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Nitration of Flavonoids and Tocopherols as Potential Modulators of Nitrosative Stress—A Study Based on Their Conformational Structures and Energy Content

José Manuel Pérez de la Lastra [1,*], Celia Andrés Juan [2], Francisco J. Plou [3] and Eduardo Pérez-Lebeña [4]

[1] Institute of Natural Products and Agrobiology, CSIC-Spanish Research Council, Av. Astrofísico Francisco Sánchez, 38206 La Laguna, Spain
[2] Faculty of Sciences, Cinquima Institute and Department of Organic Chemistry, Valladolid University, Paseo de Belén, 7, 47011 Valladolid, Spain; celia.andres.juan@uva.es
[3] Institute of Catalysis and Petrochemistry, CSIC-Spanish Research Council, 28049 Madrid, Spain; fplou@icp.csic.es
[4] Sistemas de Biotecnología y Recursos Naturales, 47625 Valladolid, Spain; info@glize.eu
* Correspondence: jm.perezdelalastra@csic.es

Abstract: Vitamin E and dietary flavonoids are natural substances with antioxidant and anti-inflammatory activities, showing little or no side effects. Fruit and vegetable diets based on flavonoids and vitamin E provide a benefit to hypertensive subjects by regulating blood pressure. However, the exact mechanism of their anti-inflammatory properties has not been chemically explained. It has been proposed that their anti-oxidant and anti-inflammatory properties may be related to their ability to scavenge free radicals. We here describe the chemical considerations that flavonoids and tocopherols required to act as potential scavengers of the $^{\bullet}NO_2$ radical, a key radical in the cellular oxidative process. Moreover, we provide a theoretical study of the energy content of the nitrated compounds in the different possible positions. With this analysis, it was predicted that five flavonoids from different families (quercetin (flavanol), naringenin (flavanone), luteolin (flavone), catechin (flavanol) and aurantinidin (anthocyanin)) and three tocopherols (β-, γ-, and δ-tocopherol, but not α-tocopherol) could act as potential scavengers of the harmful $^{\bullet}NO_2$ radical. These results may help to explain their beneficial effect on cardiovascular health through its antioxidant role. To validate our theoretical considerations, we also examined uric acid, a well-known $^{\bullet}NO_2$-scavenger. We hope this study could help to elucidate the potential scavenging activity of other dietary antioxidants.

Keywords: flavonoids; tocopherols; antioxidants; peroxynitrite; $^{\bullet}NO_2$ radical; biomolecules; $^{\bullet}NO_2$ radical; nitration mechanism

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1. Introduction

Polyphenol properties have been related to their antioxidant capacities, rendering them essential to early herbal traditional medicine for treating diseases [1].

Antioxidant is a term describing a capacity involved in the neutralisation and removal of reactive oxygen species (ROS) [2] and reactive nitrogen species (RNS) [3]. All polyphenols have reducing properties. They can donate hydrogen to oxidized cellular constituent and play a significant role against oxidative stress-related pathologies such as cardiovascular diseases, cancer and neurodegenerative disorders. The following features of a molecule influences its degree of antioxidant capacity: (i) the presence of substituents with hydrogen/electron donating capacity, associated with appropriate reduction potentials [4,5]; (ii) the ability to delocalise the resulting radical; [4] (iii) the transition metal chelation potential, which depends on the nature and arrangement of the functional groups in the molecule [6]; (iv) the accessibility of the antioxidant to the site of action [7]; and (v) the interaction potential between the radicals of the antioxidant and other antioxidant molecules [8]. For polyphenols, most of the five requirements for antioxidant activity are

met. They are effective hydrogen donor molecules; by virtue of the reducing properties of the OH groups of their aromatic rings, they can delocalise the resulting radical within their structures, they complex transition metals through their catechol structure, and they have the power to regenerate α-tocopherols through the reduction of α-tocopheryl radicals [9]. Generally, the antioxidant potential of flavonoids is dependent on the number and position of the hydroxyl groups, as well as the extent of conjugation and the presence of electron-donating groups. The greater the capacity for electron delocalisation, the more effective the flavonoid as an antioxidant. The aromatic rings of polyphenols and flavonoids are able to take up electron unpairing by displacement of the π-electron system [10]. In general, polyphenols can inhibit the oxidation of low-density lipoproteins (LDL), lipid peroxidation and prevent DNA degradation. They also act by activating key antioxidant enzymes.

Vitamin E is found in vegetable oils (such as wheat germ, sunflower, safflower, corn, and soybean oils), nuts (almonds, peanuts, and hazelnuts/filberts), seeds (sunflower seeds) and green leafy vegetables (spinach and broccoli), among others. Vitamin E exists in different forms, four of which are tocopherols (α-, β-, γ- and δ-) and the corresponding four tocotrienols. In addition, tocomonoenols and tocodienols contain unsaturation of one and two double bonds in the side chain, respectively, and are also found in nature. The following food additives are common forms of tocopherol: E 306, tocopherol-rich extract; E 307, synthetic α-tocopherol; E 308, synthetic γ-tocopherol; and E-309, synthetic δ-tocopherol. Vitamin E acts as an antioxidant scavenger of peroxyl radicals and inhibits free radical-mediated lipid peroxidation [11].

High-level oxidative stress (OS) poses a serious threat to human life and well-being. Endogenous antioxidants are critical for optimal cellular function and, as a result, for systemic health and well-being. Endogenous antioxidants may be insufficient to maintain normal cellular function in conditions that increase oxidative/nitrosative stress, necessitating the use of dietary antioxidants. Vitamins E and C, melatonin, carotenoids, and natural flavonoids are all well-known antioxidants found in dietary supplements. Fortunately, chemical antioxidants can help reduce free radical levels. They can be obtained exogenously, that is, via our food or through the use of dietary supplements. Some species have the potential to be both antioxidants and pro-oxidants. The environment can also impact this dual behaviour and the relative relevance of each property. As a result, identifying the individuals or entities responsible for the reported benefits is not a simple process. In order to develop effective approaches to address oxidative/nitrosative stress and its harmful effects, a thorough study and understanding of the chemistry involved in these processes is necessary. Chemical mechanisms and computational analysis, based on molecular mechanics, may give helpful information on the free radical scavenging activity of dietary antioxidants, such as tocopherols and flavonoids. The aim of this study is to provide the chemical basis and theoretical energy computation that can help to explain how flavonoids and tocopherols could act as scavengers of the $^{\bullet}NO_2$ radical, a peroxinitrite derivative, which may be involved in many pathological processes through a mechanism similar to the known nitration process of the amino acid tyrosine.

2. Materials and Methods

2.1. Models for the Energetic Study

The following compounds were chosen as representatives of each flavonoid family: quercetin (flavanol), naringenin (flavanone), luteolin (flavone), catechin (flavanol), and aurantinidin (anthocyanin). As proxies for vitamin E, it was calculated the energy content of β-tocopherol, γ-tocopherol and δ-tocopherol. Uric acid is a well-known endogenous scavenger of peroxynitrite [12–15] and was used as positive control of our chemical and energetic considerations.

2.2. Theoretical Analysis of Electronical Stability

The delocalisation of radicals in aromatic rings has often been associated with more electronically stable systems. The stability of the resonance forms is judged by the delocali-

sation of charge between one or more rings, as well as by the subsequent stabilization of the bonds. In particular, it has been observed that after uptake of the free radical by the aromatic ring, the resonant character of the aromatic ring is recovered.

