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Singlet oxygen quenching and radical scavenging capacities of structurally-related flavonoids present in *Zuccagnia punctata* Cav.

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Abstract

The singlet oxygen ($^1O_2$) quenching and free radical (DPPH$^\bullet$, ABTS$^{\bullet+}$ and $O_2^{\bullet-}$) scavenging ability of three structurally-related flavonoids (7-hydroxyflavanone HF, 2′,4′-dihydroxychalcone DHC and 3,7-dihydroxyflavone DHF) present in the Argentinean native shrub *Zuccagnia punctata* Cav. were studied in solution by combining electrochemical and kinetic measurements, mass spectroscopy, end-point antioxidant assays and computational calculations. The results showed that the antioxidant properties of these flavonoids depend on several factors, such as their electron- and hydrogen atom donor capacity, the ionization degree of the more acidic group, solvatation effects and electrostatic interactions with the oxidant species. The theoretical calculations for both the gas and solution phases at the B3LYP level of theory for the Osanger reaction field model agreed with the experimental findings, thus supporting the characterization of the antioxidant mechanism of the *Z. punctata* flavonoids.

Keywords: *Antioxidant capacity, flavonoid, free radicals, reactive oxygen species (ROS), singlet oxygen*

Introduction

Flavonoids are widely distributed in plants and have various physiological functions in defense mechanisms against both biotic and abiotic stress [1,2]. Structurally, flavonoids are benzo-γ-pyrone derivatives consisting of a benzene ring (A-ring) attached to a six-member heterocycle (C-ring), which at C2 carries a phenyl group (B-ring). In recent years, these natural polyphenolic compounds have received considerable attention due to their biological activity in preventive treatments against infections, inflammation, cancer and heart disease [3–6]. Most of their properties are associated with the importance of flavonoids as antioxidants against free radicals and reactive oxygen species (ROS) [7–9], including singlet molecular oxygen $^1O_2$ [10–14].

Zuccagnia punctata Cav. (FABACEAE), commonly called *jarilla macho* or *pus-pus*, is a native shrub widely distributed in the semi-arid and arid regions of western Argentina. Interestingly, since ancient times, the infusion and macerated in ethanol of the leaves and stems are used in traditional medicine as an

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antiseptic, anti-inflammatory and to relieve muscle and bone pain. The leaves of the shrub are a rich source of phenolic compounds (42 mg/g of dry plant material), with ~70% being flavonoid derivatives [15], mainly distributed as methoxylated and non-methoxylated flavanones, flavones and chalcones [16,17]. In fact, ethanolic extracts from the leaves of *Z. punctata* have been shown to have efficient antibacterial activity against antibiotic multi-resistant bacteria [15]. In addition, preventive cytoprotective effects on ethanol-induced gastroduodenal tract injuries in rats have been attributed to 2′,4′-dihydroxychalcone as active compound [18]. This chalcone, together with 2′,4′-dihydroxy-3-methoxychalcone, 7-hydroxyflavanone and a caffeoyl ester derivative isolated from *Z. punctata*, were also active against phytophogenic fungi such as *Phomopsis longicolla* and *Colletotrichum truncatum* [17]. All these studies have demonstrated that the flavonoids of *Z. punctata* are bioactive compounds and that their functionality can be associated with their antioxidant capacity against different oxidant or aggressive species. However, while several studies have indicated that certain flavonoid derivatives of *Z. punctata* are moderate quenchers of $^1O_2$ [19,20], little information is available on the antioxidant activity of *Z. punctata* flavonoids in relation to their structural pattern or interaction with given oxidant species.

In this work, we analysed the antioxidant capacity of structurally and biosynthetic related non-methoxylated flavonoids presented in *Z. punctata*, i.e. 7-hydroxyflavanone (HF), 2′,4′-dihydroxychalcone (DHC) and 3,7-dihydroxyflavone (DHF).

The role of specific charge-transfer effects in the interaction of these flavonoids with oxidant species such as anion superoxide $O_2^{\bullet -}$ and $^1O_2$ and the nitrogen-based dye radicals 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^\bullet$) and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS$^{\bullet +}$) was evaluated using kinetic and end-point antioxidant assays as well as theoretical calculations. The results indicated that the antioxidant power of these flavonoids depends on both environmental conditions (solvent and pH) and the type of oxidant species. In addition, the oxidative reaction of DHF with $^1O_2$ was investigated using direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) and high performance liquid chromatography (HPLC).

Experimental procedure

Materials

The flavonoids 7-hydroxyflavanone (HF), 3,7-dihydroxyflavone (DHF) and 2′,4′-dihydroxychalcone (DHC) were from Indofine Chemical Company (New Jersey, USA). Chemicals such as 9,10-dimethylanthracene (DMA), 1,4-diazabicyclo[2,2,2]octane (DABCO), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^\bullet$), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid), diammonium salt (ABTS), phenazine methosulphate (PMS), *p*-Nitro-Blue tetrazolium chloride (NBT), β-nicotinamide adenine dinucleotide in reduced form (NADH), $K_2S_2O_8$ and Rose Bengal as sodium salt (RB) were supplied by Sigma (Missouri, USA). The inorganic reagents NaOH, NaH_2PO_4 and Na_2HPO_4 were analytical grade and obtained from Merck Argentina (Buenos Aires, Argentina). The organic solvents were HPLC grade from Sintorgan Argentina (Buenos Aires, Argentina). Lastly, water was of Milli Q Plus quality. All the chemicals were used as received.

Methods

The UV-Vis and fluorescence spectra were monitored with an OceanOptics USB2000 diode array spectrophotometer (Dunedin, FL) and a Hitachi F-2500 fluorometer (Kyoto, Japan).

Stationary and pulsed photosensitized generations of $^1O_2$ were performed as previously described [13,14]. The bimolecular rate constant for the total quenching (physical + chemical) of $^1O_2$ by the flavonoids, namely k_t, was determined by time-resolved phosphorescence detection (TRPD) using a Peltier-cooled Ge photodiode Judson J16TE2-66G (Judson Technol. Montgomeryville, PA) placed at a right angle to the excitation laser pulse. A Continuum Minilite II Nd:YAG Q-switched laser (Santa Clara, CA) operating at 532 nm (10 ns FWHM, ~3 mJ/pulse) was used to excite the sensitizer RB. Spurious light was obstructed using a 1270 nm band pass filter (Spectrogon BP-1260, Taeby, Sweden) and the output of the detector was fed via amplifier stages ($\times 10$) to a Tektronix TDS3032B (Beaverton, OR) digital oscilloscope linked to an on-line PC for data transfer and analysis.

The bimolecular rate constant of the chemical reaction between $^1O_2$ and the flavonoids, k_c, was determined using a comparative method [21]. The chemical quencher 9,10-dimethylanthracene (DMA, $k_{c,DMA} = 4.7\times10^7$ M^{-1} s^{-1} [22]) was used as the reference compound under identical photosensitization conditions. Steady-state illumination was utilized, the excitation source being a 150 W filament lamp coupled with a red cut-off filter (> 515 nm), the beam of which was focused into the sample cell at a right angle to the analysing light of the spectrophotometer.

The scavenging kinetic of the dye radical DPPH$^\bullet$ in ethanol solution was monitored through the dye bleaching at 517 nm ($\varepsilon_{DPPH} = 11\ 500$ M^{-1} cm^{-1} [23]). The radical ABTS$^{\bullet +}$ was generated as described elsewhere [24]. The end-points of the ABTS$^{\bullet +}$ and DPPH$^\bullet$ bleaching assays after the addition of different flavonoid concentrations (0.5–15 mM) were at 6 and 60 min, respectively.

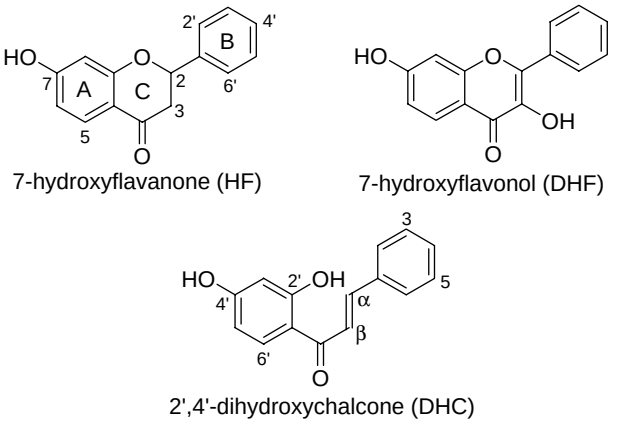


Figure 1. Chemical structure of non-methoxylated flavonoids present in *Zuccagnia punctata* Cav.

