group (lowest BDE value). So, the 4′-OH group is the preferred site of radical inactivation by hydrogen abstraction (**1** and **1a** in Fig. 3). The DFT calculated spin densities of the 4′-O·,2′-O⁻ radical anion in different media are presented in Fig. 4.

Table 1
DFT and PM6 calculated parameters of antioxidant mechanisms for morin 2′-O⁻ phenoxide anion (in kJ/mol) in different environments.

	DFT calculated					PM6 calculated				
	HAT	SET-PT		SPLET		HAT	SET-PT		SPLET	
	BDE	IP	PDE	PA	ETE	BDE	IP	PDE	PA	ETE
Gas-phase										
		324					350			
3-OH	397		1395	1769	−50	354		1325	1693	−18
5-OH	398		1395	1715	5	341		1312	1611	51
7-OH	324		1321	1617	28	300		1271	1541	80
4′-OH	310		1307	1662	−30	289		1260	1600	11
Water										
		261					304			
3-OH	365		104	164	201	332		27	101	230
5-OH	361		100	127	234	324		19	35	289
7-OH	341		80	101	240	313		8	19	293
4′-OH	332		71	118	214	306		1	51	254
DMSO										
		388					453			
3-OH	389		119	213	294	342		6	73	386
5-OH	404		134	166	356	334		−2	17	435
7-OH	344		74	110	352	324		−12	1	441
4′-OH	333		62	136	395	315		−21	29	403
Benzene										
		399					427			
3-OH	397		413	632	180	355		342	577	192
5-OH	397		413	577	234	343		331	499	259
7-OH	338		354	500	253	313		300	447	280
4′-OH	325		341	541	199	304		291	500	218

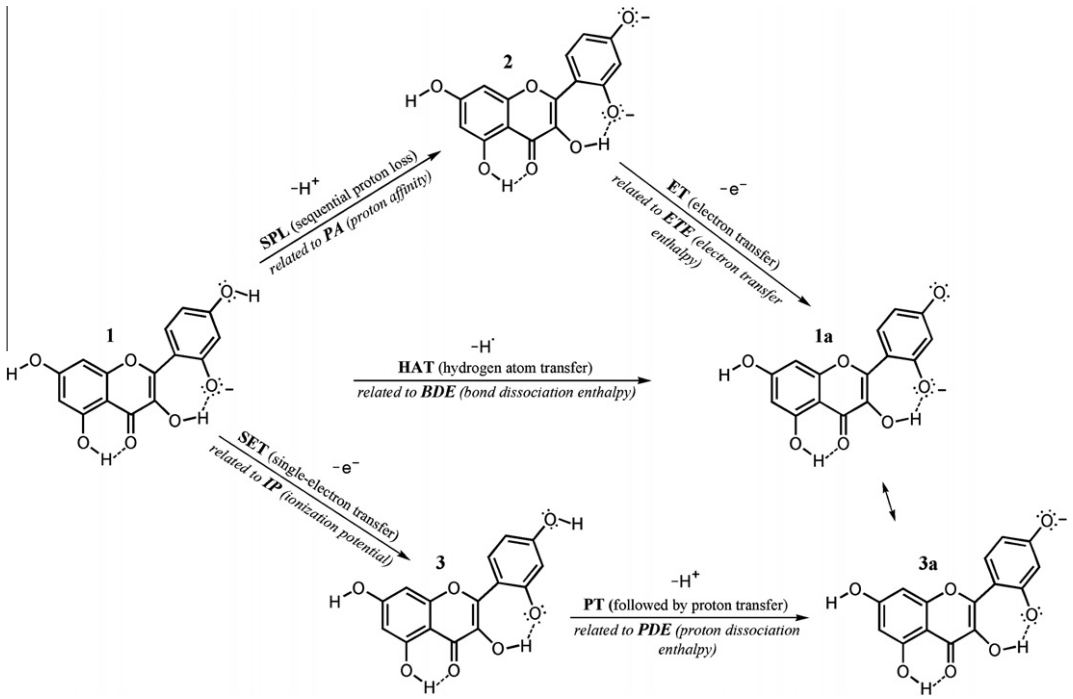

Fig. 3. Simplified presentation of HAT, SET-PT and SPLET mechanisms (Resonance structures of **1a** are presented in Fig. S3 in Supplementary material).

