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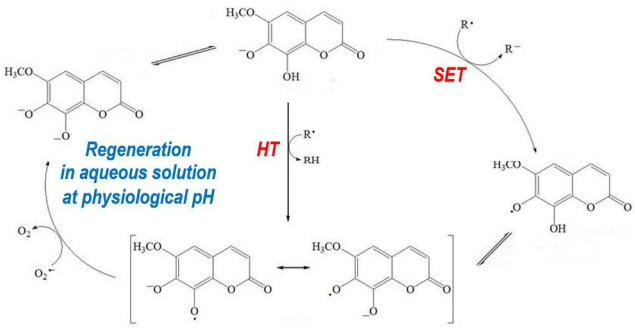

Fraxetin is an excellent and versatile antioxidant in aqueous media. In addition it regenerates, scavenging two radical equivalents per cycle

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ARTICLE

Antioxidant Activity of Fraxetin and its regeneration in Aqueous Media. A Density Functional Theory Study

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In this work, we have carried out a quantum chemistry and computational kinetics study on the reactivity of fraxetin towards two peroxyl free radicals (•OOH and •$OOCH_3$), in aqueous and lipid simulated biological environments. We have considered three reaction mechanisms: hydrogen transfer (HT), radical adduct formation (RAF), and single electron transfer (SET). Rate constants and relative branching ratios for the different paths contributing to the overall reaction, at 298.15 K, are reported. In aqueous media, fraxetin exists in two forms, depending on pH. Neutral fraxetin reacts mainly through the HT mechanism, while the deprotonated fraxetin reacts mainly through the SET mechanism. The overall reaction rate constants are 3.99 x 10^8 and 2.76 x 10^9 M^{-1} s^{-1} for reaction with •OOH and •$OOCH_3$ peroxyl radicals, respectively. In addition, we have shown that fraxetin is a versatile antioxidant in aqueous media, since it has a great scavenger activity towards other free radicals, under the same conditions. Furthermore, the possible regeneration of fraxetin after scavenging a first radical, was investigated in aqueous solution at physiological pH. It was found that regeneration is very likely to occur, which suggests that this compound has the ability to scavenge several radical equivalents (two per cycle), under such conditions. In lipid media, fraxetin reacts with the peroxyl radicals only through the HT mechanism, and the calculated reaction rate constants are 2.43 x 10^4 and 2.81 x 10^3 M^{-1} s^{-1} for •OOH and •$OOCH_3$ radicals, respectively.

Introduction

Antioxidants are molecules that decrease the concentration of reactive oxygen species (ROS). ROS are oxidant substances, such as hydroxyl (•OH) and peroxyl (•OOR) radicals, which are capable of reacting with DNA, proteins and fat acids.[1] High concentrations of ROS in biological media, also called oxidative stress, represent a health hazard that has been associated with diseases such as atherosclerosis, Parkinson, Alzheimer and cancer.[2]

Fraxetin (7,8-dihydroxy-6-methoxy coumarin) belongs to the coumarin family and it has been isolated from Aesculus hippocastanum, Actinidia deliciosa (kiwifruit) and the Fraxinus genus.[3] It has been reported to possess antioxidant, anti-inflammatory, antiviral, antitumor and neuroprotective effects.[4,5] Fraxetin has been the subject of several studies and its great antioxidant capacity has been demonstrated both in vitro and in vivo.[4-13]

In a recent work, Potapovich *et al*.[6] studied the antioxidant activity of fraxetin in the methemalbumin–H_2O_2–tetramethylbenzidine (TMB) pseudoperoxidase system, and their results showed that fraxetin inhibited the pseudoperoxidase oxidation of TMB in a noncompetitive manner. Thuong *et al*.[4] found that fraxetin has direct protective properties against low-density lipoprotein (LDL) oxidation at low concentrations, and that higher concentrations of fraxetin induce antioxidant enzymes activation; these effects suggest the potential anti-atherosclerosis effects of fraxetin. It has also been reported that fraxetin has a good radical-scavenging capacity for DPPH radicals in lipid peroxidation assays. Furthermore, it was proposed that fraxetin has selective scavenging activity for hydroxyl radicals and hydrogen peroxide, and it is equally effective as quercetin - one of the best known antioxidants - in scavenging hydrogen peroxide.[7]

It has been suggested that the presence of an *ortho* catechol moiety and the α-pyrone ring in the coumarin molecules plays an important role in the protective activities against radical-induced cytotoxicity. In contrast, the sugar moiety markedly reduces the activity of coumarin glycosides.[8] Martín-Aragón *et al*.[9] investigated the modifications in endogenous antioxidant capacity in the liver and brain supernatants of mice under fraxetin treatment; they concluded that this compound might provide an important resistance to, or protection against, free-radical-mediated events that contribute to degenerative diseases of aging. Fernández-Puntero *et al*.[10] studied the influence of the fraxetin treatment in a Drosophila melanogaster experimental model, by analyzing several parameters in normal situations and also in instances of induced oxidative stress. They showed that fraxetin prevents oxidative stress by increasing the antioxidant

reserves of glutathione (GSH), thus preventing peroxidative damage. In a study where human neuroblastoma cells were used, intracellular GSH appeared to be an important factor in fraxetin-mediated cytoprotection against rotenone-toxicity. The authors also observed that fraxetin inhibited the formation of ROS. Their conclusion was that the anti-oxidative and anti-apoptotic properties of fraxetin are responsible for its capacity to protect against rotenone-induced cytotoxicity.[11] In a later study, it was suggested that major features of rotenone-induced neurotoxicity are partially mediated by free radical formation and oxidative stress, and that fraxetin partially protects against rotenone toxicity affecting the main protection system of the cells against oxidative injury.[12]

Benedí *et al.*[13] investigated the influence of treatment with fraxetin on the lifespan and longevity of Drosophila melanogaster, introducing the treatment at different ages and different stress conditions. They found that fraxetin administration prolonged the lifespan and longevity in female flies, especially with a 2-week treatment, both in normal conditions and in a stress situation.

In order to elucidate the overall antioxidant capacity of fraxetin, in this work we have carried out a quantum chemistry and computational kinetics study of its reactivity towards two peroxyl free radicals (•OOH and •$OOCH_3$), both in water and lipid simulated biological environments. We have considered three reaction mechanisms: i) hydrogen transfer (HT), ii) radical adduct formation (RAF), and iii) single electron transfer (SET). Rate constants and relative branching ratios for the different channels contributing to the overall reaction, at 298.15 K, are reported. In addition, we have extended the study of the SET mechanism to a large set of free radicals of biological relevance with different electrophilic character.

COMPUTATIONAL DETAILS

All electronic calculations were performed with the Gaussian 09 package of programs.[14] Geometry optimizations and frequency calculations were carried out using the M05-2X[15] and the 6-311++G(d,p) basis set, in conjunction with the SMD continuum model[16] using pentylethanoate and water as solvents to mimic lipid and aqueous environments, respectively. The M05-2X functional has been recommended for kinetic calculations by its developers,[15] and it has been successfully used by independent authors for that purpose.[17] It is also among the best performing functional for calculating reaction energies involving free radicals.[18] It was found among best performing functionals in a benchmark for kinetic studies of radical-molecule reactions in solution.[19] SMD is considered a universal solvation model, due to its applicability to any charged or uncharged solute in any solvent or liquid medium for which a few key descriptors are known.[16]

Liquid phase effects on entropy loss have been included according to the corrections proposed by Okuno,[20] taking into account the free volume theory.[21] These corrections are in good agreement with those independently obtained by Ardura et al.[22] and have been successfully used by other authors.[23] In this work, the expression used to correct the Gibbs free energy is:

$$\Delta G_{sol}^{FV} \cong \Delta G_{sol}^{0} - RT\{ln[n10^{2n-2}] - (n-1)\} \qquad (1)$$

where n represents the molecularity of the reaction. According to expression (1), the entropy loss effects in solution cause ΔG to decrease by 2.54 kcal/mol for bimolecular reactions, at 298.15 K. This correction is important because the packing effects of the solvent reduce the entropy loss associated with any chemical reaction whose molecularity is equal or larger than two when compared with gas phase results.