2.3. Molecular Mechanics Calculations

The energy content was calculated using ChemBio3D Ultra 16.0 (Perkin-Elmer Inc., Waltham, MA, USA) and the molecular mechanics calculation method (MM2). Computations were performed to obtain the energy of the 3D structure of each molecule by analysing and exposing the energy of the basal state. The lower the energy, the higher the stability of the molecule. MM2 is a method for determining the geometry, molecular energies, vibrational spectra and enthalpies of formation of stable molecules in their basal state and is commonly used to determine the geometries of large molecules of biological and pharmaceutical importance. It is generally used to determine geometries in large molecules, such as those of biological and pharmaceutical importance, which were beyond the reach of more intensive methods based on molecular orbitals. For this reason, MM2 is not useful for modelling transition state of chemical processes with a large spectrum of experimental steps.

Calculations were performed to obtain the energy of different conformations of the same 3D-complex, analysing and exposing those with lower energy of formation. Different three-dimensional conformations were estimated for each flavonoid or tocopherol. The total energy content, expressed in kcal/mol, of each molecule is described by the sum of the following interactions:

$$E_{Total} = E_{Stretching} + E_{Bending} + E_{Torsion} + E_{Non\text{-}bonded\ inte}$$

It is understood that this energy is derived exclusively from molecular mechanics calculations [16].

2.4. Considerations for Acting as Potential $^{\bullet}NO_2$ Radical Scavengers

To determine whether the compounds can act as scavengers, the chemical sequence and the three-dimensional structural conformations of the nitrated molecule are studied, analysing the energy difference between the non-nitrated molecule and nitrated derivative. The lower basal energy of the nitrated compound determines the formation of more stable molecules. Therefore, those nitrated compounds with a lower energy than the non-nitrated forms were considered as potential $^{\bullet}NO_2$ scavengers, generating a structure with additional stability to that of the original.

3. Results and Discussion

In recent years, there has been considerable interest in the idea that chronic oxidative/nitrosative stress plays a role in the aetiology of human diseases, including atherosclerosis, inflammation, cancer, and neurological diseases. These chemical changes mediated by reactive nitrogen species (RNS) are detrimental to cell function but have no detectable symptoms of disease triggering. Improved protective systems against oxygen and nitrogen radicals are believed to play a key role in primate evolution, resulting in longer life spans and lower age-specific cancer rates. The scavenging of free radicals produced in cells by normal and pathological processes is an important area of biochemistry. An ideal antioxidant should be readily absorbed, neutralize free radicals, and chelate redox metals to physiologically tolerable levels. Additionally, it should be efficient in both aqueous and membrane domains and have a beneficial effect on gene expression. Antioxidants should be able to quickly penetrate physiological barriers and enter cells. So, amphiphilicity and modest size are greatly desired. The analysis of antioxidant protection in terms of the intrinsic reactivity of the antioxidant species is, by far, the most common theoretical method found in the literature. Computational techniques may give helpful information on the free radical scavenging activity of antioxidants [17].

The electron donating capacity of antioxidants is the most important aspect in their radical scavenging action. This study focuses on finding out whether the antioxidant compounds such as flavonoids and tocopherols are capable of capturing the $^{\bullet}NO_2$ radical, as a product of peroxynitrite decomposition and storing it within their molecular structure. We provide a chemical mechanism that begins with the formation of the phenoxyl radical in the aromatic ring, referable to the mechanism of tyrosine nitration, previously described in the technical literature. We studied the molecular conformational structure and energy content of five flavonoids from different families: quercetin (a flavanol), naringenin (a flavanone), luteolin (a flavone), catechin (a flavanol), and aurantinidin (an anthocyanin). These families are representative of the flavonoid universe and are widely represented in the plant world, including the human diet. In vivo, protein nitration at the tyrosine residue is recognised as a marker of oxidative stress. The nitration reaction, which occurs via a radical-radical reaction, leads to the formation of nitrotyrosine units in peptides and proteins. Tyrosine nitration proceeds through initial formation of tyrosyl radical, which is formed by oxidation of tyrosine by hydroxyl or carbonate radical. A radical-radical coupling reaction then follows in which $^{\bullet}NO_2$ adds to the C-centered tautomer of the phenoxyl radical. The proposed nitration mechanism for uric acid, flavonoids and tocopherol is based on the mechanism, already described in the technical literature, for tyrosine nitration, Figure 1. Peroxynitrite cannot react directly with tyrosine residues, as due to its reduced half-life of 10 ms, it decomposes into oxidising and nitrating species, including the $^{\bullet}OH$ and the $^{\bullet}NO_2$ radicals. The $^{\bullet}OH$ radical removes hydrogen from the phenol group of tyrosine, promoting the formation of the tyrosyl radical, which reacts with the $^{\bullet}NO_2$ to form 3-nitrotyrosine.

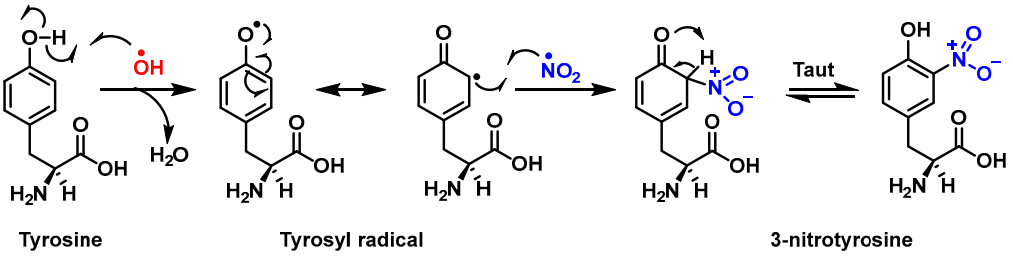


Figure 1. Mechanism of tyrosine nitration with $^{\bullet}NO_2$ to 3-nitrotyrosine.

Chemical and energetic research was focused on three complementary aspects: (i) on the antioxidant mechanics of uric acid, flavonoids and tocopherols, which allow them to act as free radical scavengers; (ii) on their ability to scavenge the $^{\bullet}NO_2$ radical, a peroxynitrite derivative that plays a key role as a cellular oxidant; and (iii) an energy computation has been performed on the potential energy associated with the original molecules and the nitrated compounds. These three aspects are analysed separately in the following sections.

3.1. Uric Acid as Antioxidant and Scavenger of $^{\bullet}NO_2$ Radical

Concerning theoretical research into free radical scavengers, our initial challenge was to identify the chemical pathways that may be involved in their anti-oxidant activity. It is based in the ability to scavenge different free radicals at different positions (Figure 2).

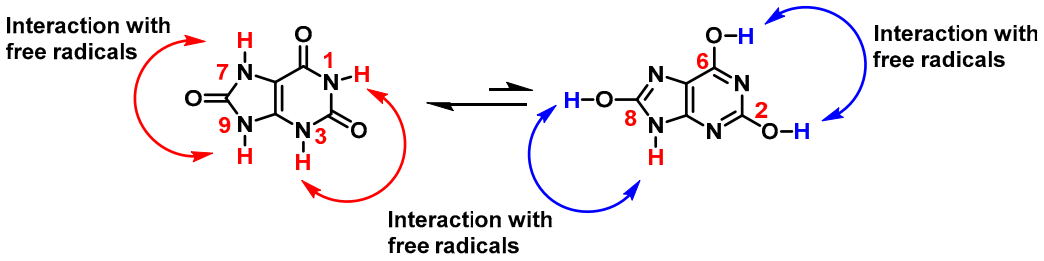


Figure 2. Possible positions for the interaction of free radicals with both tautomeric forms of uric acid.

