Chapter

Molecular Mechanism of Flavonoids Using Fluorescence Spectroscopy and Computational Tools

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Abstract

With more than 4000 compounds identified up to now, flavonoids are present in human diet since they can be found in fruits, vegetables, seeds, grains, and beverages, such as wine and tea. Over the years, medicinal properties of these polyphenolic compounds have been noticed. Consequently, the search for the biological targets and for the description of flavonoids action mechanism has been growing. Fluorescence spectroscopy and molecular docking are techniques based on physical theories which have been helping researchers to describe the interaction between flavonoids and biological targets. In this way, this chapter comes not only as an attempt to gather some works dedicated to explain flavonoid molecular mechanisms of action but also to introduce a brief theory of steady-state fluorescence spectroscopy and molecular docking.

Keywords: flavonoids, fluorescence spectroscopy, molecular docking, molecular mechanism, physical pharmacy

1. Introduction

More common than you might think, flavonoids are present in human diet since they can be found in fruits, vegetables, seeds, grains, and beverages, such as wine and tea [1]. They are most famous to beautify fruits and vegetables with vivid colors, but flavonoids most powerful actions are still unknown for most of population, which is unaware the antioxidant [2, 3], anticarcinogenic [4, 5], anti-inflammatory [6, 7], antiviral [8, 9], and antimicrobial [10, 11] effects provoked by these potent compounds. But, what are those compounds, after all?

Flavonoids, with over 4000 compounds identified until now [12], comprehend a wide group of molecules synthesized by plants as secondary metabolites responsible for ensuring vascular plant colonization and surviving on earth's environment [13]. These molecules play crucial roles to plants' life such as protection against insect attack and microbes invasion [14, 15] and by absorption of harmful ultraviolet radiation, attraction of insect pollinators by colorful anthocyanins synthesis [16], antioxidant action by inhibiting the generation of reactive oxygen species (ROS)

[17], involvement in pollen germination [13], involvement in biological communication in the rhizosphere [18] and action as regulators, involved in auxin transport and catabolism [19].

In general, flavonoids are polyphenolic compounds with the flavan nucleus as the structure skeleton, which consists of 15 carbon atoms arranged in 3 rings (C_6-C_3-C_6), labeled by A, B, and C (**Figure 1**) [3].

Flavonoids are classified into classes such as flavonol, flavone, flavanone, flavanol, isoflavone, and anthocyanidin, just to mention a few [20]. The basic structure of each class is shown in **Figure 2**. The differences between flavonoid class structures are in the oxidation level and C-ring substitution pattern, while the difference among flavonoids belonging to the same class is in the pattern of A- and B-ring substitutions [3].

As reviewed by Aidyn Mouradov and German Spangenberg [13], flavonoid synthesis in plants occurs commonly from the aromatic amino acids phenylalanine and tyrosine. At the beginning of synthesis, the amino acid is converted into coumaroyl-CoA by a number of enzymatic reactions involving *phenylalanine ammonia-lyase* (PAL), *cinnamate 4-hydroxylase* (C4H) and *4-coumarate-CoA ligase* (4CL). The *naringenin-chalcone synthase* (CHS) and *chalcone isomerase* (CHI)

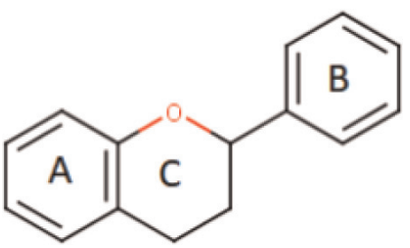

Figure 1.
Flavan nucleus, the structure skeleton of flavonoids.

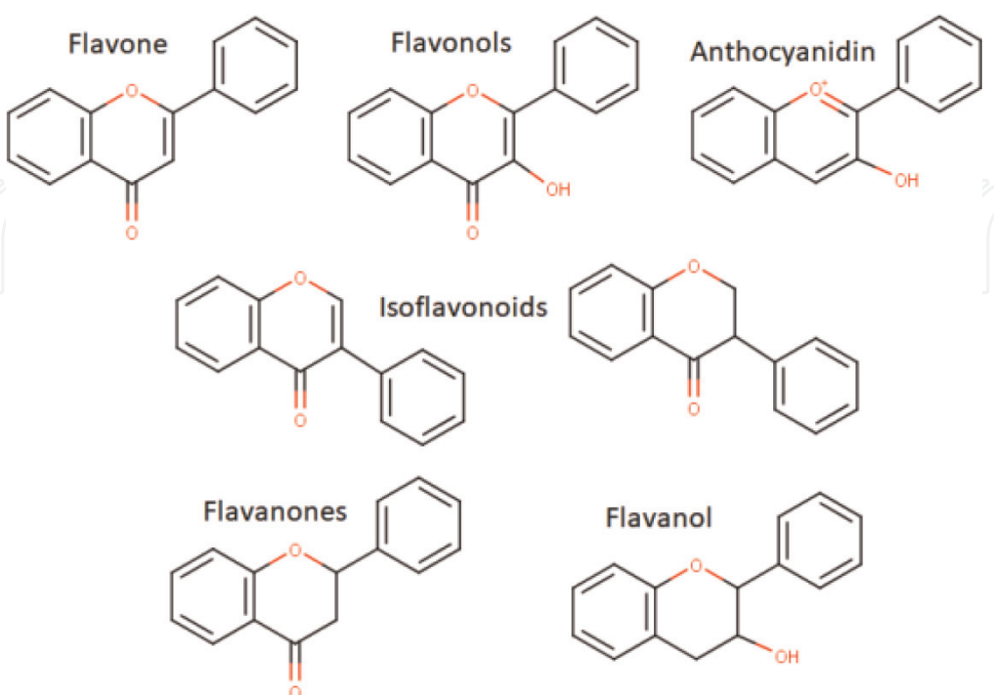

Figure 2.
Basic structures of different classes of flavonoids.

convert coumaroyl-CoA into naringenin; the pathway opens in different ways generating flavanones, dihydroflavonols, leucoanthocyanins, anthocyanidins, and flavan-3-ols by series of enzymatic reactions. These molecules can generate a couple of other compounds such as flavones and isoflavones and flavonols and anthocyanins. These flavonoids are generally found in plant epidermal cells, chloroplasts, vacuole, and nucleus [13].

