Antiradical activity of delphinidin, pelargonidin and malvin towards hydroxyl and nitric oxide radicals: The energy requirements calculations as a prediction of the possible antiradical mechanisms

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ABSTRACT

Naturally occurring flavonoids, delphinidin, pelargonidin and malvin, were investigated experimentally and theoretically for their ability to scavenge hydroxyl and nitric oxide radicals. Electron spin resonance (ESR) spectroscopy was used to determine antiradical activity of the selected compounds and M05-2X/6-311+G(d,p) level of theory for the calculation of reaction enthalpies related to three possible mechanisms of free radical scavenging activity, namely HAT, SET-PT and SPLLET. The results obtained show that the molecules investigated reacted with hydroxyl radical via both HAT and SPLLET in the solvents investigated. These results point to HAT as implausible for the reaction with nitric oxide radical in all the solvents investigated. SET-PT also proved to be thermodynamically unfavourable for all three molecules in the solvents considered.

1. Introduction

Phenolic compounds, plant secondary metabolites, are found commonly in herbs and fruits. The term phenolics encompass several thousand naturally occurring compounds, which can be subdivided into two major groups: simple phenolics and polyphenols. Flavonoids (flavones, flavanones, flavonols, flavanols, anthocyanins, etc.) are a subcategory of polyphenols. Generally, flavonoids are reported to exert a wide range of positive health benefits. This has been confirmed by numerous investigations where the consumption of food and beverages containing high percentage of polyphenolic compounds are associated with a decreased incidence of osteoporosis, cardiovascular diseases, cancer, diabetes and neurodiseases (Nothlings, Murphy, Wilkens, Henderson, & Kolonel, 2007; Song, Manson, Buring, Sesso, & Liu, 2005; Tomas-Barberan & Espin, 2001; Ness & Powles, 1996; Kamei et al., 1995; Block, Patterson, & Subar, 1992; Huang & Ferraro, 1992; Weisburger, 1992). These attributes are thought to arise mainly from their antioxidant capacity, which operates at different levels in oxidative processes induced by reactive free radical species (Swain, 1976).

Anthocyanidins and anthocyanins, hydroxylated and glycosylated flavylum compounds, are plant pigments responsible for the red, blue, and purple hues of flowers and fruits in nature (Harborne, 1967). They are found in an assortment of fruits, vegetables (cherries, raspberries, plums, apples, radishes, berries, red cabbage, etc.) and flowers (tulips, roses, orchids, etc.). Their brilliant
colours attract pollinating insects, but they also act as catalysts in the light phase of photosynthesis and as regulators of iron channels associated with phosphorylation. These compounds also have an important role in protection of higher plants against short wavelength-induced damage (especially protection of photosensitive biological targets, such as DNA, lipids, proteins and coenzymes), against microbial invasion, insects and mammalian herbivory (Swain, 1976; Harborne, 1967).

During scavenging processes, flavonoids donate hydrogen atoms from the phenoxyl groups, inactivating a range of radical species. This transfer can be visualized through at least three mechanisms characteristic not only of flavonoids but phenolics generally: hydrogen atom transfer (HAT), sequential proton loss electron transfer (SPLET), and single electron transfer followed by proton transfer (SET-PT) (Litwinienko & Mulder, 2009; DiLabio & Johnson, 2007; Foti, 2007; Litwinienko & Ingold, 2007; DiLabio & Ingold, 2005; Mayer, 2004).

Generally, there are two types of antioxidant assays in foods and biological systems; those measuring free radical scavenging capacity and those evaluating lipid peroxidation (Miguel, 2010). In this paper, we aimed to provide quantitative tools to determine antiradical activity and antiradical mechanisms for delphinidin (Dp), pelargonidin (Pg) and malvin (Mv) (Fig. 1) by combining experimental measurements and theoretical calculations of the energy requirements for the reactions of these molecules with hydroxyl and nitric oxide radicals in different media. Calculated energy requirements might indicate which radical scavenging mechanisms are thermodynamically preferred and identify active sites for radical inactivation.