The formation of nitrated form of uric acid can be explained by the abstraction of hydrogen at position N-1 or N-9 due to the attack of reactive oxygen or nitrogen species (represented as $^{\bullet}X$) yielding the formation of a free radical. The attack of a radical ($^{\bullet}X$) could displace a proton attached to the nitrogen atoms at positions 1, 3, 7, and 9 of the tri-keto tautomer, or to the phenolic hydrogens at positions 2, 6, and 8 of the enol tautomer. The radical ($^{\bullet}X$) generated a radical on the respective nitrogen or oxygen atom, which would subsequently react with the $^{\bullet}NO_2$ radical to generate the nitrated derivative. The molecular mechanism of nitration is described for both tautomers (keto and enol), Figure 3.

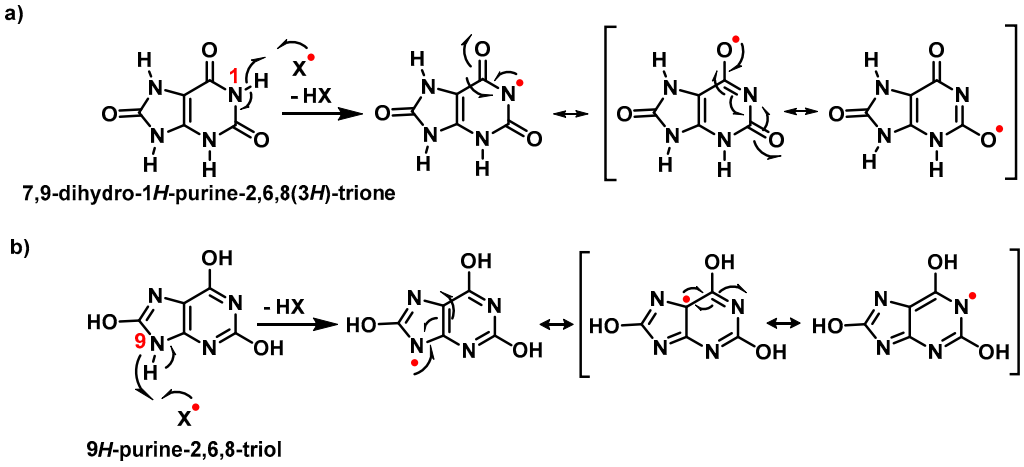


Figure 3. Interaction of a free radicals $^{\bullet}X$ with the tautomeric forms of uric acid: (**a**) keto; (**b**) enol. The radical formed is stabilised by delocalisation of the unpaired electron by resonance.

The nitrogen radical of uric acid can react with the $^{\bullet}NO_2$ radical (i.e., generated from peroxynitrite) to give the nitrated derivative, Figure 4.

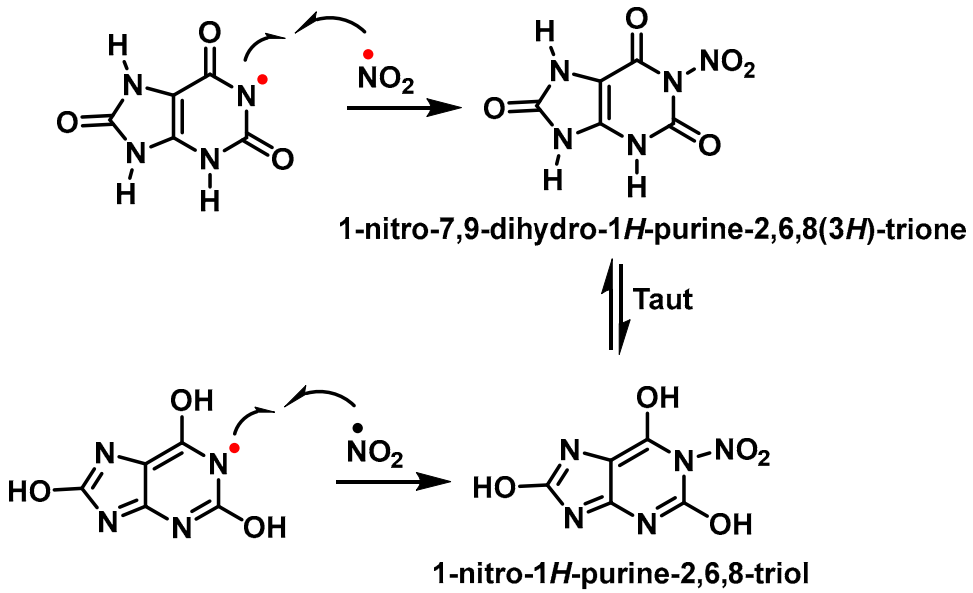


Figure 4. Mechanism of nitration of uric acid by a radical-radical reaction with $^{\bullet}NO_2$.

We next evaluated the stability of the compounds by a quantitative measure, as the favourable energy of the formed nitrated products could be proportional to the antioxidant activity of the molecules. For uric acid, according to the total energy of tautomeric forms computed by molecular mechanics, the 7,9-dihydro-1H-purine-2,6,8 (3H)-trione had 4.5 Kcal/mol lesser energy than the 9H-purine-2,6,8-triol, Figure 5.

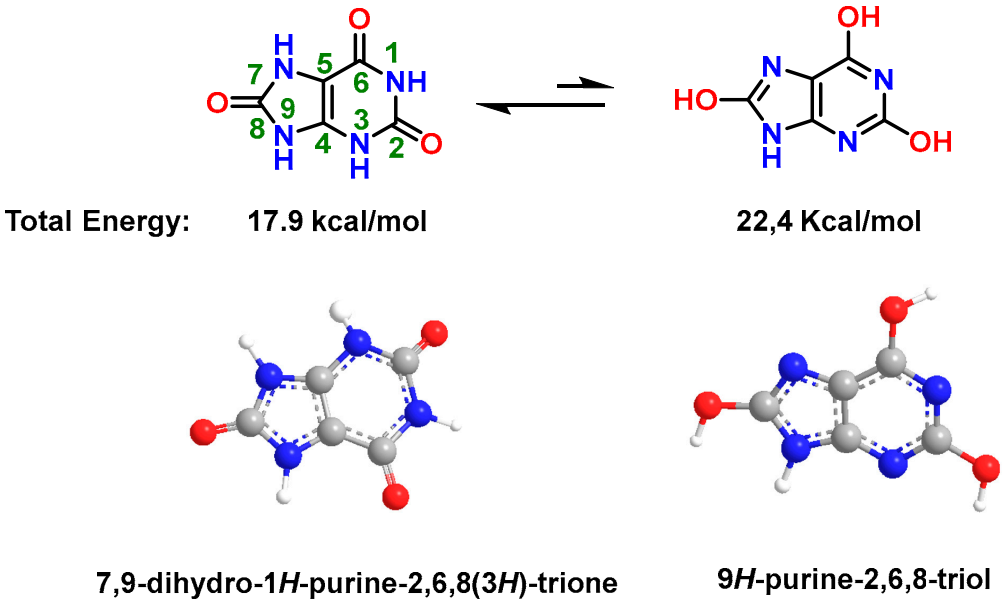


Figure 5. Energy content and three-dimensional structure of tautomeric forms of uric acid (tri-keto and enol) computed by molecular mechanics. The longer reverse arrow indicates the greater stability of the tri-keto form, versus the enol form.

In order to ascertain the most stable forms of nitrated uric acid, we computed the energy content of the different forms with different positions for nitration. A total of seven forms of nitrated uric acid were found with different energy and possible positions for nitration, Table 1.