Epidemiological studies reviewed by Romano and co-workers [21] have demonstrated an inverse relationship between dietary flavonoid intake and prevalence and risk of cardiovascular diseases and some types of cancer such as breast, colon, lung, prostate, and pancreas. Besides that, the authors emphasized the advances of the application of flavonoids in diseases related to the central nervous system, obesity, diabetes, inflammation, digestive system, and respiratory tract, as well as the effects of flavonoids in reproduction and the antimicrobial effects of these compounds.

Many efforts have been done to elucidate the mechanism of action of these compounds by using experimental approaches based on physical observable. Steady-state fluorescence spectroscopy is a technique widely used to follow the interaction of small molecules and biological targets. However, there are many others experimental techniques that have been used to describe biological systems, such as time-resolved fluorescence spectroscopy, nuclear magnetic resonance (NMR), isothermal titration calorimetry (ITC), circular dichroism (CD), and surface plasmon resonance (SPR).

Besides that, it has also used computational analysis in order to describe the interactions between flavonoids and molecular targets. Molecular docking and molecular dynamics are the most common computational techniques used to model biological systems.

In this way, this chapter comes not only as an attempt to gather some works dedicated to explain the molecular mechanisms of action of some flavonoids but also to introduce a brief theory of steady-state fluorescence spectroscopy and molecular docking.

2. Theory of steady-state fluorescence spectroscopy

The use of fluorescence spectroscopy experiments to understand the protein-ligand complex formation has become increasingly frequent in scientific community because many proteins have endogenous fluorescence probe such as tryptophan, tyrosine, and phenylalanine amino acids. Following the fluorescence signal of these probes, it is possible to characterize the molecular mechanism of complex formation [22].

As the name already suggests, this technique makes use of fluorescence as the physical observable. Fluorescence, by definition, is a photon emission mechanism which occurs by the decaying of molecule electrons from a higher energy singlet state to a lower energy singlet state with emission rate of the order of 10^8 s^{-1}, resulting in a typically fluorescence lifetime close to 10 ns [22].

The fluorescence spectroscopy, when applied in protein-ligand system, consists in analyzing the quenching of fluorescence signal in the presence of different concentrations of ligand. Quenching may be static or collisional (dynamic) depending on the nature of interactions. In collisional quenching, the fluorophore, which is in the excited state, is deactivated returning to its ground state because of diffusive encounters with some other molecule of the solution, called quencher. On the other hand, the static quenching occurs when a fundamental and non-fluorescent complex occurs [22].

In a typical experiment of fluorescence spectroscopy, the way to distinguish the quenching mechanism is analyzing the Stern-Volmer plot (Eq. (1)) in different temperatures, where F_o is the fluorescence intensity in the absence of the quencher and F is the intensity in different concentrations of the quencher, $[L_t]$ [22].

$$\frac{F_0}{F} = 1 + K.[L_t] \tag{1}$$

The increase in the constant K as a consequence of the increase in temperature is a strong indication that the quenching mechanism is collisional; in that case the constant is usually denoted by K_d. On the other hand, if the constant K decreases with the increasing in temperature, the characteristic mechanism is the static one, and the constant is usually denoted by K_{sv}. **Figure 3** shows the different behaviors of the static and collisional quenching [22].

If the mechanism determined was the static one, there are couples of models that can be applied in the system in order to obtain the association constant K_a or also called binding constant. The most common and simplest model reported in literature to study protein-ligand is the binding equilibria for a first-order reaction, where the ligands (L) are entering one by one at the binding site of the protein (P) [23].

$$\mathrm{P} + \mathrm{L} = \mathrm{PL} \rightarrow \mathrm{LP} + \mathrm{L} = L_2\mathrm{P} \rightarrow L_2\mathrm{P} + \mathrm{L} = L_3\mathrm{P} \ldots \rightarrow L_{n-1}P + L = L_nP$$

Assuming that the sites are equivalents and independents, the association constant is

$$K_a^n = \frac{[L_n.P]}{[L]^n.[P]} \tag{2}$$

where [P] is the free protein concentration, [L] is the free quencher concentration, and $[L_nP]$ is the concentration of protein-quencher complex. In this model, the protein is either free in solution or bound to quencher, then

$$[P_t] = [L_nP] + [P] \tag{3}$$

where $[P_t]$ is the concentration of total protein in the system [22]. Considering that the fluorescence intensity is only emitted by free proteins

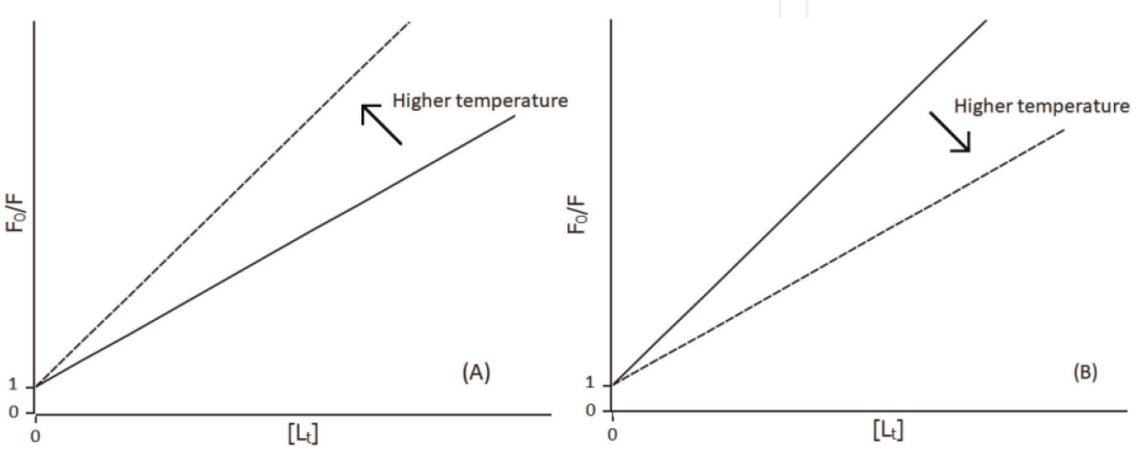

Figure 3.
Examples of different quenching mechanisms. (A) Collisional quenching and (B) static quenching.