Unrestricted calculations were used for open shell systems. Local minima and transition states were identified by the number of imaginary frequencies: local minima have only real frequencies, while transition states are identified by the presence of a single imaginary frequency that corresponds to the expected motion along the reaction coordinate. Relative energies are calculated with respect to the sum of the isolated reactants. Thermodynamic corrections at 298.15 K were included in the calculation of relative energies, which correspond to 1M standard state. In addition the solvent cage effects have been included according to the corrections proposed by Okuno,[24] taking into account the free volume theory.[25]

The rate constants (k) were calculated using the Conventional Transition State Theory (TST):[26,27,28]

$$k = \sigma\kappa\frac{k_B T}{h}e^{-\Delta G^{\neq}/RT} \qquad (2)$$

where k_B and h are the Boltzmann and Planck constants; $\Delta G^{\neq}$ is the Gibbs free energy of activation; σ represents the reaction path degeneracy, accounting for the number of equivalent reaction paths; and κ accounts for tunneling corrections. The latter are defined as the Boltzmann average of the ratio defined as the Boltzmann average of the ratio of the quantum and the classical probabilities, were calculated using the Zero Curvature Tunneling corrections (ZCT).[29]

For the electron transfer reactions the barriers were estimated using the Marcus theory.[30,31] The original classical Marcus theory for outer sphere electron transfer reactions demonstrates the importance of the solvent and leads the way to the calculation of the Gibbs free energy of activation, using the polarization properties of the solvent, the size of the reactants, the transfer distance and the Gibbs free energy of the redox reaction. It also relies on the transition state formalism, defining the SET activation barrier ($\Delta G^{\neq}_{SET}$) in terms of two thermodynamic parameters, the free energy of reaction (ΔG^{0}_{SET}) and the nuclear reorganization energy (λ)

$$\Delta G_{SET}^{\neq} = \frac{\lambda}{4}\left(1 + \frac{\Delta G_{SET}^{0}}{\lambda}\right)^2 \qquad (3)$$

The reorganization energy (λ) has been calculated as:

$$\lambda = \Delta E_{SET} - \Delta G_{SET}^{0} \qquad (4)$$

where ΔE_{SET} is the non-adiabatic energy difference between reactants and vertical products. This approach is similar to the one previously used by Nelsen and co-workers[32] for a large set of self-exchange reactions.

Some of the calculated rate constant (k) values are close to, or within, the diffusion-limit regime. Accordingly, the apparent rate constant (k_{app}) cannot be directly obtained from TST calculations. In the present work the Collins-Kimball theory[33] is used to that purpose:

$$k_{app} = \frac{k_D k}{k_D + k} \qquad (5)$$

where k is the thermal rate constant, obtained from TST calculations, and k_D is the steady-state Smoluchowski[34] rate constant for an irreversible bimolecular diffusion-controlled reaction:

$$k_D = 4\pi R D_{AB} N_A \quad (6)$$

where R denotes the reaction distance, N_A is the Avogadro number, and D_{AB} is the mutual diffusion coefficient of the reactants A (free radical) and B (fraxetin). D_{AB} has been calculated from D_A and D_B according to reference,[35] and D_A and D_B have been estimated from the Stokes–Einstein approach:[36]

$$D = \frac{k_B T}{6\pi\eta a} \quad (7)$$

where k_B is the Boltzmann constant, T is the temperature, η denotes the viscosity of the solvent, in our case water (η = 8.91 x 10-4 Pa s) and pentylethanoate (η = 8.62 x 10-4 Pa s); and a is the radius of the solute.

For the kinetic study we have not included the endergonic reaction paths, since even if they take place at significant rates, they would be reversible and, therefore, the formed products will not be observed. However, it should be noted that they might still represent significant channels if their products rapidly react further. This would be particularly important if these further stages are sufficiently exergonic to provide a driving force, and if their barriers of reactions are low. This could be the case for the SET reactions in aqueous solution since they yield reactive species, and take place at relative large reaction distances. In addition, slightly endergonic processes can be important when there are no exergonic competing paths, but such a case was not found in the present study.

The methodology used in this work has been previously proven to accurately reproduce experimental rate constants in solution.[37]

RESULTS AND DISCUSSION

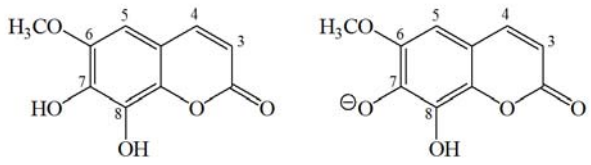
In general, in aqueous solution at physiological pH, phenolic compounds exist both as neutral and anionic species, since water is a polar protic solvent with great ionizing ability. On the contrary, in a lipid environment, phenolic compounds exist almost exclusively as neutral species, due to the low ionizing ability of the non polar environment. Thus, in water, we have considered both the neutral and the anionic species of fraxetin, while in lipid media, only the neutral form was considered.

In the first stage of this work, we studied the deprotonation of fraxetin, in order to determine the structure of the anionic form of fraxetin. In a second step, we verified the thermochemical feasibility of the considered mechanisms and their corresponding reaction channels in the reaction between fraxetin and the $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ free radicals, both in water and lipid environments. Finally, we calculated the individual rate constants and branching ratios for the relevant reaction channels, and the overall rate constants.

a. Acid-base equilibrium

We have not found in the literature experimental data on the pK_a of fraxetin. In order to identify which phenolic OH group is involved in the first deprotonation process, both the 7-OH and 8-OH sites were investigated. The Gibbs free energy of the deprotonation from site 7-OH is 2.5 kcal/mol lower than that from site 8-OH. Therefore, the anion obtained by removing a proton from 7-OH is the one used in this work for the reactions in aqueous solution, albeit the neutral form is also considered.

The chemical structures of the neutral (H_2FR) and deprotonated (HFR^-) forms of fraxetin and the sites numbering scheme are shown in Scheme 1.



Scheme 1. Neutral (H_2FR) and deprotonated (HFR^-) chemical structures of fraxetin, and numbering scheme for reaction sites.

We have calculated the pK_a of fraxetin (H_2FR) using the reaction scheme of proton transfer, according to:[38]

$$H_2FR + Ref^- \rightarrow HFR^- + HRef \quad (8)$$

where

$$pKa(H_2FR) = \Delta G/RTln(10) + pKa(HRef) \quad (9)$$

In this study, we used the esculetin molecule as a reference (pK_a=5.62).[39] According to this methodology, the calculated pK_a for fraxetin is 6.02. The molar fractions used in this manuscript are calculated using this pK_a value. Our results show that, in aqueous solution at pH = 7.4, the deprotonated form of fraxetin would predominate (96%) over the neutral form (4%).