Typically, the initial $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ concentrations were ~60 and 45 μM, respectively. The percentage of radical scavenging (%RS) by the flavonoids was calculated using the radical absorbance ratio $\Delta A/A_0$, with $\Delta A = (A_0 - A_f)$ being the absorbance change at the end-point in relation to a control solution of the radicals without flavonoids and A_0 standing for the initial absorbance of the radical.

Superoxide radical anion $O_2^{\bullet-}$ was generated via a non-enzymatic method using a phenazine methosulphate (PMS)/NADH/O_2 system [25,26]. All the reagents were dissolved in a 20 mM phosphate buffer solution (PBS) at pH 7.4. Different concentrations of flavonoids were added to test tubes. After 2 min of incubation at room temperature, the reaction was stopped by adding 0.1 M HCl and the amount of $O_2^{\bullet-}$ generated was measured by NBT assay, with the samples being incubated for 30 min at 37°C and their absorbance measured at 560 nm [25,26].

Cyclic voltammetry experiments were performed in a 1:1 mixture of ethanol and aqueous (50 mM KCl, 50 mM PBS, at pH 5.0 or 7.6) solution using an Autolab PGSTAT30 (Utrecht, NL) potentiostat with a standard three-electrode compartment cell. The reference, working and auxiliary electrodes were Ag/AgCl (3 M KCl), vitreous carbon and a Pt wire, respectively. In the present study, the base-catalysed flavanone-chalcone equilibrium [27] was observed to occur very slowly (lifetime of hours) to preclude the determination of the redox potentials.

The oxidation products formed by reaction of DHF with $^1O_2$ were determined by electrospray ionization mass spectrometry (ESI-MS/MS) experiments performed on an Esquire 4000 model (Bruker Daltonics, Bremen, Germany) equipped with an ion trap analyser, and an HPLC (Shimadzu, model LC-20AD, Kyoto, Japan) with a photo-diode array (PDA) detector (Shimadzu, model SPD-M20A) coupled to the MS. A methanol solution of 10 μM MB and 0.8 mM DHF was irradiated with a 150 W filament lamp coupled to a red–orange cut-off filter to excite the band of the sensitizer exclusively above 530 nm. Aliquots were taken after every 15 min of illumination and directly monitored by ESI-MS/MS. The samples, together with 200 μL/min of methanol/water (1:1) provided by the HPLC pump solution, were injected through a direct flow injection peristaltic pump at a constant flow rate of 4 μL/min. The MS parameters were set as follows: negative mode, capillary voltage: 1500 V, end plate offset: −500 V, capillary exit: −115 V, skimmer 1: −40.0 V, skimmer 2: −6.0 V, dry gas (N_2) temperature: 300°C and flow: 8 L/min, nebulizer: 30 psi, scan range from m/z 80–850. The MS/MS experiment was set in automatic mode to apply fragmentation energy of 1.2 V. The data acquisition and processing were carried out using Compass 1.1 software. In addition, HPLC analyses with PDA connected in series to the MS described above were conducted on a C_{18} Shim-pack CLC column (5 μm, 250 × 4.6 mm, Shimadzu, Canby, Oregon) with the linear gradient of 5% aqueous formic acid/methanol going from 85:15 to 20:80 (v/v) in 25 min, the isocratic proportion being maintained for an additional 15 min at a flow rate of 0.9 mL/min and the column temperature maintained at 29°C. The chromatograms were processed at 280 nm and the spectra monitored between 200–600 nm.

All the experiments were performed in duplicate and under a controlled temperature of 25 ± 1°C.

Computational calculations

With the aim of comparing the capabilities of the *Z. punctata* flavonoids for participating in either hydrogen-atom or electron-transfer mechanisms, geometric optimizations were performed for each FOH neutral flavonoid as well as for the $FOH^{\bullet+}$, FO^- and $FO^{\bullet}$ species. All the structures were optimized at the B3LYP level of theory [28,29], with the 6-31g(d) base set utilizing the Gaussian03 program package (Gaussian Inc., Pittsburgh, PA, 2003). For all the species, subsequent frequency analyses were performed at the same level of theory to ensure that all structures were minima and to determine the zero-point vibrational energies (ZPVEs). The ZPVEs were then scaled by the factor 0.9804 [30,31] and used to correct the computed energies. In addition, single-point energy calculations were performed with the 6-31 + g(d,p) base set. The solvent (ethanol, water) effect was computed within the framework of the Osanger reaction field model [32,33] and implemented using the Gaussian03 package. The geometric optimizations and frequency calculations of the solvated species were performed after computing the radii of the cavities occupied by the solute molecules within the solvent field, once again using tools included in the Gaussian03 program. The ionization potentials (*IP*) of both the

neutral and deprotonated flavonoids, i.e. FOH and FO^-, were calculated as the energy difference for the formation of the oxidized $FOH^{\bullet+}$ and $FO^{\bullet}$ species, respectively. The energy required to extract a single H atom from the FOH molecule, i.e. the FO-H bond dissociation energy (BDE), was calculated as the energy difference between $(FO^{\bullet} + H^{\bullet})$ and the neutral FOH molecule.

Results and discussion

Singlet oxygen quenching in ethanol solutions

After 532 nm laser excitation of RB in ethanol solutions, a typical first-order NIR phosphorescence decay of $^1O_2$ was observed with unimolecular rate constant $k_\Delta^0 = 7.1 \times 10^4\ s^{-1}$ (inset of Figure 2), as typically reported for protic solvents [22]. In the presence of flavonoids, the decay of $^1O_2$ was shortened but the initial intensity remained unchanged (inset of Figure 2). This result indicates the occurrence of parallel chemical and physical quenching reactions of $^1O_2$, with bimolecular rate constants k_c and k_q, respectively (equations 1 and 2), without changes in the efficiency of the photosensitized generation of $^1O_2$.

$$^1O_2 + FOH \xrightarrow{k_c} \text{Oxidation Products} \quad (1)$$

$$^1O_2 + FOH \xrightarrow{k_q} {}^3O_2 + FOH \quad (2)$$

In this case, the observed decay rate constant k_Δ showed a linear dependence with flavonoid concentration [FOH], equation (3) and Figure 3, which slope is the overall quenching rate constant $k_t = k_c + k_q$.

$$k_\Delta = k_\Delta^0 + k_t[FOH] \quad (3)$$

Figure 3 shows the UV-Vis spectral changes produced upon steady-state excitation of the photosensitizer

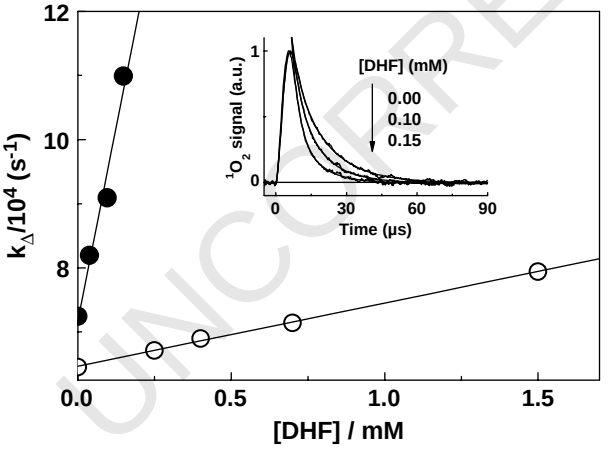


Figure 2. Dependence of the first-order rate constant for the decay of $^1O_2$, k_Δ, with the DHF concentration, equation (3), in: (○) neutral; and (●) basic (10 mM NaOH) ethanol solutions. Inset: Dependence of the $^1O_2$ phosphorescence decay with the concentration of DHF in basic ethanol solutions.

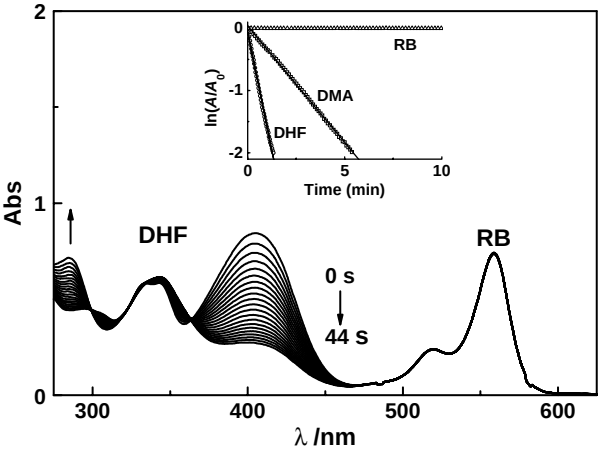


Figure 3. UV-Vis absorption changes produced for DHF (50 μM, every 2 s) upon steady-state irradiation of 8 μM RB at $\lambda > 515$ nm in aerated 10 mM NaOH ethanol solutions. Inset: first-order kinetic plots monitored at the absorption maximum of the sensitizer RB, the chemical reference DMA and the flavonol DHF.