We next performed theoretical calculations based on molecular mechanics showing that the most stable product (lower energy content) was for the 1-nitro-7,9-dihydro-1H-purine-2,6,8(3H)-trione (with a predicted energy of −77.1 Kcal/mol), whereas the most energetic nitrated form of uric acid corresponded to the substitution at position C-8 (with a total energy of 24 Kcal/mol). As this energy was higher than the non-nitrated tautomeric forms (17.9 and 22.4 Kcal/mol), it was predicted that only the nitration of uric acid at this position would not be favourable (Figure 5, Table 1). Endogenous antioxidants are critical for optimal cellular function and, as a result, for systemic health and well-being. One of these endogenous antioxidants is plasma uric acid. Uric acid was proposed as a potent antioxidant that could acts as a scavenger of free radicals and singlet oxygen preventing erythrocyte lysis [18]. Out theoretical considerations and energy calculations by molecular mechanics also pointed that uric acid can be an effective scavenger of the $^{\bullet}NO_2$ radical, particularly the tri-keto form of uric acid. Our results are in accordance with a previous report that identifies this tautomeric form as the most stable in aqueous solution [19].

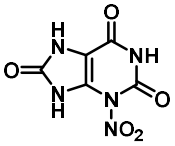
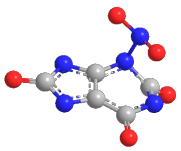
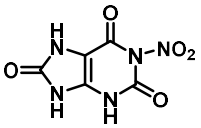
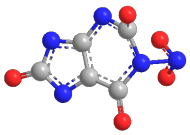
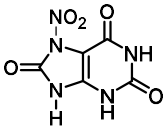
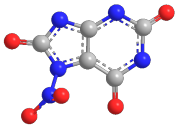
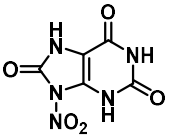
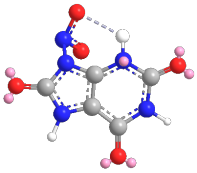
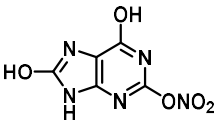
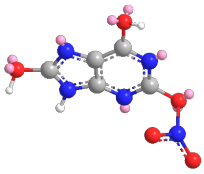
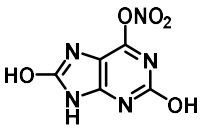
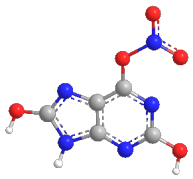
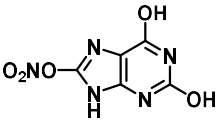
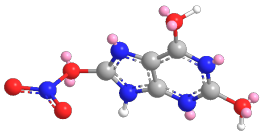
3.2. Chemical Analysis of the Antioxidant Capacity of Flavonoids and Tocopherols

The antioxidant capacity of flavonoids is based on the ability of the hydroxyl groups of the aromatic rings to donate an H^+ to various radicals, such as hydroxyl, peroxyl, etc., which lose reactivity due to their stabilisation, and on the other hand forming a relatively stable flavonoid radical [20] (Figure 6 for quercetin).

The interaction with a free radical $^{\bullet}X$ at the hydrogen in the OH of C-7 position resulted in the electron delocalisation throughout the A-ring, Figure 7. The $^{\bullet}X$ radical can be an oxygen, nitrogen and chlorine radical such as hydroxyl, peroxyl, superoxide, or peroxynitrous acid.

However, when the radical $^{\bullet}X$ was generated at the OH of the C-4′ of quercetin, there was greater delocalisation of the unpaired electron, yielding greater number of resonant forms. Derivatives of quercetin with hydroxyl groups located in the 3′ and 4′ positions of the B ring were predicted to have optimum antioxidant activity, Figure 8.

Table 1. Nitration of uric acid with the $^{\bullet}NO_2$ radical located at different positions showing different energies and three-dimensional structures.

Position	Energy Content (kcal/mol)	Developed Formula	Three-Dimensional Structure
N-3	−35.5		
N-1	−77.1		
N-7	10.9		
N-9	4.2		
O in C-2	15.4		
O in C-6	10.8		
O in C-8	24		

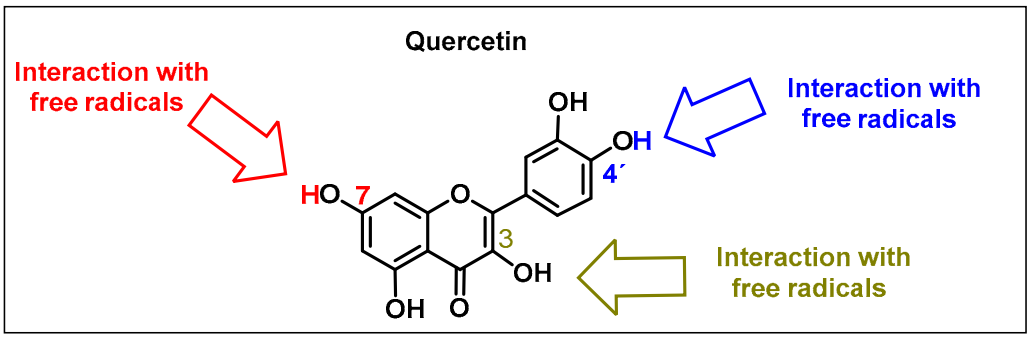


Figure 6. For quercetin, formation of the hydroxyl radical at the C-7, C-3, and C-4′ positions.

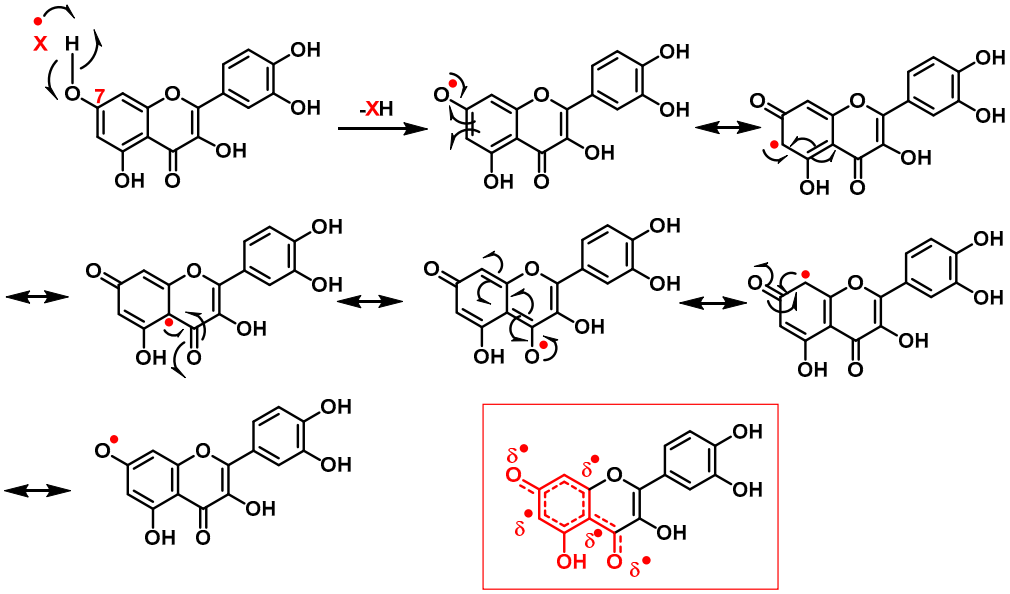


Figure 7. Quercetin capacity for electron delocalisation (in red) of a free radical •X in OH at C -7 position.