$$\frac{[P_t]}{[P]} = \frac{F_0}{F} \tag{4}$$

Eq. (2) can be rearranged to

$$K_a^n = \left(\frac{F_0 - F}{F}\right).\left(\frac{1}{[L_t] - \left(\frac{F_0 - F}{F}\right).[P_t]}\right)^n \tag{5}$$

Applying the logarithm function in Eq. (5) and rearranging it

$$\log\left(\frac{F_0 - F}{F}\right) = n.\log K_a - n.\log\left(\frac{1}{[L_t] - \left(\frac{F_0 - F}{F}\right).[P_t]}\right) \tag{6}$$

Eq. (6) is known as double-logarithm equation, where one can calculate the binding constant K_a. The binding constant is related to thermodynamic parameter through the van't Hoff Equation [24]:

$$\ln K_a = -\frac{\Delta H}{R.T} + \frac{\Delta S}{R} \tag{7}$$

where R is the universal gas constant (≈ 8.31 J/mol.K) and T is the temperature. In a graphic of ln K_a versus T, the enthalpy change (ΔH) and the entropy change (ΔS) are obtained by the slope of the linear function and the linear coefficient, respectively.

Ross and Subramanian [25] associated the enthalpy variation and entropy variation with the most predominant interactions that stabilize the complex, as showed in **Table 1**.

Besides that, Gibbs free energy variation says about the spontaneity of the complex formation process, where $\Delta G > 0$ indicates a non-spontaneity process and $\Delta G < 0$ indicates a spontaneity process. The enthalpy variation, on the other hand, indicates if the system is either an exothermic process ($\Delta H < 0$) or an endothermic process ($\Delta H > 0$) [24, 25].

Unlike the binding equilibrium method that is based upon a first-order reaction predicting a single binding site, there is another method that does not make use of a previous model, and in addition to determining the number of binding sites, it can still determine the cooperativity, if any. This method was developed by Scatchard [26], and it is not as popular as binding equilibrium method to characterize protein-ligand interaction in the scientific community yet.

This method makes use of another technique to obtain preliminary data known as binding density function (BDF) [27], where a physical observable is chosen to follow the interactions, such as absorbance and fluorescence intensity, among others. Supposing that the intensity of fluorescence emitted by the protein was chosen as physical observable, in which in an aqueous system containing protein is titrated by the ligand. Considering the system in equilibrium, the average number of quencher bound by protein $\Sigma\nu_i$ is determined from a given free quencher

ΔH	ΔS	Predominant interaction
< 0	< 0	van der Waals and hydrogen bonds
> 0	> 0	Hydrophobic interactions
≈ 0	> 0	Ionic process

Table 1.
Expected signs of contributions to ΔH and ΔS.

concentration (L); in this context, if the free quencher concentration is the same for two or more solutions of different concentrations of total protein P_t, then the average distribution of the binding density of the quencher will also be the same, reaching in the expression for the mass conservation [27]:

$$[L_t] = [L] + (\sum \nu_i).[P_t] \tag{8}$$

where $[L_t]$ is the total quencher concentration, [L] is the free quencher concentration, and $[P_t]$ is the total protein concentration. In order to obtain the average number of quenchers bound ($\Sigma\nu_i$), a graphic of total quencher concentration $[L_t]$ versus the total protein $[P_t]$ can be plotted, in which by the angular coefficient, the $\Sigma\nu_i$ is obtained and by the linear coefficient of the linear function [L] is obtained. The values for $[P_t]$ and $[L_t]$ are obtained from the graphic of ΔF versus log $[L_t]$ shown in **Figure 4**, where ΔF is the suppression percentage [27].

Once the values of $\Sigma\nu_i$ and [L] were obtained from the graphic in the BDF method, the method developed by Scatchard [26] can be used to follow protein-ligand interaction. To determine all those information from the method, a graphic of $\Sigma\nu_i/[L]$ versus $\Sigma\nu_i$ is plotted, and from the function obtained in the graphic, it can be said that the sites have cooperativity or they are equivalents and independents.

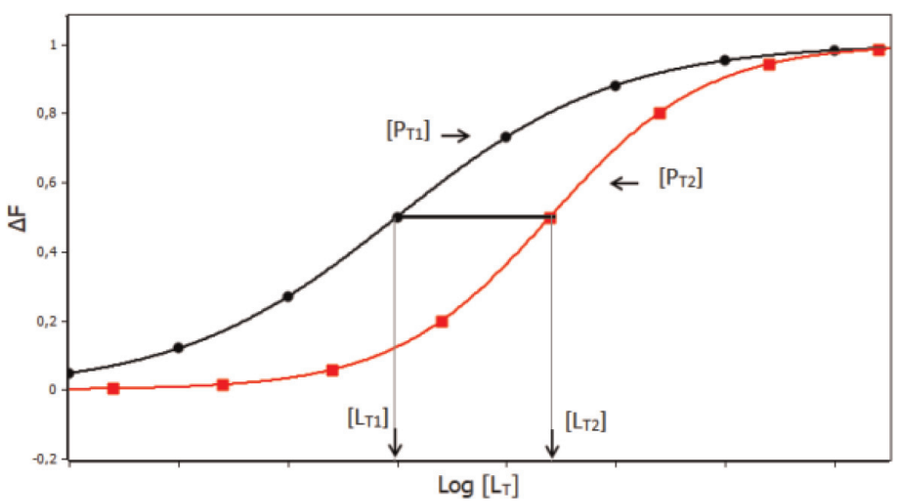

Figure 4.
Example of graph used in BDF theory. In this case, the percentage of quenching was measured in two different concentrations of protein, P_{T1} and P_{T2}.