RB at $\lambda > 515$ nm in alkaline ethanol solution of the flavonol DHF. Under these experimental conditions, no changes in either the absorption or emission spectra of RB were observed. In addition, the flavonoid photosensitized degradation was halted by the addition of DABCO, which is an efficient physical quencher of $^1O_2$ [22].

These results confirm the absence of both ground and excited state reactions of the sensitizer RB with the flavonol, as well as indicating that flavonoid degradation is principally mediated by $^1O_2$ (Type II mechanism) [10,13,14]. Under continuous illumination of RB, the concentration of $^1O_2$ remains nearly constant and the substrate depletion should follow a pseudo-first order kinetic decay, as shown in Figure 3. Comparing the observed first-order rate constant k_{obs} between the reference compound DMA and the flavonoids under identical experimental conditions allowed for calculation of the rate constant for the chemical reaction between $^1O_2$ and the flavonoids (equation 4) [21].

$$k_{c,\ FOH} = k_{c,\ DMA} \times (k_{obs,\ FOH} / k_{obs,\ DMA}) \quad (4)$$

Table I shows the calculated k_c, k_q and k_t values for the *Z. punctata* flavonoids under different acid-basic conditions in ethanol solutions, with the values following a quenching capacity trend DHF > DHC > HF. Increasing the hydroxide ion concentration of the medium (pH increases) produces a significant bathochromic shift of the flavonoid absorption bands (see Figure S1 in the Supplementary Material) due to full deprotonation of the phenolic groups of the flavonoids [34].

Differences in the $^1O_2$ quenching capacities of the *Z. punctata* flavonoids can be understood on the basis of their structural features (Figure 1), as formerly stated by Tournaire et al. [10]. For instance, the k_t value of the chalcone DHC is nearly one-order

Table I. Rate constants for the chemical reaction (k_c), and for the total total (k_t) and physical (k_q) quenching of $^1O_2$ by the flavonoids present in *Zuccagnia punctata* Cav., in acid (5 mM HCl), neutral and basic (5 mM NaOH) ethanol solutions at 25°C.

Solution		k_c (M^{-1} s^{-1})[a]	k_t (M^{-1} s^{-1})[b]	k_q (M^{-1} s^{-1})[c]	k_q/k_t
HF	Acid	$(0.2\pm0.2)\times10^5$; [275 nm]	$(0.7\pm0.2)\times10^6$	$(6.8\pm0.2)\times10^5$	0.97
	Neutral	$(0.2\pm0.1)\times10^5$; [275 nm]	$(0.9\pm0.1)\times10^6$	$(8.8\pm0.1)\times10^5$	0.98
	Basic	$(2.2\pm0.8)\times10^5$; [338 nm]	$(5.8\pm0.4)\times10^6$	$(5.6\pm0.4)\times10^6$	0.96
DHC	Acid	$(1.2\pm0.5)\times10^5$; [350 nm]	$(3.3\pm0.3)\times10^6$	$(3.2\pm0.3)\times10^6$	0.96
	Neutral	$(1.6\pm1.0)\times10^5$; [350 nm]	$(3.5\pm0.1)\times10^6$	$(3.3\pm0.1)\times10^6$	0.95
	Basic	$(2.1\pm0.2)\times10^6$; [400 nm]	$(1.6\pm0.1)\times10^7$	$(1.4\pm0.1)\times10^7$	0.87
DHF	Acid	$(5.0\pm0.6)\times10^6$; [360 nm]	$(0.8\pm0.4)\times10^7$	$(3.0\pm0.6)\times10^6$	0.63
	Neutral	$(5.9\pm0.3)\times10^6$; [360 nm]	$(1.0\pm0.3)\times10^7$	$(4.1\pm0.3)\times10^6$	0.41
	Basic	$(2.6\pm0.3)\times10^8$; [405 nm]	$(2.5\pm0.4)\times10^8$	≈0.0	≈0.0

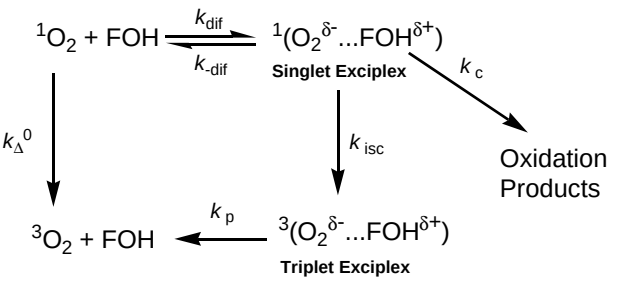
[a] From UV-Vis experiments (bracket values indicate the monitoring wavelength).
[b] From TRPD experiments.
[c] Calculated as $k_q = k_t - k_c$.

magnitude larger than its isomeric flavanone HF. This effect can be attributed to the presence of the $C\alpha = C\beta$ double bond in the chalcone, which heightens interaction with the electrophilic $^1O_2$ species [13]. In the case of the flavonol DHF, the presence of a C-ring with an extra electron-donating C3 – OH group increases the electron-donor capacity towards $^1O_2$ and the largest k_t was observed (Table I). A similar hydroxylation effect on the double bond of the C-ring was also observed for $^1O_2$ quenching by flavone ($k_t = 3\times10^5$ M^{-1} s^{-1}) and 3-OH-flavone ($k_t = 5\times10^6$ M^{-1} s^{-1}) in ethanol solutions [12]. In fact, DHF showed the largest chemical reaction efficiency, confirming that the reactivity with $^1O_2$ is mainly controlled by the presence of the –OH group in the C3 position, activating the double bond of ring C [10].

The quenching rate constants of the *Z. punctata* flavonoids strongly increased in 5 mM NaOH ethanol solutions, but slightly decreased in acidic solutions (5 mM HCl) (Table I). Close comparison of the UV-vis spectra of the flavonoids in acidic, neutral and alkaline ethanol solutions confirmed the presence of small traces of ionized flavonoids (< 10%) in neutral ethanol (Figure S1 in the Supplementary Material). In fact, the UV-vis absorbance titration of the various *Z. punctata* flavonoids in 10 mM phosphate buffer aqueous solution had similar pK_a values in relation to the deprotonation of the C7OH group in HF (pK_a = 7.1) and DHF (pK_a = 7.3) and its equivalent (C4′OH) in DHC (pK_a = 7.4). A second more basic value was observed for both DHC (p$K_a \approx 11$) and DHF (pK_a = 9.7) due to the deprotonation of the respective C2′OH and C3OH groups, which are hydrogen-bonded with the C4 = O group. Thus, the increment of the quenching rate constant with the ethanol basicity is associated with the increment of the ionized flavonoid species, as typically observed for phenolic-like compounds [11,13,21,35].

Both the structural and acid-base effects on the quenching capacity of the *Z. punctata* flavonoids can be linked to the increase in the electron-donor ability of the flavonoid series towards $^1O_2$. This assumption is supported by both the measured peak oxidation potential (E_{ox}) and the calculated ionization potential (*IP*) of the flavonoid series (Table II), both of which indicate that the electron-donor capacity of the *Z. punctata* flavonoids increases as DHF > DHC > HF. The accepted quenching mechanism of $^1O_2$ by phenolic-like derivatives (Q) involves an excited state charge-transfer complex (*exciplex*) in which the $^1O_2$ is the electron-acceptor species [35,36] (Scheme 1).