Since blood can be modeled essentially as an aqueous solution at pH = 7.4, full hydration and prevalence of the deprotonated form is expected in biological environments. In a non-polar environment, fraxetin exists almost exclusively in the neutral form. In this work, both neutral (H_2FR) and deprotonated (HFR^-) forms will be used to study the reactivity of fraxetin towards the considered peroxyl free radicals in water. In non-polar (lipid) media only the neutral form is used, since such media do not promote the necessary solvation to stabilize the ionic species.

b. Reaction mechanisms. Thermochemical feasibility

Three mechanisms were considered: single electron transfer (SET), hydrogen atom transfer (HT) and radical adduct formation (RAF), as shown below.

a) Single electron transfer (SET):

$$H_2FR + {}^{\bullet}R \rightarrow H_2FR^{\bullet+} + R^- \quad (10)$$

$$HFR^- + {}^{\bullet}R \rightarrow HFR^{\bullet} + R^- \quad (11)$$

b) Hydrogen transfer (HT):

$$H_2FR + {}^{\bullet}R \rightarrow HFR^{\bullet} + HR \quad (12)$$

$$HFR^- + {}^{\bullet}R \rightarrow FR^{\bullet-} + HR \quad (13)$$

c) Radical adduct formation (RAF):

$$H_2FR + {}^{\bullet}R \rightarrow [H_2FR\text{-}R]^{\bullet} \quad (14)$$

$$HFR^{-} + {}^{\bullet}R \rightarrow [HFR\text{-}R]^{\bullet -} \quad (15)$$

In the HT mechanism, we have considered the hydrogen atom abstraction from the hydroxyl groups at the 7-OH and 8-OH positions, and the abstraction of the hydrogen atoms from the methoxy group that is bound to carbon 6. In the RAF mechanism, we have proposed the formation of the radical adducts at all carbon atoms having sp^2 hybridization, i.e. on the positions 2-10. All these channels could occur in parallel, but at different reaction rates. One of the objectives of the present paper is to determine which mechanism has the fastest rate constant in the reaction of fraxetin with the $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ free radicals, in water and lipid media.

The thermochemical feasibility of the different mechanisms and channels of reaction was investigated first, in water and lipid environments, since it determines the viability of chemical processes. Relative reaction Gibbs free energy values (ΔG) for the HT, RAF and SET mechanisms, calculated in water and pentylethanoate at 298.15 K, are reported in Table 1.

The thermodynamic analysis of the HT reaction channels shows that the reaction at the 6-OCH_3 position, involving the abstraction of an H atom from the methyl of the methoxy group is always endergonic. The Δ*G* values of the HT reaction paths involving H_2FR are slightly less negative in aqueous solution than in lipid environment. On the other hand, in water, the HT from the non-deprotonated site in HFR^{-} (8-OH) is more exergonic,by more than 9 kcal/mol, than the corresponding channel in H_2FR. This indicates that the thermochemical viability of the HT mechanism increases with pH as the anionic form of fraxetin prevails.

In the case of H_2FR, the channel 8-OH is exergonic by -3.28 and -1.62 kcal/mol, for the •OOH and •$OOCH_3$ respectively, while the channel 7-OH is exergonic by -6.42 and 4.76 kcal/mol for the •OOH and •$OOCH_3$ respectively. In the case of HFR^{-}, the channel 8-OH is exergonic by -12.83 and -11.18 kcal/mol to the •OOH and •$OOCH_3$ radicals, respectively, most exergonic that the HT mechanism for the H_2FR previously observed. In a lipid environment, thermodynamic results indicate that channel 6-OCH_3 is endergonic, and the 7-OH channel is exergonic, and the channel 8-OH is slightly endergonic, regardless both peroxyl radicals. These results show that channels 7-OH and 8-OH could be important in the antioxidant activity of H_2FR. The reason for the higher exergonicity of the 7-OH site can be explained by the activating effect of methoxyl and hydroxyl groups in both *orto* positions as well as by the H-Bond formed with the H of the hydroxyl group. On the other hand, in the deprotonated fraxetin, the 8-OH position becomes even more activated by the neighbour phenoxide oxygen atom.

In water, all the RAF channels are endergonic for both radicals. In lipid media, similarly to what was found in water media, all the RAF reaction paths are significantly endergonic for both peroxyl radicals (Table 1). This means that independently of the environment's polarity, the RAF mechanism is not expected to contribute to the peroxyl radical scavenging activity of fraxetin.

For the deprotonated form of fraxetin, we have considered the SET mechanism for both radicals, although the calculated values of the relative reaction Gibbs free energies in the reaction with the $^{\bullet}OOH$ radical is positive. As the transfer of an electron is facilitated, this mechanism could be relevant for the fraxetine antioxidant activity. The SET mechanism of the reaction between H_2FR and the two peroxyl radicals is highly endergonic in both cases; while in the case of HFR^{-} with the $^{\bullet}OOH$ radical this mechanism is slightly endergonic and it is exergonic with eth $^{\bullet}OOCH_3$ radical.

Table 1. Gibbs free energies of reaction (ΔG, kcal/mol) for the HT, RAF and SET channels in the reaction of neutral and deprotonated fraxetin with $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals, in aqueous and pentylethanoate solution at 298.15 K.

Channel	H_2FR				HFR^{-}	
	H_2O		$AcOC_5H_{11}$		H_2O	
	ΔG ($^{\bullet}OOH$)	ΔG ($^{\bullet}OOCH_3$)	ΔG ($^{\bullet}OOH$)	ΔG ($^{\bullet}OOCH_3$)	ΔG ($^{\bullet}OOH$)	ΔG ($^{\bullet}OOCH_3$)
HT						
6-OCH_3	12.16	13.82	14.30	16.24	11.84	13.50
7-OH	-6.42	-4.76	-4.71	-2.77	-	-
8-OH	-3.28	-1.62	0.05	1.99	-12.83	-11.18
RAF						
C2	24.12	10.15	8.69	16.66	9.85	13.00
C3	13.39	15.05	12.78	15.58	13.73	15.71
C4	14.97	17.79	16.59	18.64	12.72	15.74
C5	15.83	18.03	18.14	20.85	17.24	19.47
C6	19.06	20.72	19.82	21.99	14.19	17.61
C7	11.24	13.09	11.73	14.67	13.12	17.89
C8	17.30	18.82	18.38	21.71	13.52	15.07
C9	14.28	15.58	15.94	18.44	18.08	19.24
C10	26.93	28.75	27.98	30.49	23.69	25.55
SET	26.24	23.92	75.32	76.79	0.98	-1.34

In non-polar environments the SET mechanism is not expected to contribute to the overall reactivity of fraxetin towards free radicals since, as mentioned before, such environments do not promote the necessary solvation of the intermediate ionic species yielded by this mechanism. However, just to prove this point, the Gibbs energy of reaction (ΔG) for the SET processes was calculated. It was found to be higher than 75 kcal/mol for both

peroxyl radicals (Table 1). Therefore, the viability of this mechanism in lipid media has been definitively ruled out.

As mentioned before, for the kinetic study we have not included the endergonic reaction paths because, even if they took place at significant rates, they would be reversible and, therefore, the formed products would not be observed. According to these considerations and taking into account the thermochemical feasibility of the reaction channels, it can be stated that the antioxidant activity of fraxetin, with respect to the studied peroxyl radicals in water, depends only on the HT and SET reaction mechanisms, while in a lipid environment only the HT mechanism is expected to occur.

Antioxidant activity of Fraxetin in aqueous media

The antioxidant activity of fraxetin with the studied peroxyl radicals in water depends only on the HT and SET reaction mechanisms, Therefore, we have carried out the kinetic study only for the HT 7-OH and 8-OH channels, and the SET mechanism, in the reaction of fraxetin with the $^{\bullet}$OOH and $^{\bullet}$OOCH$_3$ peroxyl radicals.

The fully optimized geometries of the HT transition states for the reactions of H_2FR and HFR^- with $^{\bullet}$OOH and $^{\bullet}$OOCH$_3$ peroxyl radicals are shown in Figures 1 and 2.

Gibbs free energy of activation ($\Delta G^{\neq}$), rate constants (k), and relative branching ratios (Γ) for the HT channels in the reaction of neutral and deprotonated fraxetin with the $^{\bullet}$OOH and $^{\bullet}$OOCH$_3$ peroxyl radicals, in water environment at 298.15 K, are reported in Table 2.