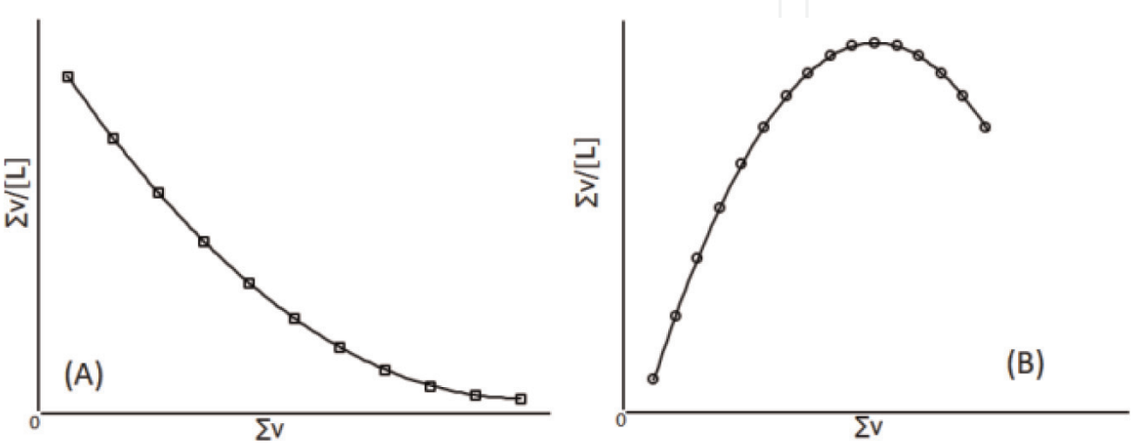

Figure 5.
Illustration of two Scatchard graphics. (A) Negative cooperativity and (B) positive cooperativity.

Bordbar and co-workers [28] reported the mathematical functions that describe the behavior of the system in the Scatchard plots with the type of cooperativity. They determined that if the function is polynomial with the positively concavity (**Figure 5(A)**), the protein has negative cooperativity between the sites. If instead there is a polynomial function with negative concavity, the protein has a positive cooperativity among the sites (**Figure 5(B)**) [28].

For these cases where the Scatchard graph shows a cooperativity function shape, one can apply the equation developed by Hill [24] to determine the number of sites n of each set of equal sites and the K_a association constant of each set of interaction sites:

$$\sum \nu_i = \frac{n_1.(K_{a1}.[L])^{H1}}{1+(K_{a1}.[L])} + \frac{n_2.(K_{a2}.[L])^{H2}}{1+(K_{a2}.[L])} \tag{9}$$

where [L] is the concentration of free quenchers and H is Hill's index. If H = 1 the system is noncooperative, H > 1 the system has positive cooperativity, and H < 1 the system has negative cooperativity [24, 28].

In the case that the function, which describes the system, is linear, the protein has no cooperativity among the sites, and all sites are identical and independent. For this behavior, Scatchard [26] developed his own mathematical model to find the number of sites n and the association constant K_a:

$$\sum \nu_i = \frac{n.K_a.[L]}{1+K_a.[L]} \tag{10}$$

To rearrange in the form of linear equation to model the Scatchard graphic, it is given as follows:

$$\frac{\sum \nu_i}{[L]} = n.K_a - K_a.\sum \nu_i \tag{11}$$

3. Theory of molecular docking

Molecular docking is a powerful technique that has been used in association with experimental data to determine ligand binding sites in targets with pharmacological interest. It can also predict the ligand conformation in the binding site and consequently the interactions that stabilize the complex. In addition, molecular docking has been also used as an efficient and a cheap technique for virtual screening large molecule databases and selects compounds which bind with specificity in pharmaceutical targets before carrying out in vitro and in vivo experiments [29, 30].

Molecular docking consists in determining the most probable conformations of the complex composed by receptor ligand based on an energy ranking of each conformation. To obtain this energy ranking, the ligand is put to interact in an environment under a protein force field including non-covalent potentials such as van der Waals force, hydrogen bonds, and electrostatic nature forces [29]. There are several softwares to perform this kind of prediction such as AutoDock, GOLD, DOCK, and FlexX, just to mention a few.

Each of those mentioned software has its own potential mathematical function to elucidate the forces involved in the complex and calculate the energy score to be ranked. The following is an example of potential utilized by AutoDock04 [31] software:

$$V = I.\sum_{i,j}\left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}}\right) + J.\sum_{i,j}E(t).\left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}}\right) + K.\sum_{i,j}\frac{q_i.q_j}{\varepsilon.r_{ij}^{2}} + \Delta W_S \qquad (12)$$

The weighting constants I, J, E(t), K, and W are those optimized to calibrate the empirical free energy based on a set of experimentally characterized complexes. The first term is the Lenard-Jones potential, in which parameters A and B are taken from the Amber force field [31]. The second term refers to the hydrogen bond in which the parameters C and D are obtained to ensure a minimum energy of 5 kcal/mol in 1.9 Å for O-H and N-H and 1 kcal/mol in 2.5 Å for S-H. The function E(t) provides directionality based on the angle t of the geometry of an ideal hydrogen bond [31]. The third term is a shielded Coulomb potential for electrostatic interaction. The last term is the desolvation potential based on the volume of the atoms surrounding a given atom and shelter it of the solvent [31].

To reduce the computational costs, the programs usually make use of a precalculation type. The software creates grid maps for each type of atom present in the ligand to be used in the docking. Grid maps consist of a three-dimensional array of regularly spaced points centered on the active site of the protein or macromolecule under study. Each point inside the grid maps records the energy interaction of a test atom with the protein [31]. The complexity of finding the best conformation requires computational methods with the potential to effectively investigate a large number of possible solutions, aiming to find the best result. The search algorithms that are usually utilized by molecular docking softwares can be classified into three categories such as systematic, deterministic, and stochastic search methods [32].