According to Scheme 1, the exciplex $^1(O_2^{\delta-}\cdots FOH^{\delta+})$ decays either via an intersystem crossing process (k_{isc}) to yield ground state $^3O_2$ and FOH, thus favouring the physical quenching pathway, or through chemical reaction (k_c) to oxygenation products, hence facilitating the chemical reaction. The intermediate singlet state exciplex is sensitive to the spin-coupling and entropic factors that modulate physical and chemical decays [37]. Within this framework, as the electron-donor ability of the flavonoid increases (i.e. as the oxidation potential E_{ox} values decrease, coupled with structural [entropic] factors) the chemical reaction pathway from the intermediate exciplex comes to be favoured. This result is supported by the linear increases of the efficiency of



Scheme 1. Exciplex-mediated mechanism for the quenching of $^1O_2$ by flavonoids (FOH).

physical quenching k_q/k_t (Table I) with the E_{ox} of the *Z. punctata* flavonoids. The ratio k_q/k_t has biological relevance, given that when this ratio tends to unity the quencher is not oxidized by $^1O_2$, thus allowing for its reutilization in consecutive interactions with either $^1O_2$ or other ROS, e.g. $O_2^{\bullet -}$ or $HO^{\bullet}$. From this point of view, both the flavanone HF and the chalcone DHC can be considered more efficient catalytic antioxidants of $^1O_2$ than the more reactive flavonol DHF (Table I). This behaviour is evidenced when the total quenching efficiency of $^1O_2$ by the flavonoids (η_q^{Δ}, equation 5) is analysed as a function of consecutive quenching steps.

$$\eta_q^{\Delta} = \frac{k_t[\mathrm{FOH}]_n}{k_{\Delta}^0 + k_t[\mathrm{FOH}]_n} \quad (5)$$

After *n*-consecutive reactions with $^1O_2$, the effective flavonoid concentration $[FOH]_n$ is depleted at each step by the factor k_c/k_t, which stands for chemical reaction efficiency. Thus, η_q^{Δ} decreases in a different fashion for each flavonoid, as Figure 4 illustrates in neutral ethanol solutions. In the initial step, the flavonol DHF was ~10- and 3-times a more efficient quencher than HF and DHC, respectively. However, in the succeeding steps, the quenching efficiency of DHF rapidly decreased to become negligible after four or five phases of the reaction. Nevertheless, in this case the oxidation products of DHF could also be quenchers of $^1O_2$ and thus contribute to the total antioxidant capacity (see next section). This effect should be more important under alkaline conditions, since for $k_t \approx k_c$ (Table I).

Thus, comparing both isomeric HF and DHC compounds, the chalcone revealed a promising catalytic quencher activity since it is characterized by a moderate k_q value and extremely limited rate constant of chemical reaction.

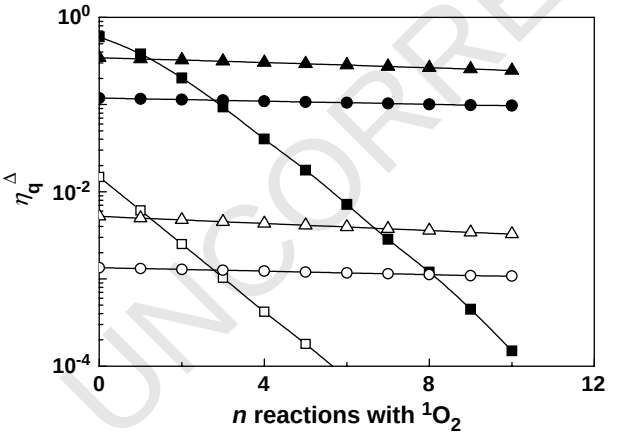


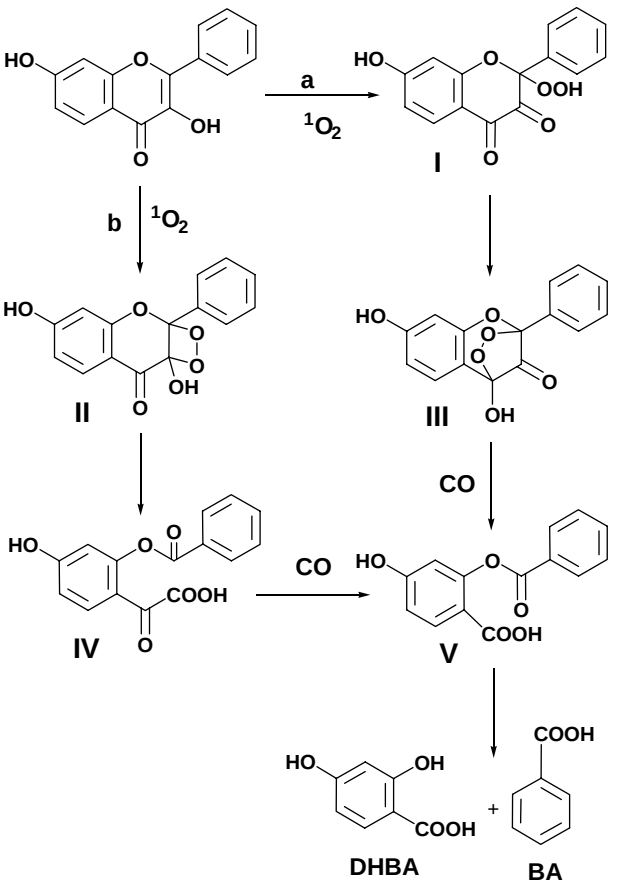
Figure 4. Variation of the total quenching efficiency of $^1O_2$ by the flavonoids (η_q^{Δ}) as a function of the number *n* of consecutive reactions with $^1O_2$, equation (6), for: (■,□) DHF, (▲, Δ) DHC and (●, ○) HF; with $[FOH]_0 = 10$ mM (solid symbols) and $[FOH]_0 = 0.1$ mM (open symbols).

Photo-oxidation products of DHF

Considering the largest chemical reactivity of the flavonol DHF with $^1O_2$, the time evolution of the photosensitized reaction was monitored by electrospray ionization mass spectroscopy (ESI-MS/MS) in order to characterize the oxidation products. Prior to illumination of the samples ($t = 0$), the mass spectrum of DHF was characterized by an intense deprotonated molecule $[M\text{-}H]^-$ peak at *m/z* 253 (see Figure S2 in the Supplementary Material). The second-order MS showed consecutive losses of water (*m/z* 235), CO (*m/z* 225), CO_2 (*m/z* 209), CO + CO (*m/z* 197) and CO + CO_2 (*m/z* 181), all from the deprotonated molecule. The other fragments, at *m/z* 165, 153 and 141 u, were probably formed through retro-Diels-Alder cyclization. Upon illumination, the mass spectra of the photosensitized samples revealed an abundant product with an $[M\text{-}H]^-$ peak at *m/z* 153, which was identified as 2,4-dihydroxybenzoic acid (DHBA, Mw = 154). Interestingly, the relative abundance of this peak compared to the main peak at 253 u increased with the illumination time, indicating that DHBA was being produced via the degradation of DHF (Figure S3 in the Supplementary Material). A second expected cleavage product was benzoic acid (BA, Mw = 122) [13], but the corresponding- ion was not detected by MS under the experimental conditions. However, the formation of BA in the photosensitized solutions was detected through HPLC-PDA analysis and confirmed by injection of standard solutions (Figure S4 in the Supplementary Material). The results observed are in agreement with those reported for the photosensitized reactions of flavonols [10,13,38]. Scheme 2 summarizes the two possible photo-oxidation mechanisms of DHF, based on previous proposed mechanisms for quercetin where the attack of $^1O_2$ occurs at the C2 = C3 double bond in the C-ring to form a hydroperoxide intermediate I by ene-type reaction [38], or by an 1,2-dioxetane II by [2 + 2]-cycloaddition on the activated C2 = C3 bond [10]. Interestingly, both pathways yield the ester V, after molecular rearrangement and decarbonylation of the respective intermediates III and IV. The intermediate V was isolated and characterized for the photosensitized oxygenation of quercetin [10]. Finally, compound V hydrolyses to yield final products DHBA and BA, both of which were detected in this study. These products can also act as quenchers of $^1O_2$, in particular the phenolic derivative DHBA with $k_t \approx 10^6 - 10^7\ M^{-1}\ s^{-1}$ [22], contributing to a secondary antioxidant effect.

Scavenging of the dye radicals DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ in ethanol solutions

Table II shows the flavonoid concentration values required to achieve 50% of radical scavenging capacity, SC_{50}. This magnitude was obtained from the



Scheme 2. Proposed mechanisms of the $^1O_2$-mediated oxidation of DHF.

slope of the linear variation of the percentage of radical scavenging (%RS) vs the flavonoid concentration (see Figure S5 in the Supplementary Material). According to these results, the reactivity of the *Z. punctata* flavonoids to both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ showed a DHF > DHC > HF trend, as also observed for the quenching of $^1O_2$ (see above). The relative increment of SC_{50} in the flavonoid series was parallel for both radicals in spite of the higher intrinsic reactivity of ABTS$^{\bullet+}$, indicating that a similar reaction mechanism was operating for the two nitrogen-centred radicals. Considering that the end-point colorimetric assay of DPPH$^{\bullet}$ can cause interference from non-related reactions due to the significantly long lifetime of the radical and complex kinetics with phenolic derivatives [29,39], the kinetic behaviour of the initial fast decay of the DPPH$^{\bullet}$ radical was also analysed. This process is related to the abstraction of the most labile H-atom attached to a hydroxyl group of the flavonoid, with k_S as the rate constant of the scavenging process [40,41] (equation 6).

$$DPPH^{\bullet} + FOH \xrightarrow{k_S} DPPH_2 + FO^{\bullet} \quad (6)$$

The scavenging kinetics were monitored under pseudo-first-order conditions, with a flavonoid/radical molar concentration ratio of $5 < [FOH]/[DPPH^{\bullet}] < 200$. Under these conditions, the rate constant k_S was obtained with equation (7) [40,41], where k_{DPPH} is the pseudo-first-order rate constant for the decay of DPPH$^{\bullet}$ (see Figure S5 in the Supplementary Material).