Hydroxyl and nitric oxide radicals are radical species of particular relevance. The hydroxyl radical is considered the most reactive (with the half-life around $10^{-9}$ s) and the most damaging. Uncontrolled actions of hydroxyl radicals can have devastating effects within the body since they react at diffusion rates with virtually any molecule including macromolecules, such as DNA, membrane lipids, proteins and carbohydrates. Reactive nitrogen species (RNS) are a family of molecules, derived from nitric oxide (NO) and the activity of inducible nitric oxide synthase 2 (NOS2), which have antimicrobial activity. Most of the cytotoxicity attributed to NO is in fact due to peroxynitrite produced by the diffusion-controlled reaction between NO and the superoxide anion radical. Peroxynitrite interacts with lipids, DNA, and proteins via direct oxidative reactions or indirect, radical-mediated mechanisms (Halliwell, 2001; Aruoma & Halliwell, 1998; Fridovich, 1978).

### 2. Experimental

#### 2.1. ESR spectra

ESR spectra were recorded at room temperature using a Bruker Elexys E540 ESR spectrometer (Bruker, Billerica, MA, USA) operating at X-band (9.51 GHz) with the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 2 mW. The spectra were recorded using EXepr software (Bruker BioSpin). Samples of Dp, Pg and Mv (all purchased from Extrasynthese, Genay, France) were drawn into 10 cm long gas-permeable Teflon tubes (Zeus Industries, Raritan, USA) (wall thickness 0.025 mm and internal diameter 0.6 mm). All measurements were performed under normal conditions, using quartz capillaries into which Teflon tubes were placed.

The ESR spin-trapping experiment was carried out as follows: a) selected reactive oxygen species (ROS) were produced using a pure chemical radical generating systems and the amounts determined based on the amplitude of the selected ESR signals, which originated from the spin-adducts formed during a 5 min period by particular trapping radical; b) the same experiment was repeated after addition of Dp, Pg and Mv, which should decrease the intensity of the ESR signal since a proportion of the radicals present will be removed.

The capacity of the selected flavonoids to eliminate free radicals was evaluated based on the difference between the relative amplitudes of the ESR signals of spin-adduct in radical generating systems, with and without the addition of these compounds.

The results are presented as oxidant scavenging (% of radical reduction), which represents the relative decrease in radical production: % of radical reduction = $100 \times (I_0 - I_1)/I_0$; where $I_0$ = relative height of the third low-field ESR peak of the spin-adduct of the control system and $I_1$ = relative height of the same ESR peak in the spectrum of the sample containing Dp, Pg and Mv.

#### 2.2. Generation of OH radical

The ability of Dp, Pg and Mv to scavenge OH radicals was tested using the Fenton reaction as a “OH producing” system. The Fenton reaction system contained 0.5 mM H$_2$O$_2$ (Sigma Aldrich, Taufkirchen Germany) and 0.075 mM FeSO$_4$ (Sigma Aldrich, Germany) and 0.025 mM H$_2$O$_2$.
2.3. Generation of ‘NO radical

The spin trapping of ‘NO radicals, generated by the reduction of nitrous acid in the form of potassium nitrite, was achieved using a standard ferro-N-dithiocarboxysarcosine (Fe(DTCS)2) spin-trapping system (Kleschyov, Wenzel, & Munzel, 2007).

2.4. Computational methods

PM7 calculations were performed using MOPAC2012™ (Stewart, 2012) and DFT calculations using the Gaussian 09 (Frisch et al., 2009).