In systematic search algorithms, each degree of freedom has a set of values, so that all degrees of freedom of the molecule are explored combinatorically during the search. Examples of systematic search algorithms are anchor-and-grow or incremental construction algorithm [32]. The deterministic search methods are characterized by the fact that, given the same initial input state, they always produce the same output. It happens because the initial state determines the possible movement to generate the next state, which has to have the same or lower energy than the previous state. Examples of deterministic search are energy minimization methods and molecular dynamics (MD) simulations [32]. Stochastic methods vary randomly in all degrees of freedom of the ligand (translational, rotational, and conformational) at each step, generating a great diversity of solutions. The solutions are evaluated according to a probabilistic criterion to decide if they will be accepted or not. In this way this method requires a large number of conformations to obtain a desired result. Monte Carlo, simulated annealing, and evolutionary methods are examples of stochastic search method, and they are the most common in molecular docking softwares [32, 33].

4. Molecular targets for flavonoids

Many efforts have been done to describe flavonoids' main biological targets involved in cellular processes such as inflammation and cancer. Flavonoid mechanism of action in cellular responses includes the inhibition of proteins in cytoplasm medium such as IκB kinase (IKK) complex and mitogen-activated protein kinases (MAPKs), as well as in extracellular medium such as interleukins and other cytokines.

In the pathway that triggers the activation of nuclear factor κB (NF-κB) in tumor necrosis factor (TNF) stimulated cells (**Figure 6**), IKK complex is a target for many flavonoids such as apinegin [34], luteolin [34], acacetin [35], ochnaflavone [35], delphinidin [35], and morin [36].

The inhibition of IKK complex results in failure in phosphorylation of Iκβ. As a consequence, NF-κB is not released to translocate to the nucleus. In the case of the flavonoids apinegin and luteolin, they can also inhibit NF-κβ activation by inhibition of MAPKs [34].

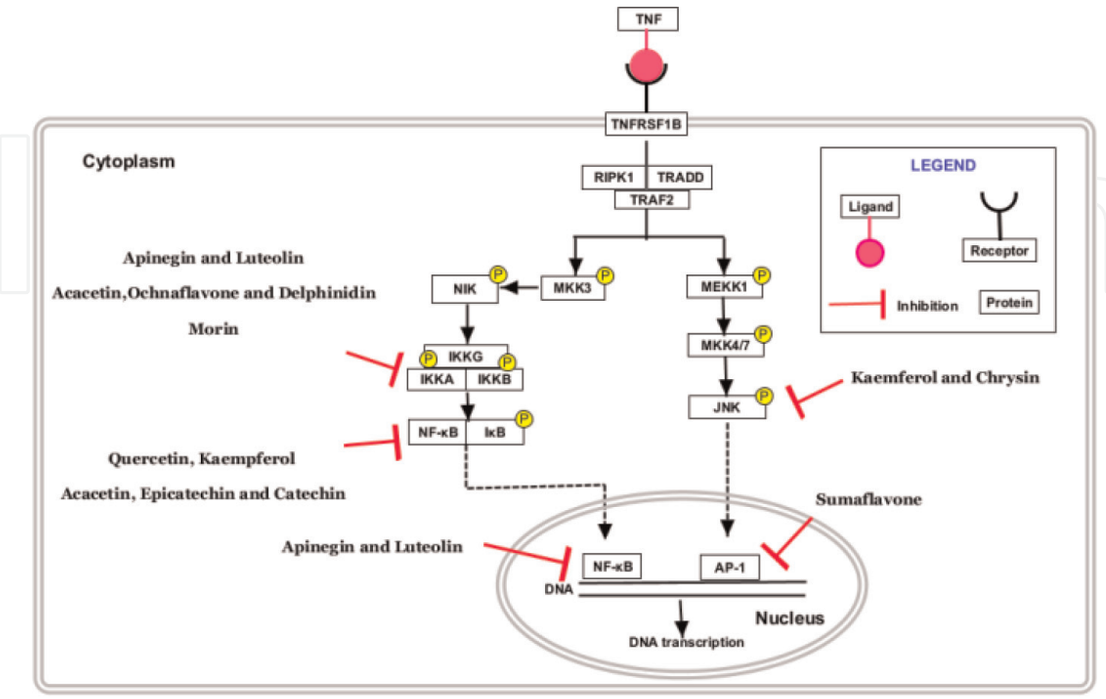

Figure 6.
NF-kB and AP-1 activation pathways induced by TNF.