For the HT mechanism, it can be observed (Table 2) that neutral fraxetin reacts with $^{\bullet}$OOH radicals mostly through the 7-OH channel (~71%), while the 8-OH channel is the minor channel (~29%). In the case of the $^{\bullet}$OOCH$_3$ radical, the 7-OH channel represents more than ~99%, while the 8-OH channel is less than 1%. The calculated individual rate constant for the 7-OH channel is larger than the one obtained for the 8-OH channel, for both radicals. Deprotonated fraxetin only reacts through the 8-OH available channel, and its calculated rate coefficients towards both peroxyl radicals are higher than the ones corresponding to the neutral fraxetin HT channels.

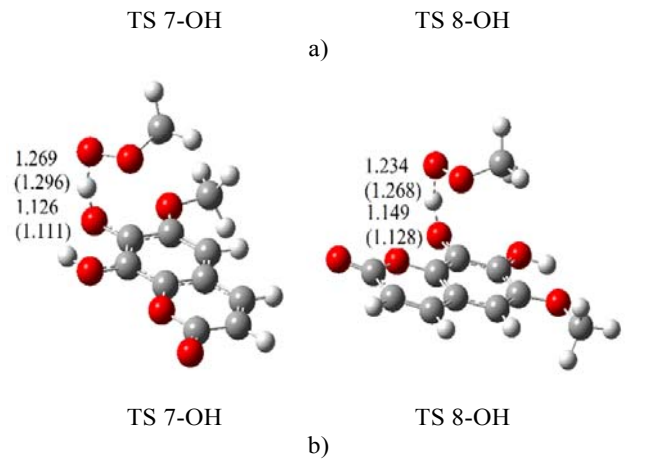


Figure 1. Optimized geometries of the transition structures for the reaction between H_2FR and the $^{\bullet}$OOH (a) and $^{\bullet}$OOCH$_3$ (b) radicals in lipid (water) media. Distances are given in Å.

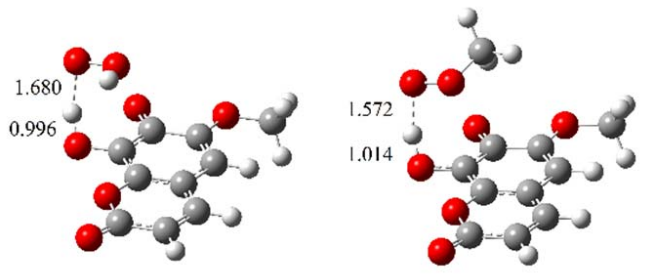


Figure 2. Optimized geometries of the HT 8-OH transition structures in the reaction between HFR^- and the $^{\bullet}$OOH and $^{\bullet}$OOCH$_3$ radicals, in water media. Distances are given in Å.

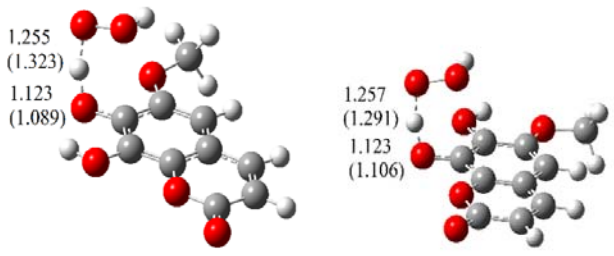


Table 2. Gibbs free energy of activation ($\Delta G\neq$, in kcal/mol), rate constants (k, in M^{-1} s^{-1}), and relative branching ratios (Γ) for the HT channels in the reaction of H_2FR and HFR^- with the $^{\bullet}$OOH and $^{\bullet}$OOCH$_3$, in water environment at 298.15 K.

Channel	H_2FR				HFR^-			
	$\Delta G^{\neq}$	tunnel	k	Γ	$\Delta G^{\neq}$	tunnel	k	Γ
				$^{\bullet}$OOH				
HT 7-OH	14.50	104.44	1.54×10^{4}	71.1	--	--	--	
HT 8-OH	15.32	170.10	6.26×10^{3}	28.9	9.60	171.52	9.81×10^{7}	100.0

							$^{\bullet}OOCH_3$		
HT 7-OH	18.91	59.28	$9.12x10^{3}$	99.2		--	--	--	
HT 8-OH	22.24	135.49	$7.55x10^{1}$	0.8		9.18	288.07	$3.35x10^{8}$	100.0
$X^{a}=3$	-0.61	25.24	25.84	6.16				$1.89x10^{8}$	1.75

[a]X is the number of water molecules to solvate the radical and anion OOH.

We have carried out the study of the SET mechanism in the reaction between neutral and deprotonated fraxetin with $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals, in aqueous media at physiological pH. The reaction products are the radical cation or a neutral radical, depending if the parent molecule is the neutral or the deprotonated form of fraxetin and the anionic species from the free radicals. In the case of the ^{-}OOH anion, in order to calculate ΔG and $\Delta G^{\neq}$, we used the experimental solvation energy, that is -97.70 kcal/mol.[40] In the case of the $^{-}OOCH_3$ anion, there are no reported experimental data on its solvation energy. In order to obtain rate constants values closer to the real ones, we have carried out a solvation model of the ^{-}OOH anion using a mix-model including the SMD model of solvation and explicit water molecules. We have used one, two and three water molecules, in order to determine the number of water molecules that stabilize the ^{-}OOH anion. This methodology can be extrapolated to the $^{\bullet}OOCH_3$ radical, in order to obtain ΔG and $\Delta G^{\neq}$ values close to the real ones. In Table 3, we show the results of the thermodynamic and kinetic studies of the reaction between H_2FR and HFR^{-} with the $^{\bullet}OOH$ radical using the experimental correction to solvation energy and using the mix-model, considering XH_2O to solvate the radical and anion OOH, where X = 0, 1, 2, 3. The geometries of the formed complexes are showing in figure A1 (of the ESI).

In the SET reaction between neutral fraxetin and the $^{\bullet}OOH$ radical, ΔG = 26.25 kcal/mol when using the experimental correction to solvation energy, The lower difference in ΔG in the SET reaction was obtained when using two water molecules. The $\Delta G^{\neq}$ obtained when using the experimental correction to the solvation energy was 26.32 kcal/mol. The lower difference in $\Delta G^{\neq}$ values was observed when using two water molecules. To comparing the rate constant obtained when using the correction to solvation energy and when this reaction was modelled with explicit water molecules, the ratio of the rate constants was calculated, k_{exp}/k_{calc}; this ratio is lower when using two water molecules. If not used the explicit water molecules, the calculated rate constants will be a million times smaller than the rate constants corrected with the experimental solvation energy.

Table 3. Thermodynamic and kinetic values of the SET mechanism for the reaction between the H_2FR and HFR^{-} with the $^{\bullet}OOH$ radical.

	ΔG	ΔE	λ	$\Delta G^{\neq}$	k	k_{exp}/k_{calc}
			H_2FR			
exp	26.25	49.86	23.61	26.32	$3.15x10^{-7}$	
$X^{a}=0$	32.36	49.86	17.50	35.52	$5.70x10^{-14}$	$5.53x10^{6}$
$X^{a}=1$	30.20	56.17	25.97	30.37	$3.41x10^{-10}$	$9.24x10^{2}$
$X^{a}=2$	25.81	53.66	27.85	25.85	$6.99x10^{-7}$	0.45
$X^{a}=3$	24.66	52.80	28.14	24.77	$4.36x10^{-6}$	0.07
			HFR^{-}			
exp	0.98	22.29	21.31	5.83	$3.31x10^{8}$	
$X^{a}=0$	7.10	22.29	15.20	8.17	$6.32x10^{6}$	52.37
$X^{a}=1$	4.93	28.60	23.67	8.64	$2.88x10^{6}$	114.84
$X^{a}=2$	0.55	26.09	25.55	6.66	$8.11x10^{7}$	4.08

According to the results above, in the study of the SET reaction mechanism of the neutral and deprotonated fraxetin with the $^{\bullet}OOH$ radical, the model that best describes the reaction is the SMD model of solvation plus two explicit water molecules. Therefore, the study of SET reaction mechanism between H_2FR and HFR^{-} with the $^{\bullet}OOCH_3$ radical was carried following the same methodology. Reorganization energy (λ), Gibbs free energy of activation ($\Delta G^{\neq}$), apparent rate constant (k_{app}) and total rate coefficients (k_{total}) for the SET reactions of neutral and deprotonated fraxetin with the studied peroxyl radicals, in aqueous solution at physiological pH, are reported in Table 4.