$$k_{DPPH} = k^0_{DPPH} + k_S[FOH] \quad (7)$$

In this case, $k^0_{DPPH} = 6 \times 10^{-7}$ s^{-1} corresponded to the unimolecular decay constant for DPPH$^{\bullet}$ in ethanol solution. This very low value for this rate constant indicates that the dye radical is practically stable in the absence of flavonoids during the time window of the scavenging experiments. Analogous to the results observed for the end-point colourimetric assay, the k_S values showed the same reactivity trend, i.e. DHF > DHC > HF (Table II). The scavenging capacity order observed in ethanol solutions can also be explained by the electron-donor ability of *Z. punctata* flavonoids when plots of log SC_{50} or log k_S vs either *IP* or E_{ox} maintain a strong linear relationship ($r \geq 0.994$) (Figure 5A), thus confirming the effect of electron-transfer processes in the scavenging reaction. This result can be explained by the '*anomalous*' *kinetic solvent effect* observed in alcohol solvents for the scavenging reaction of stable dye radicals by phenol derivatives [42]. In this case, the phenolic compounds are partially deprotonated by the solvent and the global radical scavenging reaction is a combination of classical hydrogen atom abstraction (HAT) from protonated phenol and electron-transfer (ET) from phenoxide anion, this mechanism being termed *sequential proton-loss electron-transfer* (SPLET) (see Scheme 3) [23,42,43].

As recently discussed by Litwinienko and Ingold [42], the relative contributions of the HAT and ET processes are highly sensitive to both reactants (pK_a's) and solvents (polarity, proton donor-acceptor capacity, etc.) [42,44]. Usually, HAT will dominate the scavenging process in solvents with low dielectric constants and poor H-acceptor properties such as

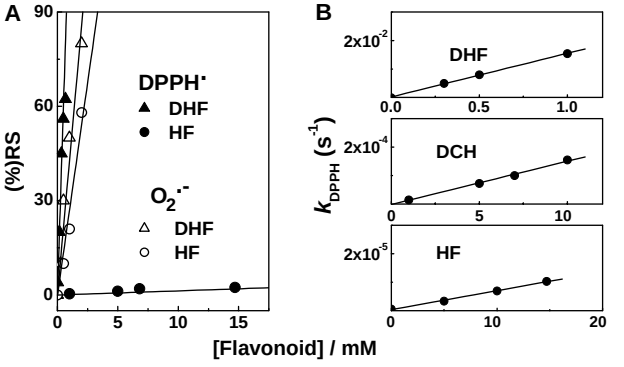
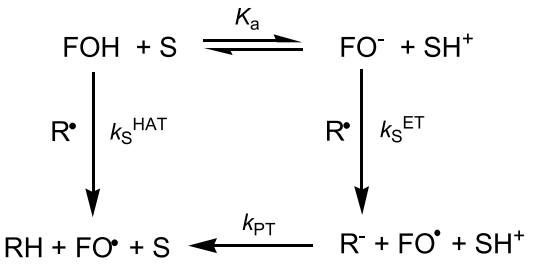


Figure 5. Linear correlations observed for (A) log k_S or (B) log SC_{50} vs the oxidation potential at pH 7.6 (E_{ox}) of the *Z. punctata* flavonoids. In (B) are also included data for quercetin (Q; 5,7,3′,4′-tetrahydroxyflavonol) and kaempferol (K; 5,7,4′-trihydroxyflavonol) from Yokozawa et al. [45].



Scheme 3. Solvent-assisted sequential proton-loss electron-transfer mechanism (SPLET).

alkanes, but the SPLET will prevail in more polar and hydrogen-bonded solvents such as alcohols and water [42]. Typically, $k_S^{ET} >> k_S^{HAT}$, so small concentrations of phenoxide anion FO^- can strongly enhance the rate of the scavenging reaction. In the present case, considering that ~10% of the *Z. punctata* flavonoids are deprotonated in neutral ethanol (see above), a dominant SPLET-like mechanism should be expected to operate, with an electron-transfer branch depending on the redox properties of the flavonoid series. This effect was confirmed by the linear relationship observed between log SC_{50} or log k_S and E_{ox} (Figure 5).

The flavonol DHF was the most efficient $DPPH^•$ and $ABTS^{•+}$ radical scavenger in the series. However, on comparing its $SC_{50} = 380$ μM for $DPPH^•$ to those reported for other more hydroxylated flavonols in ethanol [45] such as quercetin (5,7,3′,4′-tetrahydroxyflavonol, $SC_{50} = 8$ μM) and kaempferol (5,7,4′-trihydroxyflavonol, $SC_{50} = 23$ μM), DHF is seen to be a one-order magnitude weaker radical scavenger. Once again, this behaviour results from the higher E_{ox} value of DHF compared to those for quercetin and kaempferol under the same experimental conditions [45], given that these compounds have the same linear dependence of log SC_{50} vs E_{ox} as the *Z. punctata* flavonoids (Figure 5B).

Scavenging of superoxide anion in aqueous solutions

The scavenging capacity of $O_2^{•-}$ by the *Z. punctata* flavonoids in aqueous buffer solution at pH 7.4 showed a DHC > DHF > HF trend, contrary to that observed for the bulky nitrogen-based radicals in ethanol (Table II). In addition, SC_{50} was weakly correlated with both E_{ox} and *IP*, thus precluding confirmation of dominant electron-transfer processes in the scavenging reaction, despite the significant ionization degrees of the *Z. punctata* flavonoids in aqueous solutions at pH 7.4. According to the respective pK_a values for the deprotonation of the more acidic phenol group (see above), the calculated fractions of ionized flavonoids were $\alpha_{FO^-} = 0.50$, 0.56 and 0.67 for DHC, DHF and HF, respectively. The SC_{50} value of $O_2^{•-}$ increases linearly ($r \geq 0.980$) in relation to both α_{FO^-} and the calculated bond dissociation energy (BDE) for the H-atom transfer in water (Table II). This result indicates that the scavenging reaction of $O_2^{•-}$ by the flavonoids is mainly governed by the neutral FOH rather than by the phenoxyl FO^- species, suggesting the prevalence of a special type of HAT reaction called concerted *proton-coupled electron-transfer* (PCET) (equation 8) [42,46].

$$FOH + O_2^{•-}. \rightarrow (FO{\cdot}H{\cdot}O_2)^{•-} \rightarrow FO^• + HO_2^- \quad (8)$$

This type of mechanism is involved in the H-transfer in self-exchange reactions such as those between phenol and phenoxyl radical [46]. In this case, since increasing pH or decreasing pK_a reduces the concentration of FOH, the scavenging capacity of $O_2^{•-}$ by the flavonoids is reduced. Nonetheless, it has been reported that ionized species FO^- can react with $O_2^{•-}$ via a SPLET pathway, thereby producing the basic species O_2^{2-} and their subsequent neutralization (equations 9 and 10) [47].

$$FO^- + O_2^{•-} \longrightarrow FO^• + O_2^{2-} \quad (9)$$

$$O_2^{2-} + 2H_2O \longrightarrow H_2O_2 + 2HO^- \quad (10)$$

In the present case, if a SPLET mechanism is operating, the flavonol DHF should be the most efficient scavenger of $O_2^{•-}$ due to its having the lowest E_{ox}. However, the results showed that the chalcone DHC was the more efficient scavenger, with the lowest ionization degree and BDE for H-atom transfer. In addition, it has been reported that electrostatic repulsion between the anionic species FO^- and $O_2^{•-}$ can reduce the reaction rate by at least a factor of two [47]. These features support assigning the PCET pathway as the scavenging mechanism of $O_2^{•-}$ in aqueous media (equation 8). It is interesting to note that flavonols react with potassium superoxide (KO_2) in heterogeneous aprotic media yielding benzoic acid and aldehyde derivatives by opening of the ring C, while flavans, flavones and flavanones induced only the disproportionation of $O_2^{•-}$ without undergoing further oxidation [48]. The proposed mechanism involved a proton transfer from the C3-OH group to the superoxide anion and subsequent rearrangement and break bond reactions [48]. Probably, this heterogeneous phase proton-transfer mechanism does not operate in aqueous homogeneous solutions, but in any case, flavonoid degradation can be expected mainly for the flavonols by reactivity of the C2 = C3-OH group in the ring C [48].