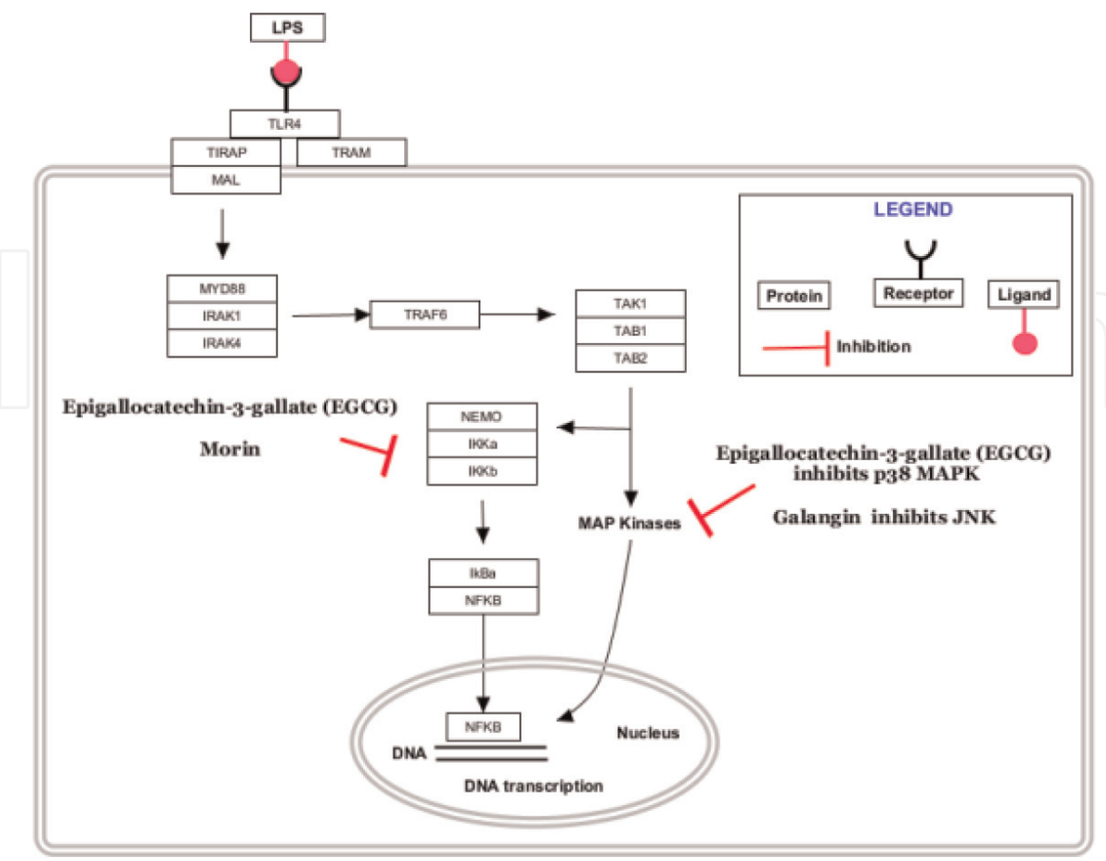

Figure 7.
NF-kB activation pathway induced by LPS.

In the pathway that triggers the activation of activator protein 1 (AP-1), c-Jun N-terminal kinase (JNK) is a target for flavonoids kaempferol and chrysin [34]. Such flavonoids bind to the protein and inhibit the activation of AP-1. Sumaflavone also suppresses the AP-1 activation, but the main target for this flavonoid has not been identified yet [35].

In LPS-stimulated cells (**Figure 7**), epigallocatechin-3-gallate (EGCG) [37] and morin [36] inhibited the activation of NF-κB activity by IKK complex inhibition. Besides that, EGCG also inhibited p38 MAP kinase activity [37]. Another molecular target in LPS-stimulated cells is JNK, which is inhibited for galangin [38].

The molecules genistein, kaempferol, quercetin, and daidzein inhibited the activation of STAT-1 and NF-kB in LPS-induced cells, while flavone, isorhamnetin, naringenin, and pelargonidin inhibited only the NF-kB activation. However the molecular targets for such flavonoids have not been identified yet [39, 40].

Cytokines can also be targets for flavonoids. As verified by Li and co-workers [41], baicalin is able to complex with a variety of chemokines such as SDF-1a, IL-8, MIP1-b, and MCP-2 and reduce the capacity of the cytokines to bind and activate their receptors.

Despite the works described above show which proteins are inhibited by flavonoids, the methodology used does not give information related to how strong is the affinity of the flavonoid for the protein, which amino acids of the protein participate in the interaction with flavonoids, which are the molecular driving forces involved in the interaction, the thermodynamic parameters of complex formation, how many binding sites there are in the protein for the flavonoids, and whether the protein presents cooperativity among their sites. In this way, the methodology of fluorescence spectroscopy and molecular docking described in Sections 2 and 3 come to help give this set of information that are of the utmost importance to drug discovery.

Among the target proteins mentioned above, NF-κβ (p50 and p65 subunits) has one tryptophan residue, while JNK have four tryptophan residues. Besides that, all the cytokines (SDF-1a, IL-8, and MCP-2) mentioned above have 1 tryptophan residue, except for MIP1-b which has 12. The presence of tryptophan makes the use of fluorescence spectroscopy possible, but the presence of more than one makes the interpretation of the spectral data more complicated. Different of the proteins mentioned above, AP-1 does not have tryptophan residue. On the other hand, there are tyrosine residues, which can also be followed in the experiments.

Besides the endogenous fluorescent probe, solubility is another important factor because fluorescence spectroscopy experiments are conducted with protein in solution. As the proteins mentioned above are found in cytoplasm or in extracellular medium, they are expected to be soluble in buffer.

5. Physical pharmacy in the description of molecular mechanism of flavonoids

As already discussed in the sections before, the stabilization of the complex is made by non-covalent interactions, such as hydrogen bonds, hydrophobic interactions, van der Waals and electrostatic forces, salt bridge, π stacking, and cation-π interactions. Consequently, the affinity and the thermodynamic parameters of each system are results of these interactions.

The complete characterization of a complex involves the description of these interactions, the affinity, and the thermodynamics. That is the reason why the use of techniques based on physical approaches in the pharmacy area has been growing.

Several authors characterized the complex formed by flavonoids and biological targets based on fluorescence spectroscopy data, as showed in Section 5.1. Some others used molecular docking to describe the complexes, as shown in Section 5.2.

Nevertheless, the most accurate description of the complex is reached by the association of data from fluorescence and docking, as one can see in Section 5.3.

5.1 The use of steady-state fluorescence spectroscopy

Based on the theory of fluorescence quenching described in Section 2, several authors investigated the molecular mechanism of interaction between flavonoids and proteins. The most basic method used to estimate the affinity of the ligand toward the protein is the Stern-Volmer plot, where one can calculate the Stern-Volmer constant (K_{sv}) and associate the magnitude of this constant with the binding affinity.

Papadopoulou and co-workers [42] used K_{sv} to evaluate the interaction of two flavonoids, quercetin and rutin, and the bovine serum albumin (BSA). The authors verified that quercetin demonstrated stronger affinity toward BSA compared to rutin. Such difference was explained based on the structure of both flavonoids, once rutin is a glucoside of quercetin. The authors concluded that hydrophobicity was an important factor in the change of affinity, once the incorporated disaccharide rutinose made the flavonoid less hydrophobic. Besides that, another important factor was the steric hindrance caused by the incorporated disaccharide.

With a similar methodology, Cao and co-workers [43] investigated the influence of glycosylation of quercetin and baicalein in the affinity toward BSA. The authors used the Stern-Volmer plot to classify the quenching mechanism as static or dynamic. Then they used the double-logarithm plot to calculate the binding affinity of the ligands studied. They verified that the glycosylation made the interaction weaker and suggested that the decrease in binding constant was an effect of steric hindrance caused by glycoside groups.