2.4.1. MOPAC calculations

The different conformations of Dp, Pg and Mv (Fig. S1) were optimized using the PM7 method included in MOPAC2012™. The eigenvector (EF) optimization procedure was carried out with a final gradient norm less than 0.01 kcal mol⁻¹ Å⁻¹. The solvent contribution to the enthalpies of formation for Dp, Pg and Mv was determined employing COSMO (Conductor-like Screening Model) calculations implemented in MOPAC 2012™ (Maia et al., 2012; Stewart, 2012; Frisch et al., 2009). This approach was used for all three flavonoids. DOH-1, POH-1 and MOH-1 were found to be the most stable conformers of Dp, Pg and Mv (Figs. S1 and S2). These conformers were also used for the geometry reoptimizations by DFT methods.

2.4.2. DFT calculations

Most theoretical studies of Dp, Pg and Mv have focused on the rings, where OH groups are located. Geometry optimizations of all species involved in radical scavenging mechanisms have been carried out using M05-2X functional, developed by Truhlar (Zhao & Truhlar, 2008), and 6-311+G(d,p) basis set implemented in the Gaussian 09 (Frisch et al., 2009). This functional has been recommended for kinetic and thermochemistry calculations by Zhao, Schultz, and Truhlar (2006) and others (Dimitrić Marković, Milenković, Amić, Popović-Bjelić, et al., 2014; Marković et al., 2012; Galano, Macías-Ruvalcaba, Medina-Campos, & Pedraza-Chaverri, 2010; Zavala-Osegua Alvarez-Idaboy, Merino & Galano, 2009).

M05-2X is among the best performing functionals for calculating reaction energies involving free radical species (Zhao & Truhlar, 2008). The influence of water, dimethyl sulfoxide (DMSO), ethanol and dimethylformamide (DMF) as solvents was approximated using the SMD solvation model (Marenich, Cramer, & Truhlar, 2009). This model has proven to be better than others in explaining solvation energies for the neutral and ionic species in aqueous solvents (Marenich et al., 2009).

The geometries were fully optimized without imposing any restrictions. Local minima were confirmed by the absence of the imaginary frequencies. Thermodynamic corrections at 298.15 K were included in the calculation of the relative energies. The vibrational frequencies were obtained from diagonalization of the corresponding M05-2X Hessian matrices. The nature of the stationary points was determined by analysing the number of the imaginary frequencies: 0 for minimum and 1 for the transition state. Therefore, the structures obtained were verified by normal mode analysis. No imaginary frequencies were obtained.

3. Results and discussion

3.1. ESR measurements

The free radical scavenging activities of Dp, Pg and Mv towards hydroxyl and nitric oxide radicals were estimated using ESR spectroscopy. It was observed that the molecules investigated quenched ESR signals to different extents, suggesting different radical scavenging activities. The antiradical activity (AA) was calculated with respect to the relative heights of selected hydroxyl and nitric oxide ESR peaks.

Signals from stable spin-adducts, formed with DEPMPO spin-trap in a standard Fenton reaction system (Yoshimoto, Furukawa, Yamamoto, Horie, & Watanabe-Kohno, 1983) (Fig. 2), were quenched by all three molecules. The results (Table 1) showed that the efficiency of the different radical species depended on their structure. Both, the number and position of hydroxyl and methoxy groups in ring B affect the stability and reactivity of these molecules and, consequently, their antiradical activity. To some extent it was noted that, the higher number of hydroxyl groups, the higher the antiradical activity.

Data in Table 1 indicate Pg and Dp are stronger antioxidants than Mv. Given the structure of Pg (Fig. 1), and the fact that it has a somewhat higher activity than Dp, it is possible to deduce that position C4 primarily contributes to the activity, before ortho-hydroxy or pyrogallol group in ring B. It is also possible that the C5-OH group in Dp and Pg, as demonstrated in structurally related flavone molecules (Dimitrić Marković, Milenković, Amić, Mojić et al., 2014), has an important role in selectivity towards hydroxyl radical. The greater activity of Pg against hydroxyl radicals was in good agreement with the results of Antal, Gárban, and Gárban (2003) who also confirmed Pg as the most efficient anthocyanidine against hydroxyl radical. The somewhat reduced
activity of Mv (Table 1) could be associated with glycosylation, which decreases the antiradical capacity of anthocyanins by reducing the coplanarity to the rings (De Lima, Sussuchi, & De Giovani, 2007). According to some authors (Antal, Gârban, & Gârban, 2003; Kähkönen & Heinonen, 2003; Wang, Cao, & Prior, 1997) the number and position of sugar residues, especially at C3, can also affect the antiradical activity of anthocyanins.