Careful analysis of the radical anion structure indicates that there has been a shift of an hydrogen atom from the 3-OH group to the 2′-O⁻ moiety, i.e., intramolecular H-atom transfer occurs. The natural charge (NC) distribution obtained by the natural bond orbital (NBO) analysis indicates that oxygen atoms O3, O4, O2′, and O4′ are more negatively charged. Almost uniform negative charge distribution between these oxygen atoms is probably a consequence of the electron delocalisation (Fig. S4). The spin density distribution of the most stable radical anion indicates that the C1′ atom is the most probable radical centre in the gas-phase and all solvents. The rest of the spin density is delocalized over O3, C3′, C3, and O4′, atoms of the rings C and B, for all solvents (Fig. 4). The geometrical data presented in Table S1 confirm that delocalisation plays an important role in the structure of the radical anion. The C2–C1′ bond length is considerably shorter than in the parent molecule of morin, indicating the formation of a partial double C=C bond between rings B and C. The radical anion is also more planar then the parent molecule because the dihedral angle τ is

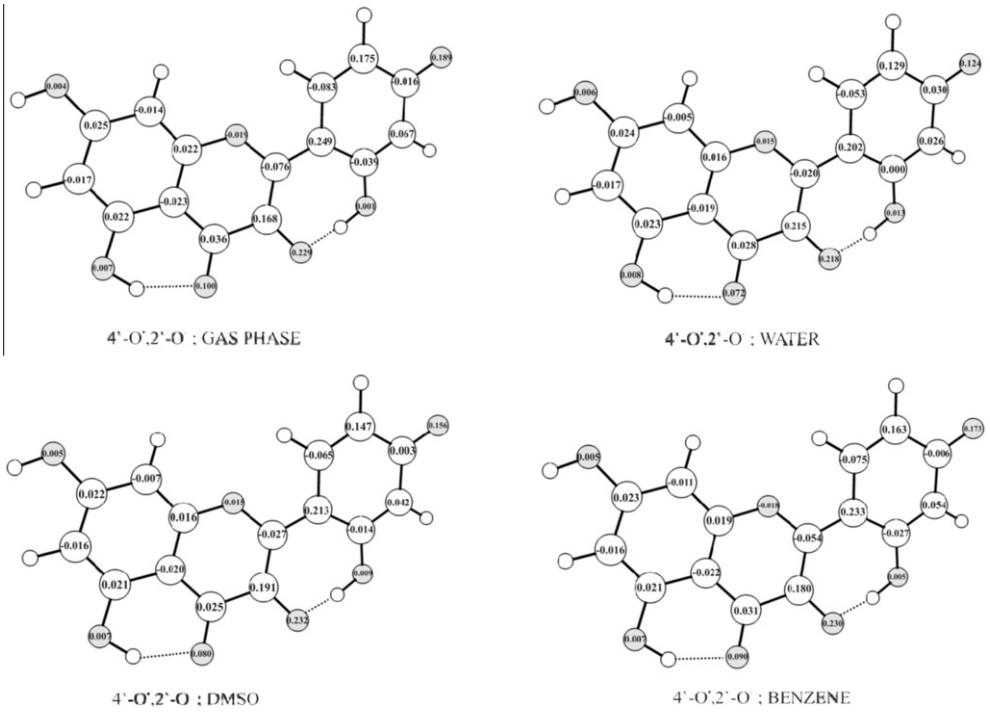

Fig. 4. DFT spin densities of 4′-O·, 2′-O⁻ morin radical anion.

reduced to −15.1°, providing the more pronounced electron delocalisation between the rings B and C.