The influence of glycosylation in the binding affinity observed by Papadopoulou and co-workers [42] and Cao and co-workers [43] was attributed to steric hindrance. The steric hindrance suggested by the authors could be verified if they had used molecular docking to model the system.

The influence of ions Cu^{2+}, Al^{3+}, Mg^{2+}, and Zn^{2+} in the interaction of flavonoids and human serum albumin (HSA) was investigated by Bi and co-workers [23]. The authors calculated the binding constant by double-logarithm plot (Eq. (6)), and they concluded that binding constants were 14.2–99.6% of the ones without these metal ions. Therefore, the ion concentration would shorten the storage time of the compounds in blood plasma and enhance the effectiveness of the flavonoids. The influence of ions in the binding constant was also observed by Hu and co-workers [44] during experiments with morin and BSA. In this case, the authors observed that the constant decreased in the presence of Ba^{2+} and Hg^{2+}, but with the addition of the ions K^{+}, Li^{+}, Mn^{2+}, Fe^{2+} and Sn^{2+}, the constant increased.

Another experimental methodology has been used to characterize the interaction between protein and small ligands. As described in Section 2, thermodynamic parameters are calculated from fluorescence quenching data by using the van't Hoff equation.

Li and co-workers [45] used fluorescence experiments to compare the inhibition ability of three flavonoids (quercetin, isoquercetin, and rutin) toward *α-glucosidase* activity. They concluded that the complexes formed by the protein and the three flavonoids were spontaneous processes, driven mainly by hydrophobic forces, once ΔS and ΔH are both positive numbers. Based on the results obtained, the authors suggested that the flavonoids studied would be useful as an inhibitor of the enzyme and would help in the treatment of hyperglycemia and obesity.

Xi and co-workers [46] studied the interaction between hemoglobin and the flavonoids quercetin and rutin by using double-logarithm and Van't Hoff plots. The authors observed that for both flavonoids, the values of ΔH and ΔS were

negative and concluded that the acting forces are hydrogen bonding and van der Waals. As one can see, the results obtained by [45, 46] reveal that molecular forces that drive quercetin and rutin into *α-glucosidase* and hemoglobin are different. This difference is noticed because the same molecules interact in cavities with different characteristics.

Another molecular target for flavonoids in biological systems is nucleic acid. The study of the interaction between flavonoids and DNA using fluorescence spectroscopy is made by following fluorescence signal from flavonoids once DNA exhibits a very weak intrinsic emission.

The article published by Jana and co-workers [47] described the affinity of 3-hydroxyflavone toward DNA, by following the flavonoid fluorescence signal. The same methodology was used by Sengupta and co-workers [48] to describe the affinity of fisetin toward DNA. The interaction of polyphenolic compounds with DNA has a protective role, once flavonoids exhibit antioxidant properties.

DNA is also a target for luteolin, as shown in the article published by Chowdhury and co-workers [49]; the interaction between luteolin and nucleic acid is one of the causes of the decrease in affinity between DNA and *topoisomerase* I.

5.2 The use of molecular docking

The influence of flavonoid structure, the addition of organic group as well as the main interaction between the small molecules and biological targets can be described through the molecular docking technique.

The potential of some flavonoids as inhibitors of *α-amylase* verified by experimental assays was explained in the work published by Lo Piparo and co-workers [50, 51] using molecular docking. The authors observed that flavonols and flavones have the same scaffolds (**Figure 2**), and both subclasses possess a carbonyl group in the position C4 of the pyrone ring. The C2-C3 double bond is conjugated to the 4-keto group and is responsible for electron delocalization between the ring C and ring A. As a consequence, flavones and flavonol form a highly conjugated π-system that confers better stability of the protein-ligand complex. The authors also described a specific pattern of OH group, which interacts with the catalytic residues and promotes the inhibition of the enzyme.

The inhibitory effect of some flavonoids was also observed for *β-secretase*; the work published by Shimmyo and co-workers [52] showed that OH groups in myricetin, quercetin, kaempherol, morin, and apigenin stabilized the binding poses of flavonoids against the *β-secretase* active center by hydrogen bonds. In many cases, the OH directly interacted with the Asp catalytic residue and enhanced the inhibitory activity of polyphenolic compounds.

Si and co-workers [53] showed that some flavonoids inhibiting CYP2C9 activity may increase the risk of toxicity from coadministered drugs that are CYP2C9 substrates. The authors used molecular docking to give details about the interaction of CYP2C9–6-hydroxyflavone complex, showing that the 6-hydroxyflavone is bound by a π-π stacking interaction with the phenyl group of Phe 100 and by two hydrogen bonds with Leu102 and Phe100.

The examples above used molecular docking to describe the interaction between flavonoids and molecular targets in order to explain the results obtained from experimental assays. However, one can also use molecular docking in order to select the best candidates and then submit the chosen molecules to the experimental assays.

In the work published by Salam and co-workers [54], the authors built a library of 200 natural compounds including some flavonoids. With virtual screening, they selected the 29 best candidates to bind to the peroxisome proliferator-activated

receptors (PPARs) in the cell culture experiments. Besides that, docking studies showed that flavonoids are predicted to occupy the hydrophobic environment formed by residues Phe282, Phe360, and Phe360. They also showed that there is one specific OH group in all the flavonoids studied that is responsible to make a hydrogen bond with the receptor, except in the case of apigenin.

5.3 The use of fluorescence spectroscopy associated with molecular docking

Data from fluorescence spectroscopy offers a description of the complex in terms of binding constant, number of binding sites, cooperativity, and the thermodynamics of the complex formation. On the other hand, molecular docking offers details about the binding site environment, the ligand conformation, and the interactions that stabilize the complex. Some authors associated the fluorescence quenching data with the results from molecular docking in order to give a complete description of the biological system.