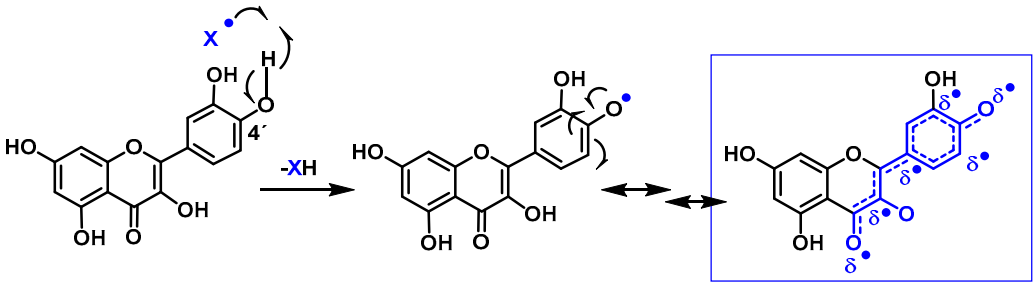


Figure 8. Resonance forms showing quercetin capacity for electron delocalisation (in blue) of a free radical located at the C-4′ position.

Following theoretical considerations, flavonoids with dihydroxyl substituents at the 3′ and 4′ positions on the B-ring were predicted to be more effective antioxidants, and this effect could be enhanced by the presence of a double bond between carbons 2 and 3, a free OH group at the 3 and 5 position, and a carbonyl group at the C-4 position. Therefore, free radical scavenging by flavonoids could be largely dependent on the presence of a hydroxyl OH at C-3, Figure 9. The OH group at the C-3 position, with C-2 and C-3 double bond, increases the resonance stabilization for electron movement across the molecule.

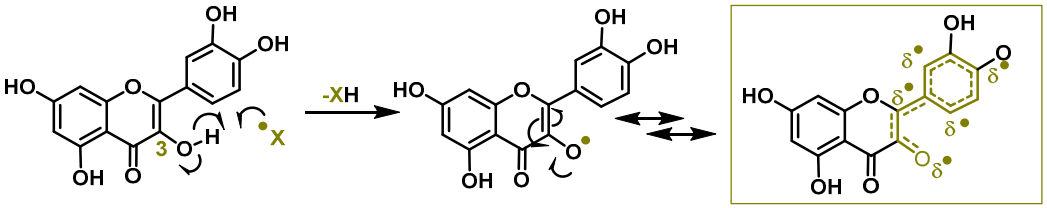
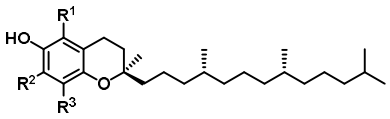


Figure 9. Quercetin capacity for electron delocalization (in green) of a free radical •X in OH at C-3 position.

Tocopherols include an aromatic ring with a hydroxyl that can donate H^+ to reduce free radicals and a hydrophobic side chain that allows penetration into biological membranes (Table 2).

Table 2. Conformational structure of tocopherols, according to the substituents R^1, R^2, and R^3.

Tocopherol Structure	R^1	R^2	R^3	Name
	CH_3	CH_3	CH_3	α-
	CH_3	H	CH_3	β-
	H	CH_3	CH_3	γ-
	H	H	CH_3	δ-

Regarding the antioxidant capacity of tocopherol, a scheme such as that occurring in the polyphenolic aromatic rings of flavonoids is presented below, delocalising the free radical charge inside the ring, in a mechanism similar to that described above for quercetin. Our theoretical considerations showed that the attacking of a radical •X was able to displace the proton from the single aromatic hydroxyl in tocopherol, which in turn delocalised its charge inside the ring (Figure 10), in a mechanism similar to that described above for quercetin. However, in this case, there is only one skilled position at which the attack could occur, whereas for quercetin, three different positions were available, Figure 9.

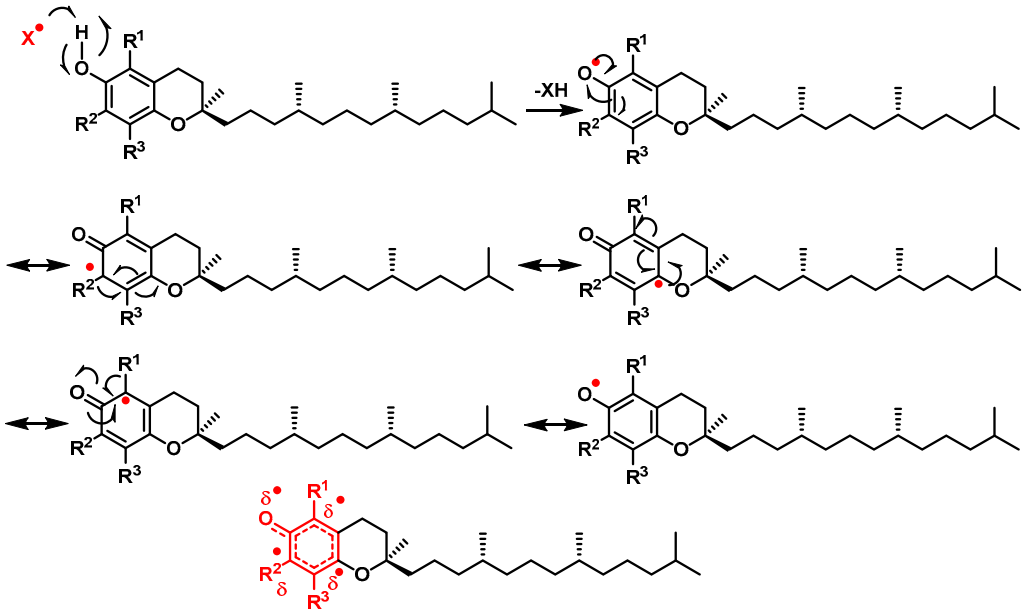


Figure 10. Tocopherol capacity for charge delocalisation (in red) of a free radical.

3.3. Proposed Mechanism for the Nitration of Flavonoids and Tocopherols

For the nitration of flavonoids, we considered a mechanism similar to that of tyrosine, Figure 1. However, there are some differences between the nitration of tyrosine and quercetin, such as the existence of numerous reactive positions in the flavonoid. On the other hand, tyrosine contains only one OH group susceptible to this modification. Figure 11 describes the mechanism of quercetin nitration at the C-2′ position (the most energetically favourable), starting with the attack of the hydroxyl radical •OH on the H of the OH at C-3, generating the alkoxyl radical •O and the delocalisation of the free radical along the C2-C3 double bond to subsequently move to the B-ring. At the C-2′ position, the $^{\bullet}NO_2$ radical is taken up and the aromatic resonance is restored. This same mechanism is repeated for all of the flavonoids studied. In general, the attack is initiated by the abstraction of a hydrogen atom by an existing radical in the phenolic OH of the A and B rings or hydroxyl of C, generating a phenoxyl or alkoxyl radical and the delocalization of the free radical in the rings. This mechanism results in the nitration of flavonoids.

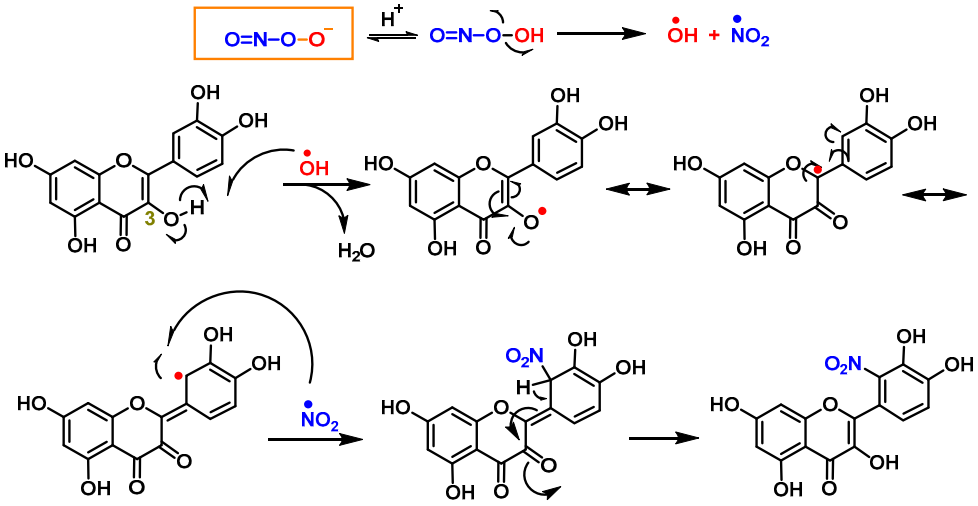


Figure 11. Nitration reaction of quercetin at the C-2′ position.