Table 1 also shows that none of the molecules showed activity towards nitric oxide radical.

### 3.2. Antiradical mechanisms of delphinidin, pelargonidin and malvin

Scavenging properties of Dp, Pg and Mv were related to their capacity to transfer hydrogen atoms to hydroxyl and nitric oxide radicals. Since neighbouring groups and geometry influenced the behaviour of different OH groups in polyphenolic structures, only the most stable structures were taken into account.

In the radical scavenging mechanisms reactive radical species were inactivated by hydrogen donation from a phenoxy group of the flavonoid (Ar-OH). By reducing radicals, flavonoids become phenoxyl radicals, which are less reactive and more stable.

The following reactions describe H atom transfer:

\[
\text{Ar} - \text{OH} + \text{HO}^- \rightarrow \text{Ar} - \text{OH} + \text{H}^- \quad (1)
\]

\[
\text{Ar} - \text{OH} + \text{ON}^- \rightarrow \text{Ar} - \text{O}^- + \text{OH}^- \quad (2)
\]

Energy requirements related to the radical scavenging mechanisms, which all have the same net result, are governed by different molecular properties: bond dissociation enthalpy (BDE) in the HAT; proton affinity (PA) together with electron transfer energy (ETE) of ArO\(^-\) in SPLET; ionization potential (IP) and proton dissociation enthalpy (PDE) of ArOH\(^+\) in SET-PT.

In HAT (Eqs. (1) and (2)), hydrogen atoms are transferred from the phenolic OH group to the free radical. \(\Delta H_{\text{BDE}}\) for HAT can be calculated using the following equations:

\[
\Delta H_{\text{BDE}} = H(\text{Ar} - \text{O}) + H(\text{HO}) - H(\text{Ar} - \text{OH}) - H(\text{H}^-) \quad (3)
\]

\[
\Delta H_{\text{BDE}} = H(\text{Ar} - \text{O}) + H(\text{ON}) - H(\text{Ar} - \text{OH}) - H(\text{ON}^-) \quad (4)
\]

where the \(H(\text{Ar-O})\), \(H(\text{HO})\), \(H(\text{Ar-OH})\), \(H(\text{H}^-)\), \(H(\text{ON})\) and \(H(\text{ON}^-)\) are the enthalpies of the phenoxyl radical, molecule obtained after hydrogen atom abstraction from the phenolic compound, starting phenolic compound and reactive radical species, respectively. Lower \(\Delta H_{\text{BDE}}\) values, indicating increased stability of Ar-O and better antioxidant capacity of Ar-OH, can be attributed to increased capacity of phenolic compound to donate hydrogen atom to hydroxyl and nitric oxide radicals.

The first step in SET-PT is the one-electron transfer from the phenolic compound to the free radical which, leads to the formation of a phenolic radical cation (Ar-OH\(^+\)) and the corresponding anion:

\[
\text{Ar} - \text{OH} + \text{HO}^- \rightarrow \text{Ar} - \text{OH}^- + \text{H}^- \quad (5)
\]

\[
\text{Ar} - \text{OH} + \text{ON}^- \rightarrow \text{Ar} - \text{O}^- + \text{NO}^- \quad (6)
\]