As has been noted previously, the preferred mechanism of antiradical activity of 2′-O⁻ phenoxide anion can be estimated from values of BDE (HAT mechanism), IP (first step of SET-PT mechanism) and PA (first step in SPLET mechanism) (Klein et al., 2007; Rimarčik et al., 2010). The lowest value indicates which mechanism is thermodynamically the most probable process. On the other hand, the preferred site of antioxidant action may be estimated from the sum of the enthalpies involved in particular free radical scavenging mechanisms. For the HAT mechanism, it is simply the value of BDE that accounts for one-step H-atom transfer. In the case of the SET-PT mechanism, this sum embraces the IP and PDE, and in the SPLET mechanism the PA and ETE. In all media, the minimal energy requirements for homolytic bond cleavage, i.e., the minimal value of BDE, is related to the O−H group on the C-4′ atom (Table 1). The heterolytic bond cleavage is preferred at the same O−H group because the minimal value of PA + ETE is associated with the C-4′ OH group. In the case of SET-PT mechanism, the minimal value of the sum of IP + PDE is also associated with this OH group. The same OH group is the preferred site for radical inactivation by all three mechanisms because this particular OH group possesses minimal energy requirements. In agreement with this finding, the performed DFT and PM6 calculations indicate 4′-O·,2′-O⁻ radical anion as the lowest energy radical anion. According to this fact and data presented in Table 1, it is clear that the formation of O3, O5 and O7 radicals is unlikely in all solvents as well as in the gas-phase. For this reason they are excluded from the discussion.

In polar solvents that support ionisation, the antioxidant reaction of 2′-O⁻ phenoxide anion occurs primarily by the SPLET mechanism. The unique properties of water enable SPLET to be the preferred mechanism therein. Kinetic measurements show that scavenging reactions in water are affected by the acidity of phenolic compounds, i.e., by the amount of accessible phenoxide anions (Litwinienko & Ingold, 2007). At a physiological pH of 7.4, the second OH group of the 2′-O⁻ phenoxide anion loses a proton. The lowest sum of PA + ETE values is characteristic of 4′-OH group of 2′-O⁻ phenoxide anion (Table 1). Consequently, the 4′-OH group of 2′-O⁻ phenoxide anion is the preferred site to enter the SPLET mechanism. The removal of a proton from that position produces a phenoxide dianion (**2** in Fig. 3), followed by electron transfer to a morin radical anion (**1a** in Fig. 3). The sum of IP + PDE has the lowest value at the 4′-OH group. If SET-PT mechanism occurs, electron transfer from the phenoxide anion **1** will result in the phenoxyl radical **3** and, after its deprotonation, radical anion **3a** will be formed. This radical anion is stabilised by intramolecular H-atom transfer and electron delocalisation (Fig. S3).

The radical scavenging mechanisms of antioxidants depend not only on their physicochemical properties, but also on the properties of the scavenged radicals (Shen, Zhang, & Ji, 2005). The DFT and PM6 values for BDE (Table S2) indicate that some prevalent radicals in food chemistry, such as lipidperoxyl radicals (LOO·) represented here by MeOO·, can abstract an H-atom from the 4′OH position of 2′-O⁻ phenoxide anion in all media under investigation. Also, significantly lower values for IP and PA of 2′-O⁻ phenoxide anion in comparison to those of MeOO· indicate that this anion is a good free radical scavenger for this radical type.

It should be noted that the theoretical predictions, based on DFT (and PM6) calculated reaction enthalpies of thermodynamically preferred scavenging mechanism of 2′-O⁻ phenoxide anion in various solvents are in general accordance with the experimentally determined kinetic solvent effects on the free radical scavenging ability of phenolic compounds (Litwinienko & Mulder, 2009), as well as with DFT predicted mechanisms from the thermodynamic point of view (Klein et al., 2007; Rimarčik et al., 2010).