The Gibbs free energy of activation are lower for the HFR^{-} than for the H_2FR, and therefore, the reaction rate constants are expected to be higher for HFR^{-}. The reaction rate constants for the HFR^{-} towards $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ peroxyl radicals were 4.29 x 10^{8} and 2.87 x 10^{9} M^{-1} s^{-1}, respectively.

Since both neutral (H_2FR) and deprotonated (HFR^{-}) forms of fraxetin are presents in aqueous media at physiological pH, the total and overall rate constants can be calculated according to the equations:

$$k_{overall} = p^{H_2FR} k_{tot}^{H_2FR} + p^{HFR^-} k_{tot}^{HFR^-} \quad (16)$$

$$k_{tot}^{H_2FR} = k_{HT(7)}^{H_2FR} + k_{HT(8)}^{H_2FR} \quad (17)$$

$$k_{tot}^{HFR^-} = k_{HT(8)}^{HFR^-} + k_{SET}^{HFR^-} \quad (18)$$

where p^{H_2FR} and p^{HFR^-} are de molar fractions of the neutral and deprotonated species of fraxetin in water at physiological pH. The overall k values corresponds to the rate constants that would be observed at physiological pH, i.e. taking into account the molar fractions of neutral and deprotonated species of fraxetin at pH=7.4.

The overall rate constants obtained for the reactions between H_2FR and HFR^{-} and the $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals, in water at physiological pH, are 4.12 x 10^{8} and 2.76 x 10^{9} M^{-1} s^{-1}, respectively.

According to the results below, the mixture between neutral and deprotonated form of fraxetine in water at physiological pH shows a very good scavenging activity towards $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals. However, the deprotonated form represents the largest contributor to the antioxidant activity of fraxetine in water at physiological pH. Relative branching ratios (Γ) of the different channels in the reaction of neutral and deprotonated fraxetin with $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals, in aqueous solution, at 298.15 K, are reported in Table 5. The HT was found to be the most important mechanism regarding the scavenging activity of neutral fraxetine towards both radicals, while the SET mechanism is the most important in the antioxidant activity of the deprotonated form.

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Table 4. Reorganization energy (λ), Gibbs free energy of activation ($\Delta G^{\neq}$, kcal/mol), apparent rate constants (k_{app}, M^{-1} s^{-1}) and total rate coefficients (k_{total}, M^{-1} s^{-1}) for the SET reactions of neutral and deprotonated fraxetin with $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals, in aqueous solution, at physiological pH.

Radical	H_2FR			HFR^-		
	λ	$\Delta G^{\neq}$	k_{app}	λ	$\Delta G^{\neq}$	k_{app}
$^{\bullet}OOH$	23.61	26.32	$3.15x10^{-7}$	21.31	5.83	$3.31x10^{8}$
$^{\bullet}OOCH_3$	22.41	23.95	$1.73x10^{-5}$	20.11	4.38	$2.54x10^{9}$

Table 5. Branching ratios (Γ) of the different channels in the reaction of neutral and deprotonated fraxetin with $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals, in aqueous solution, at 298.15 K.

Mechanism	H_2FR		HFR^-	
	Γ ($^{\bullet}OOH$)	Γ ($^{\bullet}OOCH_3$)	Γ ($^{\bullet}OOH$)	Γ ($^{\bullet}OOCH_3$)
SET	~ 0.00	~ 0.00	~ 76.39	~ 88.33
HT 7-OH	~ 71.07	~ 99.18	-	-
HT 8-OH	~ 28.93	~ 0.82	~ 23.61	~ 11.67

The neutral fraxetin reacts with both peroxyl radicals almost only through the HT mechanism, while the deprotonated form of fraxetin reacts mainly through the SET mechanism. In the case of the neutral fraxetin, the HT 7-OH channel is predominant over 8-OH, with relative branching ratios of 71.07 and 99.18% regardless the $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals. Regarding the deprotonated form of fraxetin, the SET mechanism is the most important; the preferences for this mechanism are of 76.39 and 88.33% for the $^{\bullet}OOH$ and $^{\bullet}OOCH_3$. The observed difference in reactivity between the neutral and deprotonated fraxetin can be explained, since the ionic form of fraxetin could donate an electron through the SET mechanism more easily compared to the neutral form of fraxetin.

To put these values in perspective, we have compared the $^{\bullet}OOH$ scavenging activity of fraxetin in aqueous solution, at physiological pH, with those of other antioxidants. Under such conditions, based on kinetic considerations, the protective effects of fraxetin against oxidative stress is predicted to be much higher than that of protocatechuic acid (1.3×10^7 M^{-1} s^{-1}),[51] α-mangostin (1.4×10^6 M^{-1} s^{-1}),[53] melatonin (2.0×10^1 M^{-1} s^{-1}),[56] caffeine (3.3×10^1 M^{-1} s^{-1}),[57] allicin (7.4×10^3 M^{-1} s^{-1}),[41] dopamine (2.2×10^5 M^{-1} s^{-1}),[42] hydroxytyrosol (7.49×10^5 M^{-1} s^{-1}),[54] canolol (2.50×10^6 M^{-1} s^{-1}),[43] glutathione (2.7×10^7 M^{-1} s^{-1}),[44] esculetin (1.69×10^7 M^{-1} s^{-1}),[39] sesamol (2.4×10^8 M^{-1} s^{-1}),[45] and lower than that of propyl gallate (4.56×10^8 M^{-1} s^{-1}).[46]

Since in aqueous solution at physiological pH, SET is a very important mechanism of radical scavenging processes, this mechanism could be also important in the reaction of fraxetin with other free radicals of biological relevance. Thus, we have extended the study of this mechanism to a large set of free radicals with different electrophilic character. We have included the hydroxyl radical ($^{\bullet}OH$) because it is the most electrophilic and reactive among all the oxygen-centered radicals, with a very short half-life of ~10^9 s.[47] We have also included a large series of peroxyl radicals, which are relatively low reactive species capable of diffusing to remote cellular location,[48] with half-lives of seconds.[49] Among the chosen peroxyl radicals, the $^{\bullet}OOCCl_3$ is the most electrophilic one, and therefore, is expected to present a high reactivity. An equivalent set of alkoxyl radicals has been also included, since their reactivity is expected to lie between those of $^{\bullet}OH$ radical and the peroxyl radicals. Some nitrogen centered radicals have been also included, since they are important species in biological processes. Reorganization energies (λ), Gibbs free energies of activation ($\Delta G^{\neq}$), diffusion controlled rate constants (k_D) and apparent reaction rate constants (k_{app}) in the SET reactions of neutral and deprotonated fraxetin with all the considered free radicals are reported in Table 6.