\(\Delta H_{\text{IP}}\) for the first step of the SET-PT can be calculated as follows:

\[
\Delta H_{\text{IP}} = H(\text{Ar} - \text{OH}^+) + H(\text{HO}^-) - H(\text{Ar} - \text{OH}) - H(\text{H}^-) \quad (7)
\]

where the \(H(\text{Ar-OH}^+)\), \(H(\text{HO}^-)\) and \(H(\text{NO}^-)\) 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 deprotonation of Ar-OH\(^+\) by HO\(^-\) and NO\(^-\):

\[
\text{Ar} - \text{OH}^- + \text{HO}^- \rightarrow \text{Ar} - \text{O}^- + \text{H}^- \quad (8)
\]

\[
\text{Ar} - \text{OH}^- + \text{NO}^- \rightarrow \text{Ar} - \text{O}^- + \text{NO}^- \quad (9)
\]

\(\Delta H_{\text{IPP}}\) can be calculated using the following equations:

\[
\Delta H_{\text{IPP}} = H(\text{Ar} - \text{O}^-) + H(\text{HO}) - H(\text{Ar} - \text{OH}^-) - H(\text{H}^-) \quad (10)
\]

\[
\Delta H_{\text{IPP}} = H(\text{Ar} - \text{O}^-) + H(\text{ON}) - H(\text{Ar} - \text{OH}^-) - H(\text{NO}^-) \quad (11)
\]

\[
\Delta H_{\text{IPP}} = H(\text{Ar} - \text{O}^-) + H(\text{OH}) - H(\text{Ar} - \text{OH}) - H(\text{NO}) \quad (12)
\]

The first step in SPLET is deprotonation of the phenolic compound by HO\(^-\), NO\(^-\) or another base. The outcome of this reaction is the formation of phenoxide anion Ar-O\(^-\):

\[
\text{Ar} - \text{OH} + \text{HO}^- \rightarrow \text{Ar} - \text{O}^- + \text{H}^- \quad (13)
\]

\[
\text{Ar} - \text{OH} + \text{NO}^- \rightarrow \text{Ar} - \text{O}^- + \text{NO}^- \quad (14)
\]

\(\Delta H_{\text{IPP}}\) can be calculated as follows:

\[
\Delta H_{\text{IPP}} = H(\text{Ar} - \text{O}^-) + H(\text{HO}) - H(\text{Ar} - \text{OH}) - H(\text{H}^-) \quad (15)
\]

\[
\Delta H_{\text{IPP}} = H(\text{Ar} - \text{O}^-) + H(\text{ON}) - H(\text{Ar} - \text{OH}) - H(\text{NO}) \quad (16)
\]

In the next step electron transfer from Ar-O\(^-\) to HO\(^-\) and ON\(^-\) takes place:

\[
\text{Ar} - \text{O}^- + \text{HO}^- \rightarrow \text{Ar} - \text{O}^- + \text{H}^- \quad (17)
\]

\[
\text{Ar} - \text{O}^- + \text{ON}^- \rightarrow \text{Ar} - \text{O}^- + \text{NO}^- \quad (18)
\]

\(\Delta H_{\text{ETE}}\) can be determined from the equations:

\[
\Delta H_{\text{ETE}} = H(\text{Ar} - \text{O}^-) + H(\text{HO}) - H(\text{Ar} - \text{O}^-) - H(\text{H}^-) \quad (19)
\]

\[
\Delta H_{\text{ETE}} = H(\text{Ar} - \text{O}^-) + H(\text{NO}) - H(\text{Ar} - \text{O}^-) - H(\text{ON}) \quad (20)
\]

The reaction enthalpies associated with the three radical scavenging mechanisms (HAT, SET-PT and SPLET) were calculated using M05-2X/6-311G(d,p) level of theory. Species necessary for these calculations were generated from the most stable conformations for Dp, Pg and Mv (Fig. S2 Supplementary material). The calculations were performed for the aqueous phase, DMSO, ethanol and DMF. The most favourable mechanism was determined based on the lowest values for \(\Delta H_{\text{BDE}}\), \(\Delta H_{\text{IP}}\) and \(\Delta H_{\text{IPP}}\). The parameters calculated are presented in Tables 2–4. The corresponding free energies are listed in Tables S1–S3.

