A novel approach to trypsin inhibition by flavonoids

Alejandra I. Martinez-González, Ángel G. Díaz-Sánchez, Laura A. de la Rosa, Ismael Bustos-Jaimes and Emilio Alvarez-Parrilla*

Department of Chemistry, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Ciudad Juárez 32310, México

Department of Biochemistry, Facultad de Medicina, Universidad Nacional Autónoma de México, México D.F. 04510, México

Corresponding author: Emilio Alvarez-Parrilla, Departamento de Ciencias Químico Biológicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Ciudad Juárez 32310, México. E-mail: ealvarez@uacj.mx

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Abstract

Trypsin is a key protease related to digestion and absorption of proteins, which its inhibition must be studied when natural compounds, such as flavonoids, are used as part of alternative treatments for obesity and diabetes mellitus type 2, since trypsin and other pancreatic enzymes work at small intestine. Considering that flavonoids are good lipase and amylase inhibitors, trypsin-flavonoids interactions were analyzed through UV-Vis, intrinsic and extrinsic fluorescence spectroscopies, circular dichroism, and molecular docking. The interaction between porcine pancreas trypsin and five flavonoids: hesperetin (HES), luteolin (LUT), quercetin (QUE), catechin (CAT), and rutin (RUT) was evaluated. Most of them exhibited a mixed-type inhibition mode. LUT was the best trypsin inhibitor (e.g., lower IC50, 45.20 ± 1.00 µM). All flavonoids-trypsin complexes showed static quenching, and QUE and LUT exhibited higher affinity (associative binding constant, K_a values, 0.90 ± 0.10 and 1.60 ± 0.20·10^−1 mM^−1, respectively). Hydrophobic interactions between trypsin and flavonoids were predominant.

Keywords: Trypsin; Flavonoids; Inhibition; Structure-activity relationship.

1. Introduction

Noncommunicable diseases and their risk factors, such as diabetes mellitus type 2 and obesity, respectively, are being a target of alternative treatments for their control (Chang et al., 2020; Pelvan et al., 2021). In this way, the prevention and treatment of obesity is important to reduce the prevalence of diabetes mellitus type-2 (Liu et al., 2020). One of these treatments is related to the inhibition of catalytic activity of two relevant pancreatic enzymes: lipase and α-amylase. For example, pancreatic lipase inhibitors can diminish its catalytic activity, and can control fat levels in blood, since this enzyme is responsible for the hydrolysis of approximately 70% of dietary fats (Birari and Bhutani, 2007; Liu et al., 2020). Polysaccharides including flavonoids have been analyzed as good inhibitors of these enzymes, in comparison to FDA approved drugs such as acarbose for pancreatic α-amylase (Martinez-Gonzalez et al., 2019).

Polyphenols are secondary metabolites of plants, and they are involved in their reproduction, development, and defense against pathogens agents, among others (Huang et al., 2009). Flavonoids correspond to the biggest polyphenolic family. Most of them are derivatives of the 2-phenil-benzo-γ-pyran, they possess a common structure of two aromatic rings ("A" and "B"), and one heterocyclic ring ("C") (Harborne, 1964; Ribeiro et al., 2015). Flavonoids can be grouped in several subfamilies, depending on the number of hydroxyls present in their structure, presence of double bonds and oxidation level (Gonzales et al., 2015). Among them we have flavanones such as HES, flavones such as LUT, flavonols such as QUE, and flavanols such as CAT (Figure 1). Flavonoids can possess glycosylated groups as substituents of hydroxyl groups, such as RUT with presents a rutinoside group substituent at C3 in QUE (Figure 1).
Together with lipase and α-amylase, trypsin is produced by pancreas, and located at small intestine (Unajak et al., 2012). Trypsin is an enzyme of approximately 24 kDa within 231 amino-acid residues, mainly with β-sheet structures (Ibarz et al., 2009; Maximova and Trylska, 2015). It is one of the main proteases that catalyzes the hydrolysis of peptides and ester bonds on L-Lys and L-Arg; it possesses a catalytic triad (His57-Asp102-Ser195) within one subsite (Asp189), located inside of a pocket called S1, to make possible a salt bridge with the positively charged group from these amino acids residues (L-Lys and L-Arg); and it contains a Calcium ion (Ca2+) to stabilize the structure (Ibarz et al., 2009; Ma et al., 2005). Trypsin is synthetized as a zymogen with a molecular weight of 24.1–24.7 kDa (Kay et al., 1961), and it is stored separately from the rest of the pancreatic products, until it is released into the pancreatic duct that carries them to the duodenum (Rawn, 1989).