For the mechanism of δ-tocopherol nitration at the C-5 position, a similar mechanism to the previous one is proposed, which is initiated by the attack of the •X radical at the hydrogen in the OH group of the aromatic ring, Figure 12.

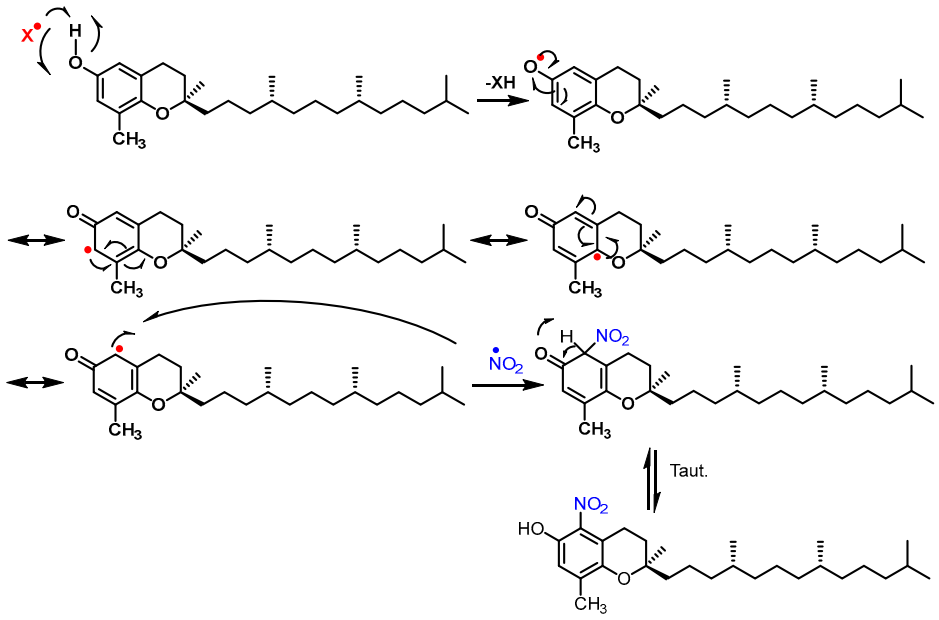


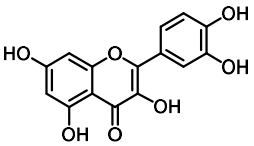
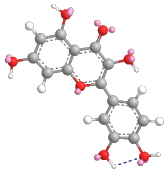
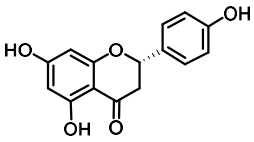
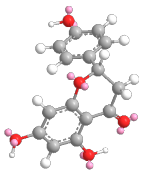
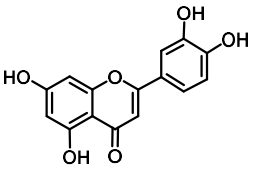
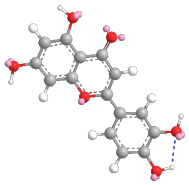
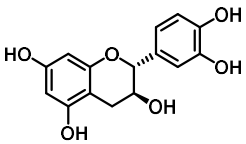
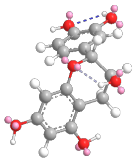
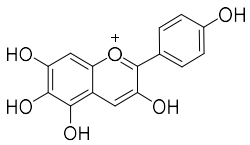
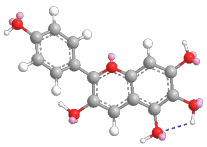
Figure 12. Nitration reaction of δ-tocopherol at the C-5 position.

3.4. Energetic Analysis of Flavonoids, Tocopherols and the Nitrated Compounds

Flavonoids and tocopherols are antioxidants with anti-inflammatory and anti-cancer properties, which can modulate cell signalling pathways [21]. The antioxidant contribution of flavonoids and polyphenols in the human diet is generally higher than that provided by other types of antioxidants, such as vitamins C and E, or carotenoids. We evaluated the stability of the compounds by a quantitative measure, as the favourable energy of the formed nitrated products could be proportional to the antioxidant activity of the molecules.

We next computed the energy of the different nitrated and un-nitrated forms of the flavonoids, quercetin, naringenin, luteolin, catechin and aurantinidin. For these, the energy content, developed formula, and spatial structure are depicted in Table 2. The lowest energy value was found for catechin (−6.6 kcal/mol), whereas the highest value was obtained for quercetin (14.9 kcal/mol). Naringenin, luteolin, and aurantinidin showed similar values between 6 and kcal/mol, Table 3.

Table 3. Energy content, developed formula, and spatial structure of selected flavonoids.

Flavonoid Energy Content (kcal/mol)	Developed Formula	Spatial Structure
Quercetin 14.9		
Naringenin 6.5		
Luteolin 7.7		
Catechin −6.6		
Aurantinidin 7.6		

In the following tables, the energy content of the same flavonoids was studied at the positions where nitration with the $^{\bullet}NO_2$ radical is possible.

For quercetin, nitration can occur at 5 different positions, at the C-6, C-8, C-5′, C-6′, and C-2′ carbon, Table 4, and the energy content has been computed for each molecule. The lowest energy value was found for the C-2′ (−26 kcal/mol) and the C-8 positions (−11 kcal/mol).

In naringenin, nitration can occur at three different positions: at the C-6, C-8, and C-3′ carbon, Table 5. For naringenin, at C-8, a hydrogen bridge is formed between the oxygen of the C=O and OH of C-5. The lowest energy value was found for the C-8 (−8.5 kcal/mol) and the C-3′ (−1.9 kcal/mol) positions.

The fact that for all of the flavonoids studied we found more energetically favourable positions indicates that nitration could preferentially take place at these positions. For lutein, at the C-2′ position, intermolecular hydrogen bonds are formed between the oxygen of the NO_2 group and the OH of C-3′ and between the OH of C-3′ and C-4′. For catechin, the C-6 position is very stable due to the formation of intermolecular hydrogen bridges between the O of position 1 and the OH of C-3, which are easily formed due to the configuration of C-2 and C-3 and which allows in this case the approach, as well as the intermolecular hydrogen bridge between the OH of C-3′ and C-4′. For aurantinidin, a hydrogen bridge is formed between the OH of C5 and OH of C-6. The energy content of the new nitrated compounds was calculated to assess whether their energy content was lower than that of the original molecule, which would indicate a greater molecular stability. Following

our theoretical considerations, the nitration of these compounds in these positions would be favourable. In all of the cases studied, the balance of energy obtained comparing the nitrated form and their corresponding free molecules were positive, except for lutein in the C-6 position and for aurantinidin in the C-3′ position. It is noteworthy that at several nitration positions of the flavonoids, the energy was particularly low, which indicates a more energetically stable molecule. During ingestion, the nitrate salt NO_3 (present in green vegetables such as green beans, spinach, broccoli and Mediterranean diet in general, can be reduced to nitrite NO_2) by commensal bacteria from the oral cavity by mixing with saliva [22]. Nitrite can easily reach the stomach, triggering the production of several nitrogen oxides, such as HNO_2, $^{\bullet}NO$, $^{\bullet}NO_2$, and N_2O_3. The $^{\bullet}NO_2$ radical can be reduced to $^{\bullet}NO$ in the presence of thiocyanate, ascorbic acid, dietary polyphenols or those present in red wine (resveratrol and flavonoids), extra virgin olive oil (hydroxytyrosol) or green tea (catechins) [23].