The results in Table 6 show that the neutral fraxetin reacts with the $^{\bullet}OH$, $^{\bullet}OCH_2Cl$, $^{\bullet}OCHCl_2$ and $^{\bullet}OCCl_3$ radicals at diffusion controlled rate constants, whereas the reactions involving the $^{\bullet}N_3$, $^{\bullet}NO_2$, $^{\bullet}OCH_3$, $^{\bullet}OOCHCl_2$ and $^{\bullet}OOCCl_3$ radicals are much slower. The radicals $^{\bullet}OOH$, $^{\bullet}OOCH_3$ and $^{\bullet}OOCH_2Cl$ do not react with the H_2FR through the SET mechanism, probably due to their low electrophilicity. In conclusion, the neutral fraxetin is a very efficient radical scavenger for substituted alcoxyl radicals more electrophilic that $^{\bullet}OCH_3$, i.e. halogen substituted and is a poor radical scavenger against the peroxyl and nitrogen centred radicals.

Table 6. Reorganization energy (λ), Gibbs free energy of activation ($\Delta G^{\neq}$, kcal/mol), apparent rate constant (k_{app}, M^{-1} s^{-1}) for H_2FR and HFR^-; and overall rate coefficient ($k_{overall}$, M^{-1} s^{-1}) at physiological pH for SET reactions with all the considered free radicals, in aqueous solution, at pH = 7.4.

Radical	H_2FR			HFR^-			$k_{overall}$
	λ	$\Delta G^{\neq}$	k_{app}	λ	$\Delta G^{\neq}$	k_{app}	
•OH	13.27	4.22	3.11 x 10^{9}	10.97	3.62	5.12 x 10^{9}	5.04 x 10^{9}
$•OCH_3$	19.09	14.92	7.16 x 10^{1}	16.79	0.57	7.80 x 10^{9}	7.49 x 10^{9}
$•OCH_2Cl$	24.58	3.84	4.23 x 10^{9}	22.28	0.74	7.59 x 10^{9}	7.46 x 10^{9}
$•OCHCl_2$	26.29	0.08	7.56 x 10^{9}	23.99	6.38	1.30 x 10^{8}	4.27 x 10^{8}
$•OCCl_3$	23.85	0.26	7.53 x 10^{9}	21.55	12.29	6.05 x 10^{3}	3.01 x 10^{8}
•OOH	23.61	26.32	3.15 x 10^{-7}	21.31	5.83	3.31 x 10^{8}	3.31 x 10^{8}
$•OOCH_3$	22.41	23.95	1.73 x 10^{-5}	20.11	4.38	2.54 x 10^{9}	2.54 x 10^{9}
$•OOCH_2Cl$	17.93	27.66	3.31 x 10^{-8}	15.63	4.61	1.94 x 10^{9}	1.86 x 10^{9}
$•OOCHCl_2$	18.67	17.35	0.12 x 10^{1}	16.37	1.08	7.44 x 10^{9}	7.14 x 10^{9}
$•OOCCl_3$	18.25	12.51	4.21 x 10^{3}	15.96	0.11	7.44 x 10^{9}	7.14 x 10^{9}
$•N_3$	4.97	14.90	7.42 x 10^{1}	2.67	10.06	2.64 x 10^{5}	2.53 x 10^{5}
$•NO_2$	31.05	14.40	1.72 x 10^{2}	28.75	1.89	7.66 x 10^{9}	7.35 x 10^{9}

The results for the deprotonated fraxetin indicate that their reaction with the •OH, $•OCH_3$, $•OCH_2Cl$, •OOH, $•OOCH_3$, $•OOCH_2Cl$, $•OOCHCl_2$, $•OOCCl_3$ and $•NO_2$ radicals have rate constants close to the diffusion limited rate constant. In the case of $•OCHCl_2$ and $•OCCl_3$ radicals, the corresponding reactions are much slower, with rate constants similar to the other alcoxyl radicals, this is because the $•OCHCl_2$ and $•OCCl_3$ are in the inverted region of Marcus parabola. The HFR^- is a very efficient radical scavenger towards $•NO_2$, •OH, $•OCH_3$, $•OCH_2Cl$, •OOH, $•OOCH_2Cl$, $•OOCHCl_2$, $•OOCCl_3$, and is an efficient radical scavenger to $•N_3$, $•OCHCl_2$, $•OCCl_3$ and $•OOCH_3$.

Total rate constants were calculated for The SET reactions of fraxetine with all the considered radicals, taking into account the molar fractions of the neutral and deprotonated species, in water solution at physiological pH. The calculated values show that fraxetine is an excellent free radical scavenger via SET. Its total rate constants were found to be diffusion controlled, in the order of 10^{8}-10^{9} $M^{-1}s^{-1}$, with a large variety of free radicals, which indicates that this compound is a versatile free radicals scavenger. The only exception is the $•N_3$ free radical, for which the total rate constant is 2.53 x 10^{5} $M^{-1}s^{-1}$.

Regeneration and multi-scavenging activity

Once an antioxidant neutralizes a free radical, it usually loses its antioxidant ability. In living systems, however, antioxidants can be regenerated, often with the help of other antioxidants. Glutathione, for example, can regenerate vitamin C, and vitamin C can in turn regenerate vitamin E. The antioxidant regeneration in water media at physiological pH has been previously reported for protocatechuic acid[51] and esculetin.[39] Following the same methodology, in this work we have carried out the study of the regeneration of fraxetin in aqueous media.

Taking into account that, at physiological pH, the dominant form of fraxetin is the HFR^- anion (96%), which is also the most active species in water, it has been used for the investigation of the antioxidant regeneration. The proposed reaction routes involved in the regeneration process are shown in Scheme 2.

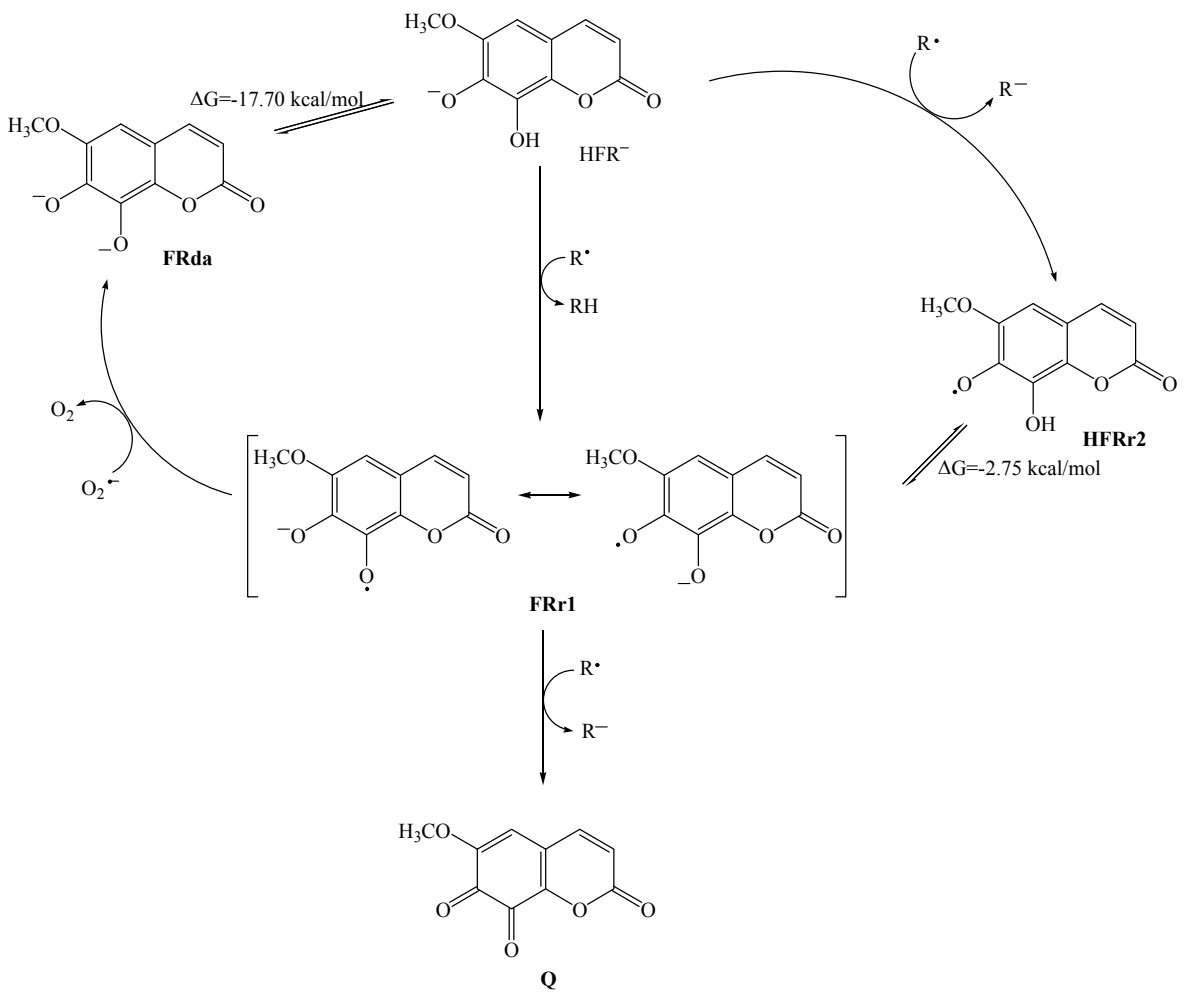
The fraxetin regeneration was proposed to occur in two steps (Scheme 2). The first step corresponds to the SET reaction from HFR^- to R• which yields the radical intermediate HFRr2. The deprotonation of the latter is an exergonic process (ΔG = -2.75 kcal/mol at physiological pH under standard conditions) that produces the radical-anion FRr1. However, since this reaction actually corresponds to an acid-base equilibrium:

$$HFRr2 \leftrightarrows FRr1 + H^+ \quad (19)$$

The results indicated that the route that involves the SET mechanism and the acid-base equilibrium are the most important ones to obtain the FRr1 intermediate.

The acid-base equilibrium reaction of the HFRr2 under standard condition have a ΔG = 7.35 kcal/mol.

$$K = \frac{[FRr1][H^+]}{[HFRr2]} = e^{-\Delta G^{\neq}/RT} \quad (20)$$



Scheme 2. Schematic representation of the overall oxidation and regeneration mechanisms for the radical scavenging activity of fraxetin in aqueous solution at physiological pH.

The rate constant can be calculated taking into account the pH of the environment. In this case, at physiological pH, the conditional equilibrium constant can be obtained according to:

$$K' = \frac{K}{[H^+]} = \frac{e^{-\Delta G^0/RT}}{10^{-pH}} = e^{-\Delta G'/RT} \quad (21)$$

Hence, the conditional Gibbs energy of reaction at each particular buffered pH would be:

$$\Delta G' = \Delta G^0 - 2.303RT(pH) \quad (22)$$

At physiological pH, ΔG = -2.75, indicating that the acid-base reaction is feasible under these conditions. This reaction occurs without barrier of activation and depends only on the $[H^+]$ environment.

The FRr1 intermediate can react through two routes. In one of these, the antioxidant regenerates by reacting with $O2^{\bullet-}$ and forming the FRda intermediate. This reaction has a rate constant of 4.30 x 10^9 M^{-1} s^{-1}, which is limited by diffusion (Table 8). The FRda product regenerates the antioxidant by means of an acid-base equilibrium, according to:

$$FRda + H^+ \rightarrow HFR^- \quad (17)$$

The Gibbs free energy of reaction for acid-base equilibrium at physiological medium was calculated according to Eq. 22. At physiological pH, $\Delta G'$= -17.70 kcal/mol, this means that the acid-base reaction of FRda intermediate protonation is feasible, under these conditions.

The second route is the reaction of FRr1 with radicals through the SET mechanism to yield the quinone that is the oxidation product of the antioxidant fraxetin. Reorganization energies (λ), Gibbs free energies of activation ($\Delta G^{\neq}$), diffusion controlled rate constants (k_D), and apparent rate constants (k_{app}) for the SET reactions of FRr1, in water at 298.15 K, are reported in Table 7.

It can be observed that the radicals $^{\bullet}N_3$, $^{\bullet}NO_2$, $^{\bullet}OH$, $^{\bullet}OCH_3$, $^{\bullet}OCH_2Cl$, $^{\bullet}OCHCl_2$, $^{\bullet}OOCH_3$, $^{\bullet}OOCH_2Cl$, $^{\bullet}OOCHCl_2$ and $^{\bullet}OOCCl_3$ react with FRr1 intermediate radical at a rate constant limited by diffusion. The reaction of FRr1 with the $^{\bullet}OOH$ radical has a rate constant of 3.30 x 10^8 M^{-1} s^{-1}; and finally, the reaction of FRr1 with the $^{\bullet}OOCCl_3$ radical has a rate constant of 5.58 x 10^5 M^{-1} s^{-1}, which is relatively slow because the reaction with this radical is very exothermic and therefore is in the inverted region of Marcus parabola.

Table 7. Reorganization energies (λ), Gibbs free energies of activation ($\Delta G^{\neq}$, kcal/mol), diffusion controlled rate constants (k_D, M^{-1} s^{-1}), and apparent rate constants (k_{app}, M^{-1} s^{-1}) for the SET reactions of FRr1, in water at 298.15 K.

Radical	λ	$\Delta G^{\neq}$	k_D	k_{app}
		FRr1 + $O_2^{\bullet}$ – → FRda + O_2		
$O_2^{\bullet-}$	19.03	3.83	7.80 x 10^9	4.30 x 10^9
		FRr1 + R$^{\bullet}$ → Q + R^-		
$^{\bullet}N_3$	5.99	3.18	7.78 x 10^9	6.13 x 10^9
$^{\bullet}NO_2$	32.07	2.08	7.89 x 10^9	7.56 x 10^9
$^{\bullet}OH$	14.30	2.11	8.11 x 10^9	7.76 x 10^9
$^{\bullet}OCH_3$	20.12	0.76	7.82 x 10^9	7.79 x 10^9
$^{\bullet}OCH_2Cl$	25.60	0.41	7.62 x 10^9	7.60 x 10^9
$^{\bullet}OCHCl_2$	27.32	4.89	7.54 x 10^9	1.34 x 10^9
$^{\bullet}OCCl_3$	24.88	9.61	7.52 x 10^9	5.58 x 10^5
$^{\bullet}OOH$	24.65	5.81	7.83 x 10^9	3.30 x 10^8
$^{\bullet}OOCH_3$	12.69	1.83	7.53 x 10^9	7.33 x 10^9
$^{\bullet}OOCH_2Cl$	18.96	4.56	7.64 x 10^9	2.06 x 10^9
$^{\bullet}OOCHCl_2$	19.70	1.28	7.49 x 10^9	7.41 x 10^9
$^{\bullet}OOCCl_3$	19.28	0.24	7.45 x 10^9	7.43 x 10^9

The reaction profiles, in terms of relative Gibbs free energies, for the regeneration and quinone formation routes involving $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals, are shown at Figure 5.

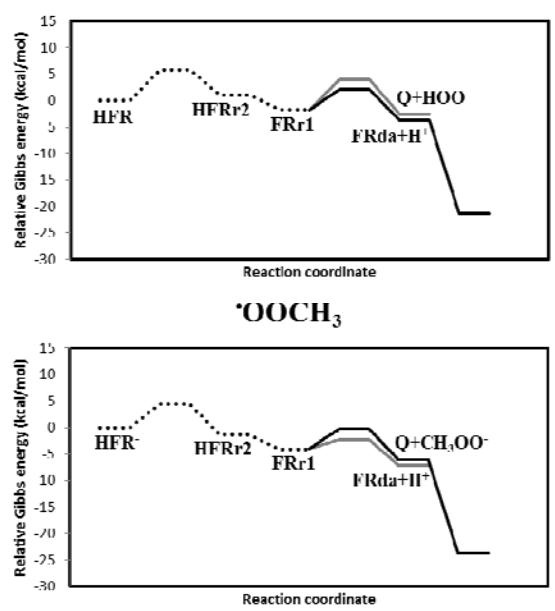


Figure 5. Reaction profiles of fraxetin regeneration (black line) and quinone formation (grey line), in water at physiological pH; for the reactions involving $^{\bullet}OOH$ and $^{\bullet}OOCH_3$ radicals.