4. Conclusion

On the basis of DFT and semiempirical PM6 calculations performed it appears that deprotonation of morin at 3-OH or 2′-OH

group results in the 2′-O$^-$ phenoxide anion. Actually, the low activation barriers for interconversions between 2′-O$^-$ and 3-O$^-$ phenoxide anions are found for the processes taking place in water. The results of geometrical optimisation, obtained using the M05-2X/6-311++G(d,p) level of theory and semiempirical PM6 method, imply that the 2′-O$^-$ morin anion is the most stable. This anion is the dominant species at a physiological pH of 7.4. This form, which is significantly non-planar in gaseous phase and other solvents, is additionally stabilised by two internal hydrogen bonds (IHBs).

The reaction enthalpies related to HAT, SET-PT and SPLET mechanisms of free radical inactivation by 2′-O$^-$ phenoxide anion are calculated by the same methods. The results indicate that the 4′-OH group of 2′-O$^-$ phenoxide anion is the active site for radical inactivation because of the lowest energy requirements. The H-atoms of 3-OH and 5-OH groups do not figure in the antioxidant mechanism since they are involved in IHB with 2′-O$^-$ and 4-C=O, respectively. Entering the SPLET mechanism represents the thermodynamically preferred reaction pathway in polar solvents (water and DMSO), where PAs of OH groups are considerably lower than corresponding BDEs. In the gas-phase and non-polar benzene HAT is the most probable pathway. Results indicate that the PM6 method, at least for the systems under study, constitutes a valid alternative to the DFT. In addition, the PM6 calculations are performed in a significantly shorter time than the more accurate DFT calculations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.05.119.

References

Basile, A., Sorbo, S., Giordano, S., Ricciardi, L., Ferrara, S., Montesano, D., et al. (2000). Antibacterial and allelopathic activity of extract from Castanea sativa leaves. *Fitoterapia, 71*(S1), S110–S116.

Binbuga, N., Hasty, J. K., Gwaltney, S. R., Henry, W. P., & Schultz, T. P. (2007). Determination of the stability constants for the binding of sulfonated morin with Fe^{2+}. *Inorganica Chimica Acta, 360*(7), 2339–2344.

Cody, V., & Luft, J. R. (1994). Conformational analysis of flavonoids: crystal and molecular structures of morin hydrate and myricetin (1:2) triphenylphosphine oxide complex. *Journal of Molecular Structure, 317*(1–2), 89–97.

Estevez, L., Otero, N., & Mosquera, R. A. (2010). A computational study on the acidity dependence of radical-scavenging mechanisms of anthocyanidins. *Journal of Physical Chemistry B, 114*(29), 9706–9712.

Foti, M. C., Daquino, C., & Geraci, C. (2004). Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH· radical in alcoholic solutions. *Journal of Organic Chemistry, 69*(7), 2309–2314.

Fraga, C. G., Galleano, M., Verstraeten, S. V., & Oteiza, P. I. (2010). Basic biochemical mechanisms behind the health benefits of polyphenols. *Molecular Aspects of Medicine, 31*(6), 435–445.

Frankel, E. N. (2007). *Antioxidants in food and biology: facts and fiction*. Bridgewater: The Oily Press.

Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Chesseman, J. R., et al. (2009). *Gaussian 09, revision A.1-SMP*. Wallingford, CT: Gaussian, Inc.

Galvez, J., Coelho, G., Crespo, M. E., Cruz, T., Rodriguez-Cabezas, M. E., Concha, A., et al. (2001). Intestinal anti-inflammatory activity of morin on chronic experimental colitis in the rat. *Alimentary Pharmacology and Therapeutics, 15*(12), 2027–2039.