He and co-workers [55] verified the tryptophan fluorescence quenching of HSA caused by alpinetin. The authors used the van't Hoff model to calculate the thermodynamic parameters and concluded that the hydrophobic interactions were predominant to stabilize the complex. With molecular docking technique, the authors selected the best binding site of alpinetin in the HSA and showed that the binding site was close to tryptophan, which explains the static quenching. The authors also showed that the binding site was a hydrophobic cavity, which explains the data obtained by van't Hoff equation.

With a similar methodology, Kim and co-workers [56] showed the relationship between the number of OH groups in flavonoids and the affinity for mushroom *tyrosinase* by fluorescence quenching. Molecular docking studies showed that the dicopper catalytic site of *tyrosinase* is a preferential binding site for flavonoids, which explains the inhibitory activity of the polyphenolic compounds.

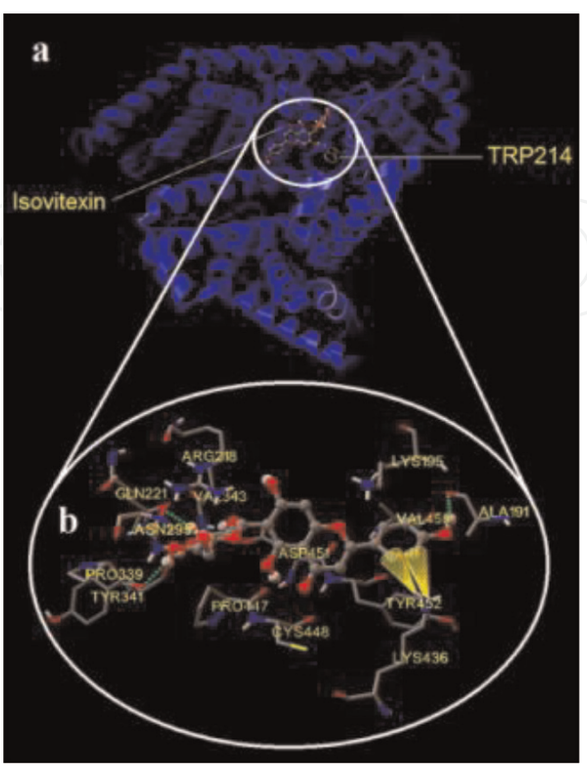

Figure 8.
(a) Binding site of isovitexin in subdomain IIA of HSA (b) Binding site microenvironment [57].

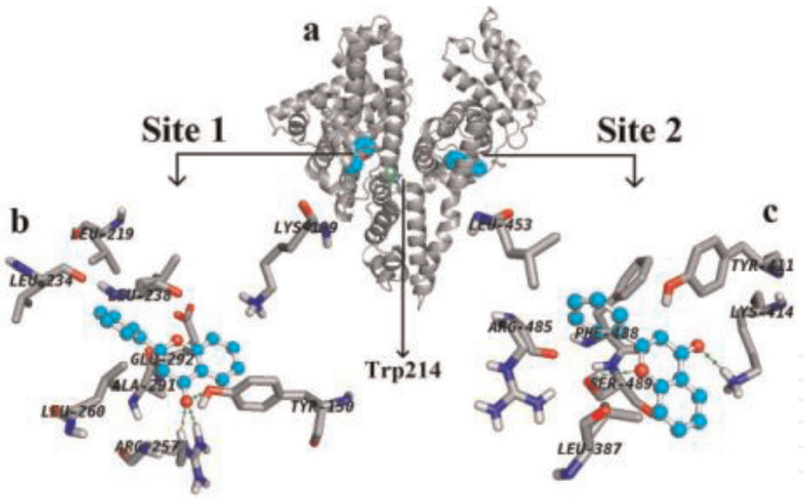

Figure 9.
(a) Location of the 2-phenylchromone molecules in HSA (b) Microenvironment of site 1, subdomain IIA of HSA (c) Microenvironment of site 2, subdomain IIIA of HSA.

Caruso and co-workers [57] used fluorescence spectroscopy to describe the interaction between isovitexin and HSA. The authors calculated the contribution of enthalpy and entropy by van't Hoff equation and concluded that the process was enthalpically driven. The complete description of the system was reached with molecular docking, as one can see in **Figure 8**. The oxygen atoms of Ala191 and Pro339 form hydrogen bonds with isovitexin, while neutral polar side chain residues like Gln221, Asn295, Tyr341, and Tyr452 are mainly taking part in electrostatic interactions (charge neutralization).

In this last article, the authors used the Scatchard method, which has been recently used to calculate the number of binding sites and to verify allosterism. With the Scatchard method, the authors concluded that there was one binding site for isovitexin in HSA, which was in agreement with the results obtained by the authors when they used the binding equilibrium model.

In another article, Caruso and co-workers [58] used a similar methodology in order to describe the interaction between 2-phenylchromone and HSA. In this case, the contributions of enthalpy and entropy calculated by van't Hoff equation showed that the process was entropically driven. With the Scatchard method, the authors concluded that there were two cooperative binding sites for 2-phenylchromone in HSA with two different binding constants. Note that this type of information could not be obtained from Stern-Volmer or double-logarithm models; only the Scatchard method can offer information about the system without the use of any binding model a priori.

Also in this study, molecular docking showed that 2-phenylchromone binds at subdomain IIA (Site 1) and subdomain IIIA (Site 2). As shown in **Figure 9**, at Site 1 amino acids Lys199, Leu (219, 234, 238, and 260), Ala291, and Glu292 are residues involved in the interactions with the molecule. At Site 2 amino acids Leu387, Tyr411, Leu453, Arg485, and Phe488 compose the microenvironment of interaction.

6. Conclusion

This chapter presents the real possibilities of combinations, between physical experimental techniques with computational tools which can contribute significantly to the advancement of the proposal of new drugs within the point of view of molecular action. Moreover, it has been demonstrated how to carry out such work,

combining the knowledge between fluorescence spectroscopy and molecular docking. These techniques may be added to pharmacology, pharmacokinetics, and pharmacognosy research fields, which represent the cradle of the search for new medicinal products and design.

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Conflict of interest

The authors declare that no conflict of interests exists.

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