Based on the thermodynamic values (\(\Delta H_{\text{BDE}}\), \(\Delta H_{\text{IP}}\)) corresponding \(AG\) values are presented in Supplementary section, Tables S1–S3, presented in Tables 2–4, it was clear that Dp, Pg and Mv reacted with hydroxyl radical via both HAT and SPLET in all the solvents under investigation. Since all \(\Delta H_{\text{BDE}}\) and \(\Delta H_{\text{IP}}\) values were close both mechanisms are equally probable.

According to Wang (Wang, Cao, & Prior, 1997), the highest antiradical activity is expected for molecules with two OH groups in B ring. The additional OH group, as in Dp, or the presence of a single OH group and some other groups, as in Pg and Mv, decreases the antiradical activity. As can be seen from Table 2, the C4′–OH group of Dp was the most favoured site for homolytic and heterolytic O–H breaking in all solvents followed by C5′–OH, C3′–OH, C5–OH and C7–OH. The most reactive position in Dp, via SPLET, was C4′–OH followed by C5–OH > C7–OH, C3′–OH, C3–OH and C5′–OH.

According to literature data (Dimitrić Marković, Milenković, Amić, Mojović, & et al., 2014), the reactivity of Dp via HAT is similar.
Calculated reaction enthalpies (kJ mol\(^{-1}\)) for the reactions of Dp with hydroxyl and nitric oxide radicals.

<table>
<thead>
<tr>
<th>Dp</th>
<th>Water (\varepsilon = 78.35)</th>
<th>DMSO (\varepsilon = 46.83)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\Delta H_{\text{HAT}})</td>
<td>(\Delta H_{\text{SPLET}})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Ethanol (\varepsilon = 24.85)</th>
<th>DMF (\varepsilon = 37.22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DpOH-3+NO</td>
<td>162</td>
<td>75</td>
</tr>
<tr>
<td>DpOH-3+NO</td>
<td>161</td>
<td>–76</td>
</tr>
<tr>
<td>DpOH-4+NO</td>
<td>135</td>
<td>–103</td>
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<tr>
<td>DpOH-5+NO</td>
<td>160</td>
<td>–78</td>
</tr>
<tr>
<td>DpOH-5+NO</td>
<td>175</td>
<td>–63</td>
</tr>
<tr>
<td>DpOH-7+NO</td>
<td>181</td>
<td>–57</td>
</tr>
</tbody>
</table>

Table 3
Calculated reaction enthalpies (kJ mol\(^{-1}\)) for the reactions of Pg with hydroxyl and nitric oxide radicals.

<table>
<thead>
<tr>
<th>Pg</th>
<th>Water (\varepsilon = 78.35)</th>
<th>DMSO (\varepsilon = 46.83)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\Delta H_{\text{HAT}})</td>
<td>(\Delta H_{\text{SPLET}})</td>
</tr>
<tr>
<td>PgOH-3+OH</td>
<td>–131</td>
<td>–236</td>
</tr>
<tr>
<td>PgOH-4+OH</td>
<td>–113</td>
<td>–218</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Ethanol (\varepsilon = 24.85)</th>
<th>DMF (\varepsilon = 37.22)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>161</td>
<td>–83</td>
</tr>
<tr>
<td>PgOH-4+NO</td>
<td>178</td>
<td>–65</td>
</tr>
<tr>
<td>PgOH-5+NO</td>
<td>173</td>
<td>–70</td>
</tr>
<tr>
<td>PgOH-7+NO</td>
<td>178</td>
<td>–66</td>
</tr>
</tbody>
</table>

Table 2
Calculated reaction enthalpies (kJ mol\(^{-1}\)) for the reactions of Dp with hydroxyl and nitric oxide radicals.