Herrero-Martinez, J. M., Repolles, C., Bosch, E., Roses, M., & Rafols, C. (2008). Potentiometric determination of aqueous dissociation constants of flavonols sparingly soluble in water. *Talanta, 74*(4), 1008–1013.

Herrero-Martinez, J. M., Sanmartin, M., Roses, M., Bosch, E., & Rafols, C. (2005). Determination of dissociation constants of flavonoids by capillary electrophoresis. *Electrophoresis, 26*(10), 1886–1895.

Ji, H.-F., Zhang, H.-Y., & Shen, L. (2006). Proton dissociation is important to understanding structure–activity relationships of gallic acid antioxidants. *Bioorganic & Medicinal Chemistry Letters, 16*(15), 4095–4098.

Jovanovic, S. V., Steenken, S., Tosic, M., Marjanovic, B., & Simic, M. G. (1994). Flavonoids as antioxidants. *Journal of the American Chemical Society, 116*(11), 4846–4851.

Klein, E., Lukeš, V., & Ilčin, M. (2007). DFT/B3LYP study of tocopherols and chromans antioxidant action energetics. *Chemical Physics, 336*(1), 51–57.

Leopoldini, M., Russo, N., & Toscano, M. (2011). The molecular basis of working mechanisms of natural polyphenolic antioxidants. *Food Chemistry, 125*(2), 288–306.

Lian, T.-W., Wang, L., Lo, Y.-H., Huang, I.-J., & Wu, M.-J. (2008). Fisetin, morin and myricetin attenuate CD36 expression and oxLDL uptake in U937-derived macrophages. *Biochimica et Biophysica Acta, 1781*(10), 601–609.

Litwinienko, G., & Ingold, K. U. (2007). Solvent effects on the rates and mechanisms of reaction of phenols with free radicals. *Accounts of Chemical Research, 40*(3), 222–230.

Litwinienko, G., & Mulder, P. (2009). Comment on "Temperature and solvent effects on radical scavenging ability of phenols". *Journal of Physical Chemistry A, 113*(50), 14014–14016.

Makris, D. P., & Rossiter, J. T. (2001). Comparison of quercetin and a non-orthohydroxy flavonol as antioxidants by competing in vitro oxidation reactions. *Journal of Agricultural and Food Chemistry, 49*(7), 3370–3377.

Manna, S. K., Aggarwal, R. S., Sethi, G., Aggarwal, B. B., & Ramesh, G. T. (2007). Morin (3,5,7,2′,4′-pentahydroxyflavone) abolishes nuclear factor-κB activation induced by various carcinogens and inflammatory stimuli, leading to suppression of nuclear factor-κB–regulated gene expression and up-regulation of apoptosis. *Clinical Cancer Research, 13*(7), 2290–2297.

Marenich, A. V., Cramer, C. J., & Truhlar, D. G. (2009). Universal solvation model based on solute electron density and on a continuum model of the solvent defined by the bulk dielectric constant and atomic surface tensions. *Journal of Physical Chemistry B, 113*(18), 6378–6396.

Marković, Z., Milenković, D., Đorović, J., Dimitrić Marković, J. M., Stepanić, V., Lučić, B., et al. (2012). PM6 and DFT study of free radical scavenging activity of morin. *Food Chemistry, 134*(4), 1754–1760.

McPhail, D. B., Hartley, R. C., Gardner, P. T., & Duthie, G. G. (2003). Kinetic and stoichiometric assessment of the antioxidant activity of flavonoids by electron spin resonance spectroscopy. *Journal of Agricultural and Food Chemistry, 51*(6), 1684–1690.

Mendoza-Wilson, A. M., Santacruz-Ortega, H., & Balandran-Quintana, R. R. (2011). Relationship between structure, properties, and radical scavenging activity of morin. *Journal of Molecular Structure, 995*(1–3), 134–141.

MOPAC2009. Available on-line from http://openmopac.net/MOPAC2009.html.