Computational evaluation of antioxidant capacity

As previously mentioned, both the calculated ionization potentials (*IP*) and the bond-dissociation energies (BDE) of the FO – H bond of the flavonoids in solution agreed with the experimental data. Additional geometric and energy parameters, such as

solvent stabilization energy and length and angle of bonds (see Figure S6 in the Supplementary Material), were also obtained through computational calculation, thus allowing for confirmation of the antioxidant properties of the *Z. punctata* flavonoids.

In the gas phase, the most stable structure of the neutral flavanone HF is characterized by the perpendicular orientation of the B-ring in relation to the 4-γ pyranone ring ($\Psi = 93.2^{\circ}$). This value indicates that, due to the saturation of the C-ring, there is no possibility of conjugation of the rings for this hydroxyflavanone. In contrast, the structures of both DHF and DHC are characterized by the formation of intra-molecular hydrogen bonds between the carbonyl oxygen and the C3-OH and C2′-OH, respectively, giving rise to five- and six-member rings having H-bond distances of 1.93 Å and 1.66 Å, respectively. In the case of the flavonol DHF, the planarity of the molecule is evidenced by the dihedral angle of 180° between rings B and C. In accordance with this structure, the good electronic delocalization and conjugation of the π electrons involving rings A and B is expected. For the chalcone DHC, the *trans* orientation of the carbonyl group in relation to the C1′–C2′ bond presents one imaginary frequency, which indicates that the form is not stable. In more stable structures, ring B is nearly coplanar with $C\alpha = C\beta$. However, in the case of DHC, the dihedral angle between this double bond and C = O ($\Psi = 141.1^{\circ}$) suggests that there is a relatively low probability of conjugation involving rings A and B due to lack of planarity in the molecule.

For the radical cation $FOH^{\bullet +}$ species, the main stabilization factor for HF is the delocalization of the odd electron on aromatic ring A, which together with the contribution of the ether oxygen increases the dihedral angle Ψ ($= 108.3^{\circ}$). In contrast, for DHF, the odd electron appears to be delocalized over the entire molecule with a slight shortening (0.05 Å) of the C4 = O···HO-C3 bond. However, radicalization of the DHC more strongly reinforces the hydrogen bond interaction because the C = O···HO-C2′ distance is shorter at 1.54 Å and the dihedral angle Ψ is 8° higher.

With regard to the neutral radical $FO^{\bullet}$ species in the gas phase, HF shows nearly insignificant changes in the geometric parameters in relation to the neutral molecule. However, in the case of DHF, this radical is well stabilized by electronic delocalization over the three rings thanks to the planarity of the species. The only relevant difference from the neutral molecule refers to the quinoid-like structure formed on ring A (Figure S6 in the Supplementary Material). For DHC, the neutral radical slightly weakens the hydrogen bond (H bond distance of 1.70 Å), as well as somewhat modifying the dihedral angles involving the $C\alpha = C\beta$ instauration and the B-ring. The parameters calculated for the gas phase therefore indicate that, of the *Z. punctata* flavonoid series, the flavonol DHF has the strongest antioxidant capacity, given its lower *IP* and *BDE* values (Table II).

However, the calculations suggest that in protic solvents, e.g. ethanol and water, the flavonoid species are sensitive to the environment in different proportions. None of the flavonoids FOH are well stabilized by solvatation (< − 1.1 kcal/mol), nor is the radical cation $FOH^{\bullet +}$ of DHF (− 1.6 kcal/mol) or DHC (− 1.3 kcal/mol) effective in this regard. This indicates that their molecular stability and *IP* values are mainly due to strong hydrogen bond intramolecular interaction. In contrast, the radical cation of HF leads to a higher degree of solvent stabilization (∼ − 3.30 kcal/mol). Thus, different from what is observed for DHF and DHC, the *IP* of HF in solution is much lower than in vacuum.

In ethanol solution, the neutral radical $FO^{\bullet}$ of HF presented an extra rotation of ∼10° of the B-ring, with the stabilization of the radical being mainly due to electron delocalization on the A-ring. For the radical of DHF, almost no structural differences were observed in comparison to the gas phase. However, for the neutral radical of DHC, the C = O···HO-C2′ distance was shorter, thus reinforcing the importance of hydrogen bond interaction and larger distortions of the dihedral angles as stabilizing factors. In addition, the solvent stabilization effect calculated for the neutral radical of DHC (− 4.5 kcal/mol) was higher than those for HF (− 1.4 kcal/mol) and DHF (− 2.2 kcal/mol), thus indicating that it comprises the most stable radical species formed after H-atom abstraction in protic solvents, such as ethanol and water.

Finally, the structure of the deprotonated flavonoid anion (FO^{-}) and its electron-donor ability in both the gas and ethanol phases were also analysed. As expected, for DHF, the anion is a highly stable species due to delocalization of the negative charge over the entire molecule and to strong hydrogen-bond interaction (H-bond distance of 1.79 Å in vacuum), being a poor electron-donor in gas phase. However, in protic polar solvents, the hydrogen bond relaxes slightly and the stabilization energy decreases significantly (− 11.48 kcal/mol). Although the phenoxyl radical $FO^{\bullet}$ has also been observed to be a stable species, in the present case the energy difference between the solvated and gas-phase radical is only − 1.54 kcal/mol. The *IP* is therefore significantly lower for the anion in the ethanol than in the gas phase. In turn, the phenoxyl anion of DHC (FO^{-}) in the gas phase showed that the dihedral angle defined by groups C = O and $C\alpha = C\beta$ changes, but the level of hydrogen interaction is maintained, as in the neutral molecule. This structural distortion makes this anion the best electron-donor candidate of the *Z. punctata* flavonoid series. In ethanol solution, however, the anion is stabilized by relaxing the

dihedral angles and allowing the six-member ring produced through hydrogen interaction to reach the most stable conformation (H bond distance of 1.49 Å). The solvent stabilization for the species is estimated to be ~ 26 kcal/mol. Consequently, a high *IP* is predicted for this FO^- in solution. Lastly, the delocalization of the negative charge of the phenoxyl anion of HF in the A-ring is extended to the carbonyl group in *para*-position. The solvent helps to stabilize the charge. However, the *IP* calculated for this anion indicates that this species is the least stable of the series.

Conclusions

The antioxidant properties and mechanisms of three structurally-related flavonoids of *Z. punctata*, specifically flavanone HF, chalcone DHC and flavonol DHF, depend on their intrinsic electron-donor capacity, acid-base properties and interactions with oxidant species, such as electrostatic effects. In the case of the quenching of $^1O_2$ in ethanol solutions, the quenching efficiency was controlled by the electron-donor properties of the flavonoids. With the lowest oxidation potential E_{ox}, the flavonol DHF was the most reactive species, yielding benzoic acid (BA) and 2,4-dihydroxybenzoic acid (DHBA) as final oxidation products. In contrast, both the chalcone DHC and the flavanone HF performed well as catalytic quenchers of $^1O_2$, practically maintaining their total quenching efficiency η_q^Δ after several consecutive reactions with $^1O_2$ due to their high physical quenching fraction, i.e. $k_q/k_t \approx 1$.

The scavenging of the bulky radicals $DPPH^\bullet$ and $ABTS^{\bullet+}$ by the *Z. punctata* flavonoids in ethanol solution was dominated by a sequential proton-loss electron-transfer (SPLET) mechanism, favoured by $\sim 10\%$ of the flavonoids being deprotonated. The radical scavenging reactivity trend was DHF > DHC >> HF, which was correlated with the electron-donor capacity of the flavonoids, i.e. E_{ox} and *IP*.

However, the scavenging of $O_2^{\bullet-}$ in aqueous buffered solution was significantly controlled by the fraction of neutral flavonoids FOH through concerted proton-coupled electron-transfer (PCET). In this case, the radical scavenging reactivity trend was DHC > DHF > HF.

The assignment of the different scavenging mechanisms was supported also by theoretical calculations, which agreed with most of the experimental findings. The calculated ionization potentials *IP* of the *Z. punctata* flavonoids in ethanol solution were well correlated with both the quenching rate constants of $^1O_2$ and the scavenger capacity of the nitrogen-centred radicals $DPPH^\bullet$ and $ABTS^{\bullet+}$.

In addition, the calculated *BDE* for H-atom abstraction of the flavonoids and the solvent stabilization energies of the neutral flavonoid radical $FO^\bullet$ formed in aqueous media were correlated with the scavenging capacity of $O_2^{\bullet-}$, hence supporting the assignment of the PCET mechanism for the scavenging of superoxide anion. In the case of the neutral radical of DHC, the calculations revealed that the key radical stabilization can be associated with the formation of a six-member ring structure induced by the hydrogen bonding of carbonyl oxygen and the C2′-OH group of the chalcone and its higher solvent stabilization effect. The above results emphasize the suitability of using standard computational calculations to elucidate antioxidant mechanisms.