The profile of the reaction between fraxetin and $^{\bullet}OOH$ radical shows that the regeneration is an exergonic route; in addition, this route has the lowest Gibbs free energy of activation. Thus, the preference for regeneration route is feasible in the reaction between the fraxetin and $^{\bullet}OOH$ radical in aqueous media at physiological pH. The profile of the reaction between fraxetin and $^{\bullet}OOCH_3$ radical shows that the quinone formation is also an exergonic reaction and has the lowest Gibbs free energy of activation. However, the regeneration route considering the superoxide radical that is in a great concentration, yield the FRda intermediate that reacts quickly to regenerate the antioxidant. Taking into account these considerations, is feasible to propose the regeneration route for the reaction between fraxetin and $^{\bullet}OOCH_3$ in aqueous media at physiological pH.

In summary, the FRr1 may be obtained through two different routes. In the first one, an HT mechanism is proposed, while for the second one, the SET mechanism followed by the acid-base equilibrium would occur. The second route has the highest rate constant, and therefore it is suggested that this route represents the main channel to obtain the FRr1 intermediate.

In turn, the FRr1 intermediate can react through two routes: i) in the first one the SET reaction with the $O_2^{\bullet-}$ was proposed and after the acid-base equilibrium the antioxidant was regenerated, and ii) in the second route, the SET mechanism was proposed, to form the quinone. As it can be observed from data in Table 8, the rate constant of the regeneration and the quinone formation are both diffusion controlled, therefore which one will prevail is driven by the $O_2^{\bullet-}$/$^{\bullet}OOR$ concentration ratio. At physiological pH, the calculated $O_2^{\bullet-}$/$^{\bullet}OOH$ concentration ratio is 166.66 and therefore, the regeneration route would be much more favoured under these conditions. Regarding other peroxyl radicals, we assume that they are in lower concentrations than $O_2^{\bullet-}$, because the latter is an essential aerobic respiration intermediate, while other peroxyl radicals have not such important physiological role.

Antioxidant activity of Fraxetin in lipid media

In order to study the antioxidant activity of fraxetin in lipid media, we have considered only the HT mechanism, through the 7-OH and 8-OH channels. We have identified the transition structures for these reactions with both radicals. The geometrical features of these structures are similar to the ones obtained in water. Gibbs free energy of activation ($\Delta G^{\neq}$), rate constants (k), and relative branching ratios (Γ) for the HT channels in the reaction of H_2FR with the $^{\bullet}OOH$ and $^{\bullet}OOCH_3$, in lipid

environment at 298.15 K, are reported in Table 8. The overall rate constants that determines the antioxidant activity of fraxetin towards •OOH and •$OOCH_3$ peroxyl radicals in lipid media, have been calculated by summing up the individual rate constants for the HT 7-OH and 8-OH reaction channels.

It can be observed that the Gibbs free energy of activation for the 7-OH channel is slightly lower than for the 8-OH channel. The trends on the $\Delta G^{\neq}$ values are directly reflected in the rate coefficients, so that the HT 7-OH reaction channel has the highest rate constant with both peroxyl radicals. On the basis of the calculated rate constants, the relative branching ratios for the HT 7-OH channel are 98.2% and 87.7% for the •OOH and •$OOCH_3$ radicals, respectively. The calculated overall rate constants for the reactions of fraxetin with •OOH and •$OOCH_3$ radicals in lipid media are 2.43 x 10^4 and 2.81 x 10^3 M^{-1} s^{-1}, respectively.

The antioxidant activity of fraxetin towards •OOH radical in lipid environment may be compared to that of other antioxidants. It is clear that the antioxidant activity of fraxetin is higher than that of the sinapinic acid (1.7 × 10^4 M^{-1} s^{-1});[50] protocatechuic acid (5.1 x 10^3 M^{-1} s^{-1}),[51] capsaicine (6.5 x 10^3 M^{-1} s^{-1}),[52] α-mangostin (7.8 × 10^3 M^{-1} s^{-1});[53] tyrosol (7.1 × 10^2 M^{-1} s^{-1}),[54] uric acid (1.85 x 10^2 M^{-1} s^{-1}),[55] melatonin (3.1 × 10^2 M^{-1} s^{-1})[56] and caffeine (3.2 × 10^1 M^{-1} s^{-1}).[57]

Table 8. Gibbs free energy of activation ($\Delta G^{\neq}$, in kcal/mol), rate constants (k, in M^{-1} s^{-1}), and relative branching ratios (Γ) for the HT channels in the reaction of H_2FR with the •OOH and •$OOCH_3$, in lipid environment at 298.15 K.

Channel	$\Delta G^{\neq}$	$\Delta H^{\neq}$	tunnel	k	Γ	$k_{overall}$
			•OOH			
HT 7-OH	18.92	7.21	159.76	$2.39x10^4$	98.2	$2.43x10^4$
HT 8-OH	21.01	9.26	97.62	$4.31x10^2$	1.8	
			•$OOCH_3$			
HT 7-OH	19.79	7.39	71.77	$2.46x10^3$	87.7	$2.81x10^3$
HT 8-OH	20.87	8.66	62.12	$3.47x10^2$	12.3	

Conclusions

In this work, we have carried out a theoretical study on the overall antioxidant activity mechanisms of fraxetin in aqueous media at physiological pH and lipid media, within the DFT methodologies framework. We have considered three reaction mechanisms: single electron transfer (SET), hydrogen transfer (HT) and radical adduct formation (RAF). Rate constants have been calculated using the Transition State Theory.

In aqueous media, fraxetin reacts with peroxyl radicals following mechanisms that depend on the molecule's acid-base form. Thus, neutral fraxetin reacts mainly by the HT mechanism, while its anion reacts through the SET mechanism. The calculated rate constants towards the •OOH and •$OOCH_3$ radicals are 3.99 x 10^8 and 2.76 x 10^9 M^{-1} s^{-1}, respectively. In addition, we have shown that fraxetin is a versatile antioxidant, since it has a great scavenger activity for other free radicals, under the same conditions. It was found that the anion (HFR^-) is the species that contributes most to the overall reactivity of fraxetin towards the studied peroxyl radicals. Therefore, the phenoxide anion seems to be the key species in the peroxyl radical scavenging activity of fraxetin. The reactions with •OOH and •$OOCHCH_2$ were found to be rather fast, with overall rate constants in the order of 10^7 M^{-1} s^{-1}. On the basis of the calculated rate constants, we can safely conclude that fraxetin is an excellent and versatile antioxidant in aqueous media at physiological pH.

In lipid media, our results show that fraxetin reacts with peroxyl radicals only through the HT mechanism. The calculated rate constants towards the •OOH and •$OOCH_3$ radicals are 2.43 x 10^4 and 2.81 x 10^3 M^{-1} s^{-1}, respectively. Thus, fraxetin is a good antioxidant in lipid media, with a relatively good peroxyl radicals scavenging activity.

In addition, we have investigated the possible regeneration of fraxetin after its reaction with an equivalent of free radical in aqueous media at physiological pH. It was found that fraxetin regeneration is very likely to occur, which suggests that this compound has the ability of scavenging several radical equivalents (two per cycle), under these conditions.

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