<table>
<thead>
<tr>
<th>M05-2X/6-311+G(d,p)</th>
<th>Water (\varepsilon = 78.35)</th>
<th>DMSO (\varepsilon = 46.83)</th>
</tr>
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<tbody>
<tr>
<td>Dp</td>
<td>(\Delta H_{\text{HAT}})</td>
<td>(\Delta H_{\text{SPLET}})</td>
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<table>
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<tr>
<th></th>
<th>Ethanol (\varepsilon = 24.85)</th>
<th>DMF (\varepsilon = 37.22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DpOH-3+NO</td>
<td>162</td>
<td>75</td>
</tr>
<tr>
<td>DpOH-3+NO</td>
<td>161</td>
<td>–76</td>
</tr>
<tr>
<td>DpOH-4+NO</td>
<td>135</td>
<td>–103</td>
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<td>DpOH-5+NO</td>
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<td>DpOH-5+NO</td>
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<td>–63</td>
</tr>
<tr>
<td>DpOH-7+NO</td>
<td>181</td>
<td>–57</td>
</tr>
</tbody>
</table>

to the reactivity of flavones, baicalein and fisetin. Reactivity of Dp via SPLET, in all the solvents investigated, was more probable in comparison with the same flavones.

The BDEs of individual hydroxyl groups in Pg differed by up to 20 kJ mol\(^{-1}\), suggesting different reactivity of the individual positions. If the reaction proceeded via HAT, C3–OH group in Pg would
be the most reactive followed by C3’–OH, C5–OH and C7–OH (Table 3).

If the reaction proceeded via SPLET, the most reactive (most acidic) positions in Pg, in all solvents under investigation, would be C5–OH and C7–OH (Table 3). Mv might also react with hydroxyl radical via both HAT and SPLET. C4–OH group was more reactive via HAT while C7–OH was more reactive when the reaction proceeded via SPLET (Table 4). Malvin has two methoxy groups at the ortho position with respect to 4’-OH. This arrangement affects 4’-OH BDE and PA values, decreasing the antiradical activity of Mv in comparison with Dp (Vaganek, Rimarčík, Dropkova, Lengyel, & Klein, 2014; Vaganek, Rimarčík, Lukeš, & Klein, 2012) (Table 4). Comparing BDE values for baicaline and fisetin (Dimitrić Marković, Milenković, Amić, Mojović, et al., 2014) with BDE values for Pg and Mv, it is evident that the flavones were underwent HAT mechanism more readily. On the other hand, PA values for Pg and Mv were significantly lower in all solvents, indicating their activity via SPLET was enhanced in comparison with the flavones (Dimitrić Marković, Milenković, Amić, Mojović, et al., 2014).

SET-PT proved to be thermodynamically unfavourable for all three molecules reacting with hydroxyl radical in all the solvents investigated.

In the case of nitric oxide radicals, HAT reactions were endothermic in all the solvents (Tables 2–4, and S1–S3), indicating that HAT is not possible for Dp, Pg and Mv. On the other hand, some AH_{PA} values for Dp, Pg and Mv reacting with nitric oxide radical were slightly negative or positive indicating SPLET as hardly plausible. The results point to the newly formed radical as less stable implying the polar solvents are not suitable for Dp, Pg and Mv to react with nitric oxide radicals via SPLET.

These results are in good agreement with the ESR results obtained. SET-PT was thermodynamically unfavourable for all three molecules in all the solvents investigated.