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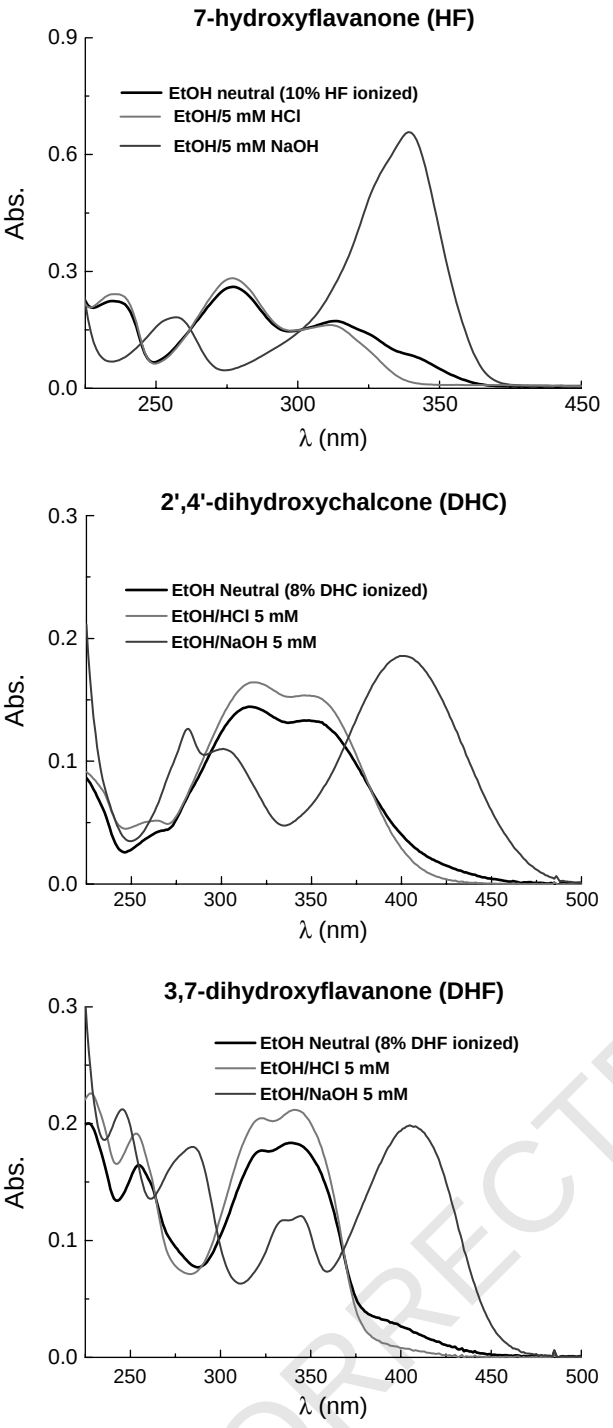
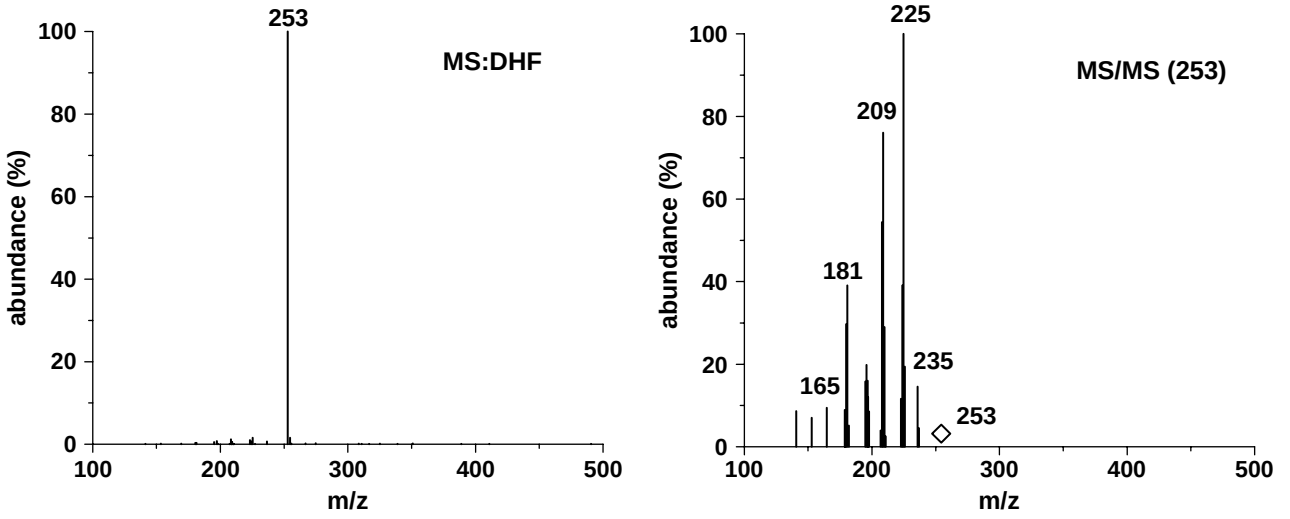
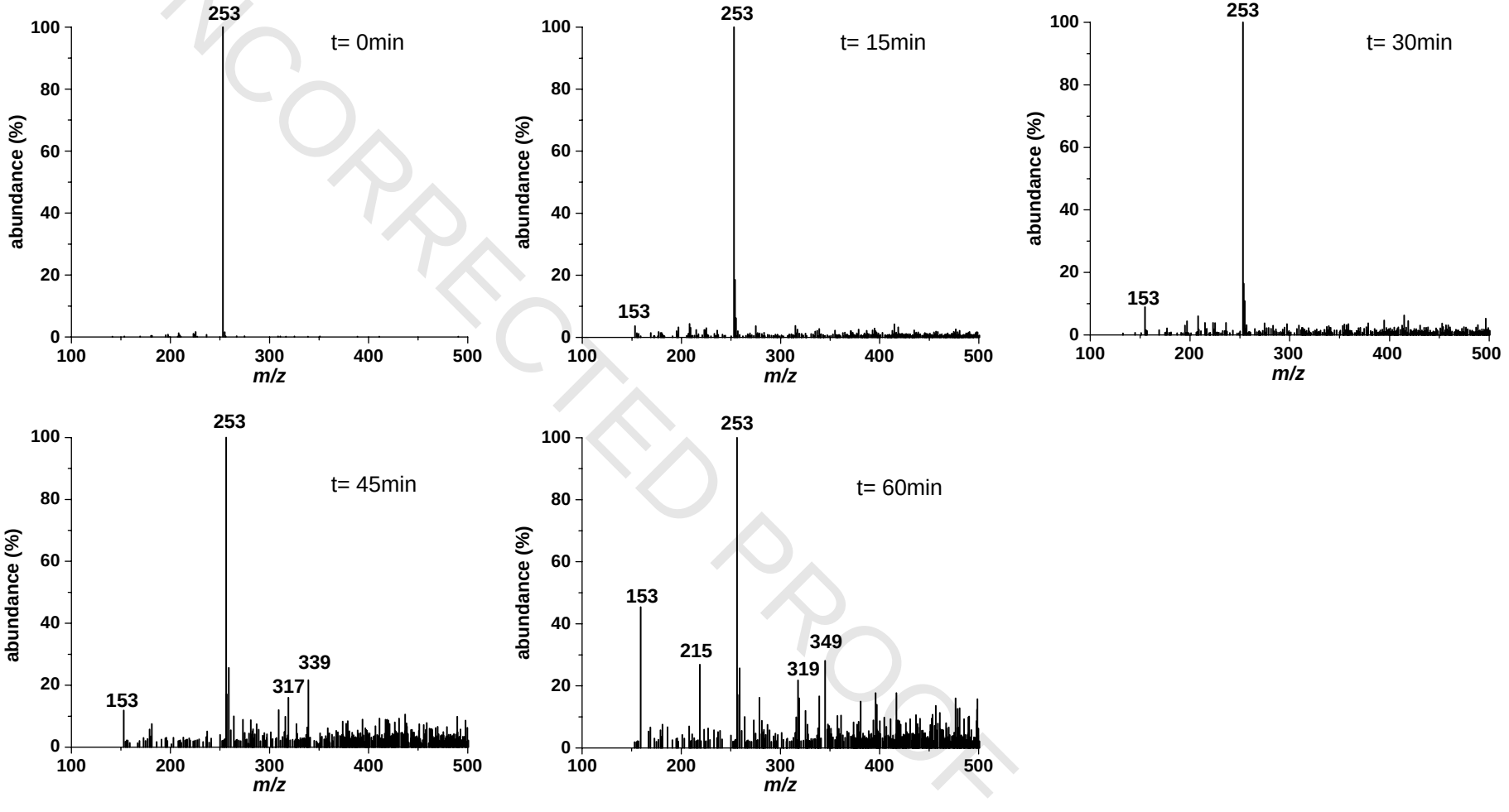