In luteolin, nitration can occur at five different positions: at the C-6, C-8, C-5′, C-6′, and C-2′ carbon, Table 6. The lowest energy value was found for the C-2′ (−27.9 kcal/mol), C-6′ (−27.7 kcal/mol), and the C-8 (−12.6 kcal/mol) positions.

In catechin, nitration can occur at five different positions: at the C6, C8, C5′, C6′, and C2′ carbon, Table 7. The lowest energy value was found for the C-6 (−59.1 kcal/mol), C-6′ (−34.1 kcal/mol), and the C-8 positions (−13.6 kcal/mol).

Table 4. Nitration of quercetin with $^{\bullet}NO_2$.

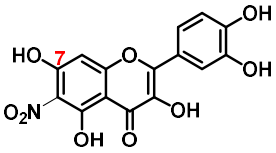
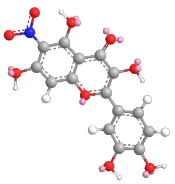
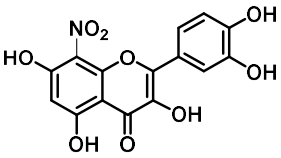
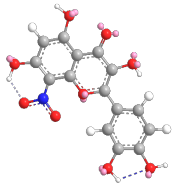
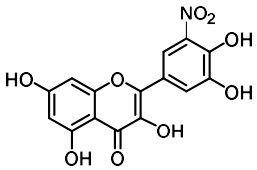
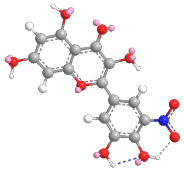
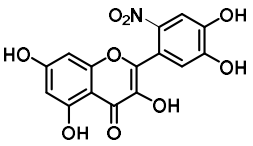
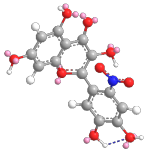
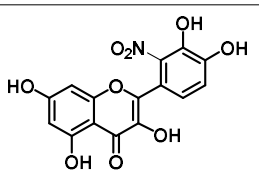
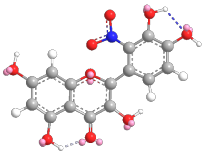
C-Position	Energy Content (kcal/mol)	Developed Formula	Spatial Structure
C-6	14.2		
C-8	−11		
C-5′	5.5		
C-6′	−4.7		
C-2′	−26		

Table 5. Nitration of naringenin with $^{\bullet}NO_2$.

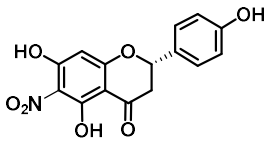
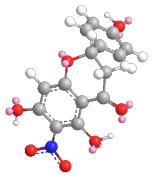
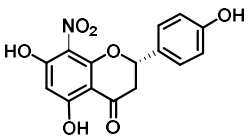
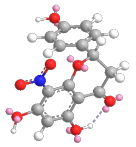
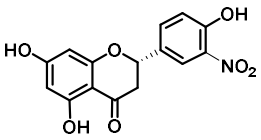
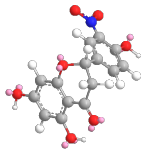
C-Position	Energy Content (kcal/mol)	Developed Formula	Spatial Structure
C-6	4.7		
C-8	−8.5		
C-3′	−1.9		

Table 6. Nitration of luteolin with $^{\bullet}NO_2$.

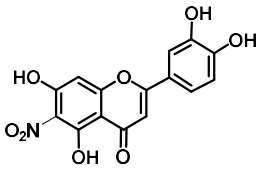
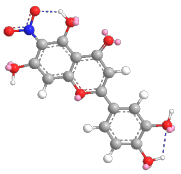
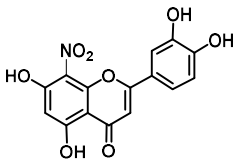
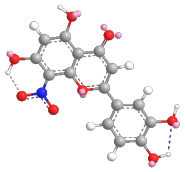
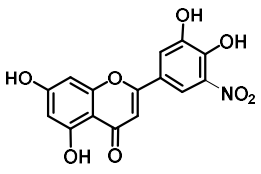
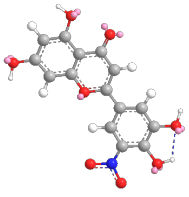
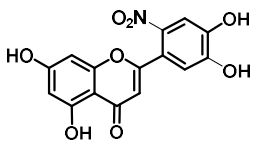
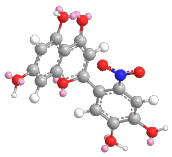
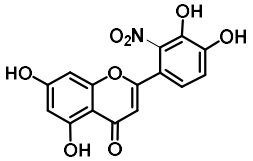
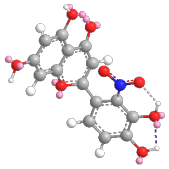
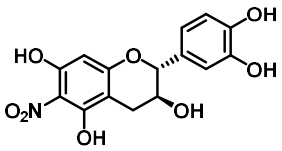
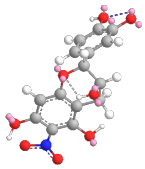
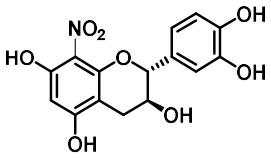
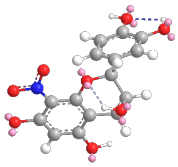
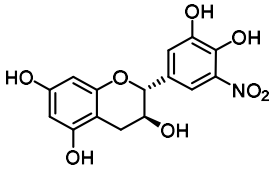
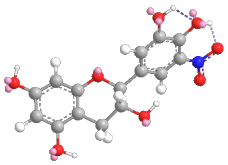
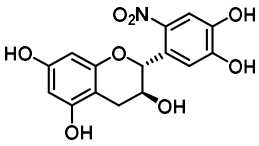
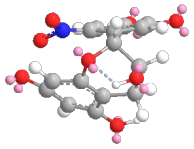
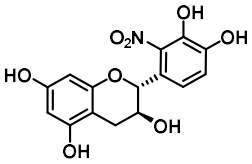
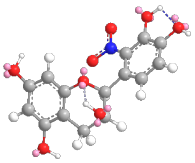
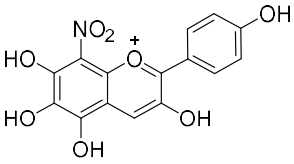
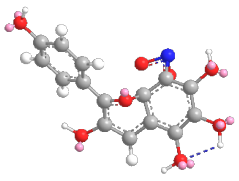
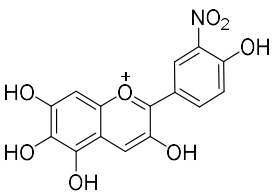
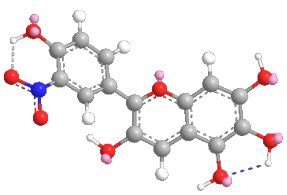
C-Position	Energy Content (kcal/mol)	Developed Formula	Spatial Structure
C-6	8.5		
C-8	−12.6		
C-5′	3.0		
C-6′	−27.7		
C-2′	−27.9		

Table 7. Nitration of catechin with $^{\bullet}NO_2$.