### 4. Conclusions

An experimental and theoretical study regarding antiradical activity of Dp, Pg and Mv was performed using ESR spectroscopy and M05-2X/6-311+G(d,p) level of theory. The results indicated that different antiradical activities for the molecules investigated against hydroxyl and nitric oxide radicals. According to the ESR measurements, Dp, Pg and Mv were selective towards hydroxyl radical. Pg and Dp both reduced hydroxyl radical to a greater extent (85% and 83%, respectively) while Mv was less active (61%). Regarding the radical scavenging sequence obtained, it is possible to deduce that C4’-OH is functional and, most probably, renders these flavonoids hydroxyl radical scavengers in advance of ortho-hydroxyl or pyrogallol groups in ring B. This assumption would be supported by Wang et al. (1997) where the highest antiradical activity is expected for molecules with two OH groups in B ring. Dp and Pg could be taken as cases “in between”. The increased activity of Pg against hydroxyl radical correlates well with the literature while the somewhat reduced antiradical activity of Mv could be considered a consequence of glycosylation, which reduces the number of free hydroxyl sites. The higher antiradical activity of Dp and Pg, in comparison to Mv, could also be explained by the influence of the 3-OH group that, when not substituted, interacts with ring B through a hydrogen bond, thus maintaining its coplanarity to rings A and C. The lower activity of Mv against hydroxyl radicals is a consequence of reduced conjugation due to the loss of ring B coplanarity with respect to the rest of the molecule. According to the ESR measurements, none of the molecules showed activity towards the nitric oxide radical, which was confirmed by our theoretical calculations.

Calculated energy requirements indicate thermodynamically plausible radical scavenging mechanisms and identify active sites for radical inactivation. Activity of each OH group is determined based on the values of the thermodynamic parameters: BDE, PA and ETE. Results indicated the effect of substitution patterns that altered the activity of the molecules through different mechanisms, specifically inductive, resonance, and steric. Since all AH_{BDE} and AH_{PA} values were negative and very close both HAT and SPLET were competitive in all the solvents under investigations. As indicated in the literature, the number of hydroxyl groups was not essential, albeit important, because the antiradical activity was dependent on the BDE of individual hydroxyl groups. This implies an efficient antiradical molecule as one having a similar relative electron density on the oxygen atom), had nearly the same BDE an efficient antiradical molecule as one having a similar relative electron density on the oxygen atom), had nearly the same BDE

### Table 4

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<tr>
<th>Mv</th>
<th>Water ε = 78.35</th>
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<td>HAT</td>
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<td>HAT</td>
<td>SET-PT</td>
<td>SPLET</td>
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<td>288</td>
<td>142</td>
<td>–108</td>
<td>–1</td>
<td>307</td>
<td>–108</td>
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<th>Ethanol ε = 24.85</th>
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<td>–104</td>
<td>–5</td>
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<td>306</td>
<td>–109</td>
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<td>306</td>
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<tr>
<td>MvOH-4’+NO</td>
<td>142</td>
<td>–108</td>
<td>–1</td>
<td>307</td>
<td>–108</td>
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<td>DMF ε = 37.22</td>
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<td>MvOH-4’+NO</td>
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</table>
(HAT and SPLIT). These sites in Pg are C3-OH (HAT) and C7-OH (SPLIT), and in Mv C4-OH (HAT) and C7-OH (SPLIT) (also in all solvents considered). The results obtained for BDE and PA led to the ease of the homolytic and heterolytic O–H breaking in the same hierarchy of molecules, but different active positions in rela-
tants considered). The results obtained for BDE and PA led to the ease of the homolytic and heterolytic O–H breaking in the solvents investigated. The hierarchy for HAT (BDA values) was Dp (C4-OH) > Mv (C4-OH) > Pg (C3-OH) and for SPLIT (PA values) Dp (C4-OH) > Mv (C7-OH) > Pg (C7-OH). The resulting sequence of activities vary relate to the one obtained using ESR measurements (Pg < Dp < Mv).

HAT and SET-PT are thermodynamically unfavourable for Dp, Pg, and Mv with the nitric oxide radical in the polar solvents investi-
gated. Small negative ΔH0 values indicate SPLIT was hardly plau-
sible for the reaction with nitric oxide radicals.

Acknowledgement

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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.2016.09.106.

References