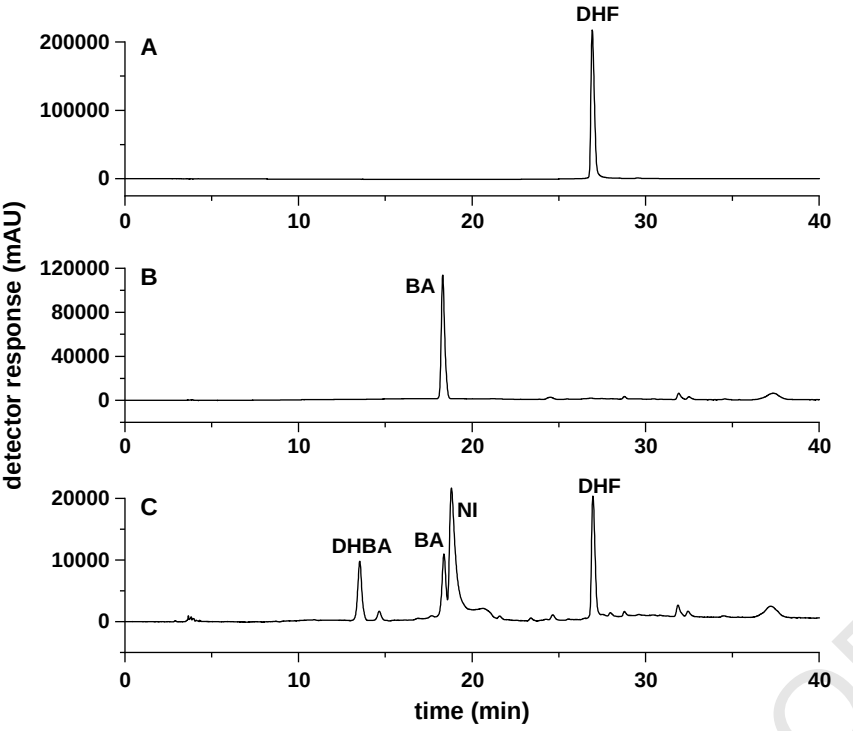
Supplementry Figure 1. UV-Vis absorption spectra of *Z. punctata* flavonoids in acid (5 mM HCl), neutral, alkaline (5 mM NaOH) ethanol solutions.



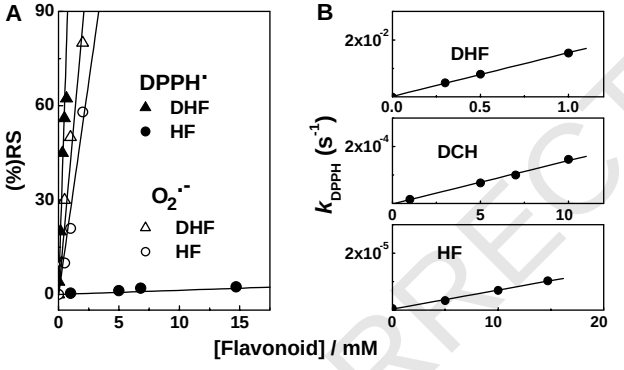
Supplementry Figure 2. MS and MS/MS spectra of DHF obtained by ESI in negative mode.



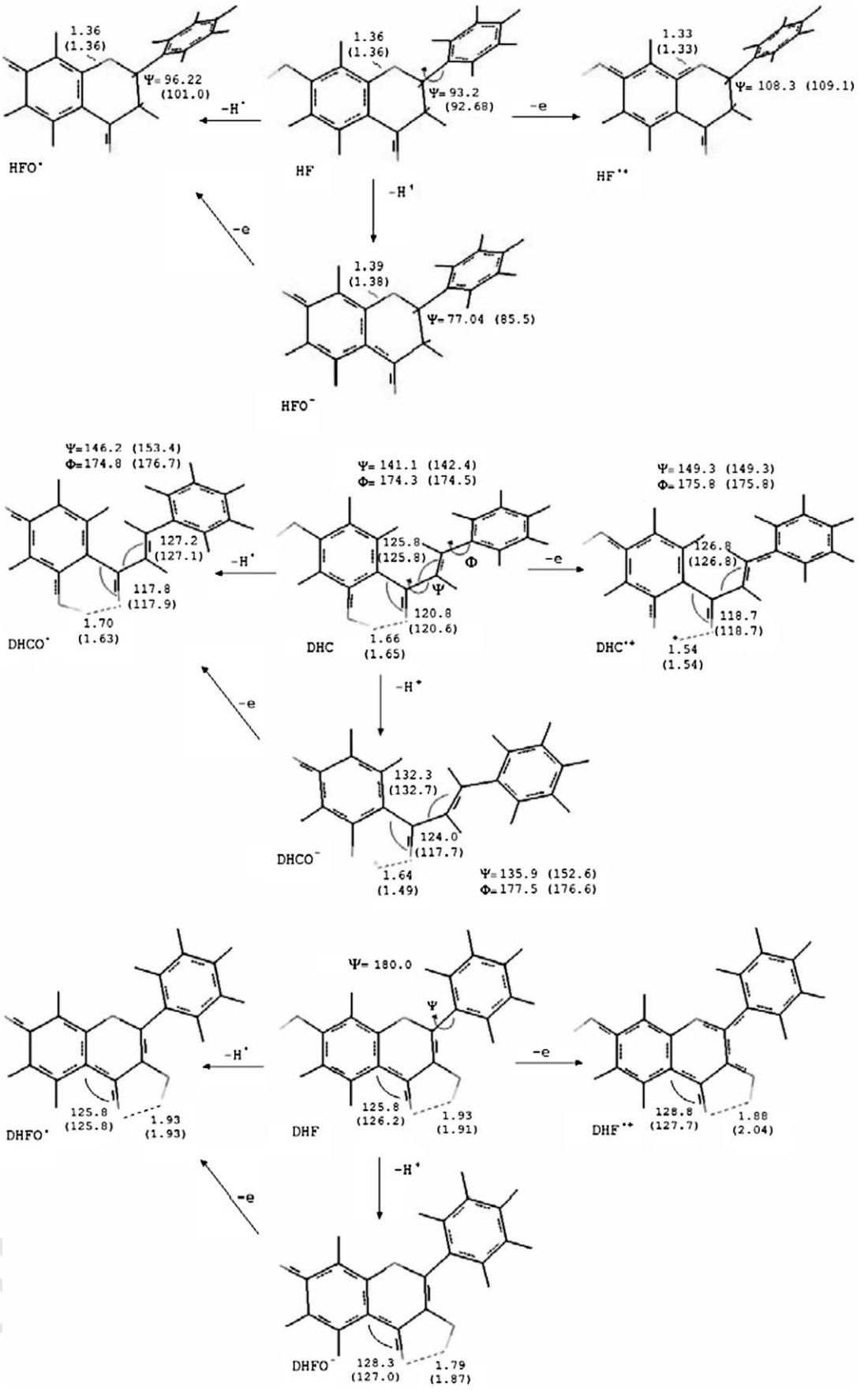
Supplementry Figure 3. MS spectra obtained by ESI in negative mode of the photosensitized solution of DHF at different times of illumination.



Supplementry Figure 4. Chromatograms, obtained by HPLC-PDA-MS/MS, processed at 280 nm.**A**) dihydroxyflavone standard (DHF), **B**) benzoic acid standard (BA), **C**) photosensitized solution: dihydroxy benzoic acid (DHBA), benzoic acid (BA), 3,7-dihydroxyflavone (DHF), not identified (NI) with $[M-H]^-$ at *m/z* 305 and MS/MS fragments at *m/z* 147, 175, 192, 239, 277.Chromatographic conditions: C_{18} Shim-pak CLC column, mobile phase: linear gradient of 5% aqueous formic acid/methanol, going from 85:15 to 20:80 (v/v) in 25 minutes, the isocratic proportion being maintained for a further 15 minutes, at a flow rate of 0.9 mL/min and column temperature set at 29°C.



Supplementry Figure 5. (**A**) Variation of the percent of radical scavenging (%)RS as a function of the flavonoid (HF or DHF) concentration for $DPPH^{\bullet}$ in ethanol solutions and for $O_2^{\bullet-}$ in aqueous PBS (pH 7.4). Solid lines represent linear fitting with zero intercept.(**B**) Dependence of the observed pseudo-first order rate constant for the scavenging of $DPPH^{\bullet}$, k_{DPPH}, with the flavonoid concentration in ethanol, eq. 7.



Supplementry Figure 6. Structural calculations of the *Z. Punctata* flavonoids and subspecies in gas-phase.