C-Position	Energy Content (kcal/mol)	Developed Formula	Spatial Structure
C-6	−59.1		
C-8	−13.6		
C-5′	−10.3		
C-6′	−34.1		
C-2′	−7.7		

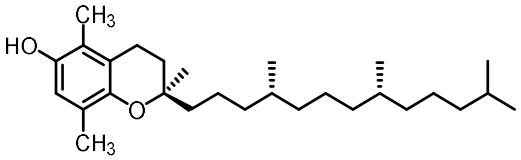
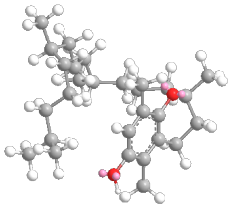
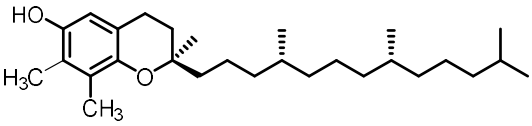
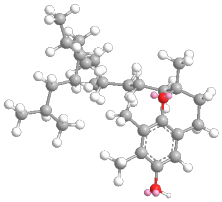
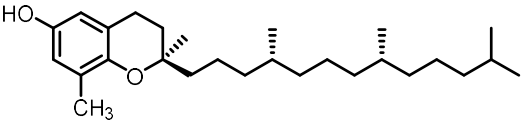
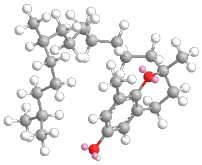
In aurantinidin, nitration can occur at five different positions: at the C8 and C3′ carbon, Table 8. The lowest energy value was found for the C-8 position (−64.1 kcal/mol).

Table 8. Nitration of aurantinidin with $^{\bullet}NO_2$.

C-Position	Energy Content (kcal/mol)	Developed Formula	Spatial Structure
C-8	−64.1		
C-3′	11.8		

We next computed the energy of the different tocopherols, β-tocopherol, γ-tocopherol, and δ-tocopherol. For these, the energy content, developed formula and spatial structure are depicted in Table 9.

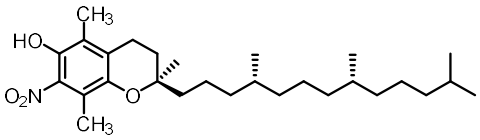
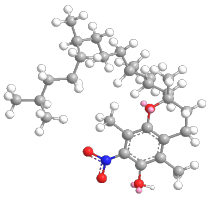
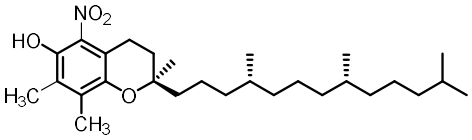
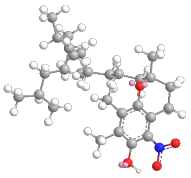
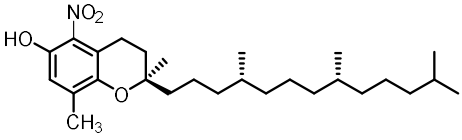
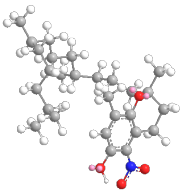
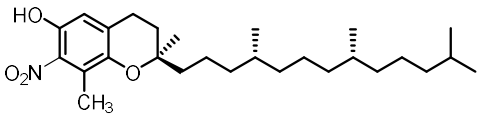
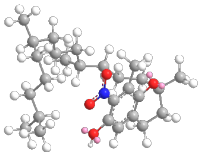
Table 9. Energy content, developed formula, and spatial structure of β-tocopherol, γ-tocopherol, and δ-tocopherol.

Name Energy Content (kcal/mol)	Developed Formula	Spatial Structure
β-tocopherol23.7		
γ- tocopherol21.9		
δ- tocopherol22.6		

In the following table, the energy content of the β-tocopherol, γ-tocopherol and δ-tocopherol was computed at the positions where nitration was possible. For β-tocopherol and δ-tocopherol it is possible at C-7 position and for γ -tocopherol and δ-tocopherol it is possible at C-5 position, Table 10. The energy content has been computed for each molecule. The lowest energy value was found for δ-Tocopherol in the C-5 position (22.3 kcal/mol).

As compounds commonly present in vitamin E, we also calculated the energy content of β-, γ- and δ-tocopherol, with the ability to scavenge the $^{\bullet}NO_2$ radical. In contrast to α-tocopherol, there is no position available in the aromatic ring, in which the $^{\bullet}NO_2$ radical can enter and be stably stored as a NO_2 group. When computing the energies for the nitrated compounds, we observed that for tocopherol, the energetic study showed that out of the 3 nitrated molecules, β- and γ-tocopherol only displayed one position for nitration, whereas δ-tocopherol could be nitrated at C-7 and C-5 positions. Only at the latter position, the energy content was slightly lower. In all other possibilities, the nitrated tocopherols molecules had a lower energy stability than the un-nitrated forms. In all four possible cases, no hydrogen bridge bond formation was observed that could significantly reduce their potential energy, as flavonoids. Nitration of δ-tocopherol is feasible, but not as feasible as the flavonoids, where nitration potential was found to be higher in all five cases studied. Etsuo Niki reported the same feature in his recent article [24], where same function of the tocopherols is argued, but it is not explained from a theoretical chemical point of view. Hydrogen abstraction has been postulated as a mechanism of action for all three vitamins against these reactive radicals. The high degree of agreement between the theoretical prediction and the experimental data demonstrated the validity of our theoretical framework.

Table 10. Energy content, developed formula and spatial structure of β-tocopherol, γ-tocopherol and δ-tocopherol, nitrated at C-5 and C-7 positions.

Name Nitration Position	Energy Content (kcal/mol)	Developed Formula	Spatial Structure
β Tocopherol C-7	37.5		
γ Tocopherol C-5	23.9		
δ-Tocopherol C-5	22.3		
δ-Tocopherol C-7	34.9		

4. Conclusions

In this study, research has focused on three complementary aspects: (i) on the chemistry of the antioxidant capacity of flavonoids and tocopherols, which allows them to act as free radical scavengers and to delocalise the charge along the aromatic rings; (ii) on the capacity of these compounds for scavenge the $^{\bullet}NO_2$ radical, which is a by-product of peroxynitrite decomposition, and therefore plays a key role as a cellular oxidant. At the same time, a multi-step biochemical mechanism is proposed to explain this feature of the studied compounds, a related mechanism to tyrosine nitration; and (iii) on the potential energy associated to the original molecules and the nitrated compounds, which has been calculated by molecular mechanics.

Under our chemical considerations, five flavonoids studied from different families (quercetin, naringenin, luteolin, catechin and aurantinidin) and three tocopherols (β-, γ-, and δ-tocopherol, but not α-tocopherol) can capture and stably store the $^{\bullet}NO_2$ radical. In most of the positions of the nitrated flavonoids, their final energy was lower to that of the original molecules indicating their potential to be scavengers of the $^{\bullet}NO_2$ radical.

This research might explain, from a theoretical perspective, the proven beneficial effect of flavonoids and tocopherols on cardiovascular health and their role as antioxidants. The scavenging of the $^{\bullet}NO_2$ radical by flavonoids and tocopherols could act as a metabolic modulator of great relevance. Further studies are necessary to elucidate the possible role of flavonoids and tocopherols as scavengers of peroxynitrite derivatives. We hope our preliminary study will help to stimulate the scientific community to find new solutions to strengthen the possible role of flavonoids and tocopherols as scavenger of the $^{\bullet}NO_2$ radical and their biological implications.

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