Musialik, M., Kuzmicz, R., Pawlowski, T. S., & Litwinienko, G. (2009). Acidity of hydroxyl groups: an overlooked influence on antiradical properties of flavonoids. *Journal of Organic Chemistry, 74*(7), 2699–2709.

Muzolf, M., Szymusiak, H., Gliszczynska-Swiglo, A., Rietjens, I. M. C. M., & Tyrakowska, B. (2008). pH-dependent radical scavenging capacity of green tea catechins. *Journal of Agricultural and Food Chemistry, 56*(3), 816–823.

Ochterski, J. W. (2000). *Thermochemistry in Gaussian*. Wallingford, CT: Gaussian, Inc.

Oteiza, P. I., Erlejman, A. G., Verstraeten, S. V., Keen, C. L., & Fraga, C. G. (2005). Flavonoid-membrane interactions: a protective role of flavonoids at the membrane surface? *Clinical & Development Immunology, 12*(1), 19–25.

Panhwar, Q. K., Memon, S., & Bhanger, M. I. (2010). Synthesis, characterization, spectroscopic and antioxidation studies of Cu(II)–morin complex. *Journal of Molecular Structure, 967*(1–3), 47–53.

Payan-Gomez, S. A., Flores-Holguin, N., Perez-Hernandez, A., Pinon-Miramontes, M., & Glossman-Mitnik, D. (2011). Computational molecular characterization of the flavonoid morin and its Pt(II), Pd(II) and Zn(II) complexes. *Journal of Molecular Modeling, 17*(5), 979–985.

Prochazkova, D., Boušova, I., & Wilhelmova, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia, 82*(4), 513–523.

Rimarčik, J., Lukeš, V., Klein, E., & Ilčin, M. (2010). Study of the solvent effect on the enthalpies of homolytic and heterolitic N-H bond cleavage in p-phenylenediamine and tetracyano-p-phenylenediamine. *Journal of Molecular Structure (Theochem), 952*(1–3), 25–30.

Shen, L., Zhang, H.-Y., & Ji, H.-F. (2005). Successful application of TD-DFT in transient absorption spectra assignment. *Organic Letters, 7*(2), 243–246.

Webb, M. R., & Ebeler, S. E. (2004). Comparative analysis of topoisomerase IB inhibition and DNA intercalation by flavonoids and similar compounds: structural determinates of activity. *Biochemical Journal, 384*(3), 527–541.

Wijeratne, S. S. K., Abou-Zaid, M. M., & Shahidi, F. (2006). Antioxidant polyphenols in almond and its coproducts. *Journal of Agricultural and Food Chemistry, 54*(2), 312–318.

Wright, J. S., Johnson, E. R., & DiLabio, G. A. (2001). Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *Journal of the American Chemical Society, 123*(6), 1173–1183.

Wu, T.-W., Fung, K.-P., Zeng, L.-H., Wu, J., Hempel, A., Grey, A. A., et al. (1995). Molecular properties and myocardial salvage effects of morin hydrate. *Biochemical Pharmacology, 49*(4), 537–543.

Xie, M.-X., Long, M., Liu, Y., Qin, C., & Wang, Y.-D. (2006). Characterization of the interaction between human serum albumin and morin. *Biochimica et Biophysica Acta, 1760*(8), 1184–1191.

Zhao, Y., Schultz, N. E., & Truhlar, D. G. (2006). Design of density functionals by combining the method of constraint satisfaction with parametrization for thermochemistry, thermochemical kinetics, and noncovalent interactions. *Journal of Chemical Theory and Computation, 2*(2), 364–382.

Zielinska, A., Paradowska, K., Jakowski, J., & Wawer, I. (2008). ^{13}C CP MAS NMR and GIAO-CHF/DFT calculations of flavonoids: morin, kaempferol, tricin, genistein, formononetin and 3,7-dihydroxyflavone. *Journal of Molecular Structure, 873*(1–3), 109–116.