The inhibitory effect of flavonoids on trypsin has not been completely studied (Li et al., 2014; Maliar et al., 2004). There are some studies about the interactions between trypsin and polyphenols (Wu et al., 2013; Xiao et al., 2015), and only few once on the trypsin-flavonoids interactions (Li et al., 2014; Maliar et al., 2004). Authors mainly calculated inhibition percentages or IC_{50}, observing that QUE, LUT and apigenin had presented 46%, 33% and 27% inhibition percentages, respectively (Li et al., 2014). However, the structure-activity relationship (SAR) of trypsin-flavonoids interactions behind these inhibitions has not been fully elucidated. Only some structural features were explained, such as flavanones had been pointed out as less effective trypsin inhibitors (Maliar et al., 2004). Trypsin inhibitory studies by polyphenolics compounds have been carried out together with pancreatic lipase or glucosidase inhibitory studies, in order to evaluate the possible antinutritional properties of these compounds (Xiao et al., 2015). Considering that previous studies have shown that flavonoids are good lipase and α-amylase inhibitors (Martinez-Gonzalez et al., 2019; Martinez-Gonzalez et al., 2020), in the present study, different flavonoids will be tested as inhibitors of trypsin.
trypsin inhibitors, to determine which structural features favors trypsin inhibition by flavonoid. For this, the effect of flavonoids on trypsin catalytic activity, intrinsic fluorescence, ANS-trypsin complex extrinsic fluorescence of trypsin-ANS, circular dichroism and molecular docking will be carried out to evaluate the SAR of trypsin-flavonoids interactions.

2. Materials and methods

2.1. Samples and reagents

Pancreatic porcine trypsin (EC 3.4.21.4), and its chromophore substrate, Nα-benzoi-DL-arginine p-nitroanilide (BAPNA) were purchased from Sigma-Aldrich (Merck, Germany). Chloride acid, sodium hydroxide, bovine serum albumin (BSA), Bradford reagent, p-nitroaniline, sodium acetate, sulfoxide dimethyl (DMSO), ANS, monobasic sodium phosphate (KH₂PO₄), dibasic sodium phosphate (K₂HPO₄), sodium chloride (NaCl), imidazole, acrylamide, and BAPNA solution in sulphate dodecyl (SDS), ammonium persulphate (PSA), 2-mercaptoethanol, Coomassie blue G250, N,N,N′,N′-tetramethylethylendiamine (TEMED), Tris, the flavonoids (HES, LUT, CAT, QUE and RUT) were also from Sigma-Aldrich. Methanol and Ethanol were purchased from J.T. Baker TM (Thermo Fisher Scientific, Spain). All samples were assayed by triplicate.

2.2. Enzymatic solution preparation

Trypsin purification process was carried on a SuperdexMR 75 size exclusion column (Sigma-Aldrich, Germany), using phosphate buffer solution (20 mM, NaCl 150 mM, pH 7.0). Protolytic activity fractions were concentrated through an Amicon Ultra-0.5 mL centrifugal filters (Millipore, USA), and purity was calculated to be 99% (data not shown). All employed solutions (enzymatic, flavonoids, ANS and buffer) were separately filtered through a 0.45 μm DuraporeTM filter before each assay.

2.3. Trypsin activity assay

Trypsin proteolytic activity was assayed through UV-Visible microplate spectroscopy according to (Onra-Tamayo et al., 2013; Unjak et al., 2012) with some modifications. 0.5 mg/mL trypsin solution (dissolved in Tris buffer, 50 mM, pH 7.0) was employed for enzymatic and flavonoids solution in a final volume of 250 µL. The BAPNA solution was added to start the reaction. For inhibition studies, different concentrations (1–50 µM) of each flavonoid (HES, LUT, CAT, and RUT) dissolved in methanol, except QUE which was dissolved in ethanol were added prior to the addition of BAPNA. Inhibition percentage was calculated from endpoint absorbance values, and IC₅₀ was calculated from an inhibitor concentration versus inhibition percentage plot (Martinez-Gonzalez et al., 2019). All samples were assayed by triplicate.

The apparent catalytic parameters, maximal reaction rate (V_max) and Michaelis-Menten constant (K_M) were calculated by both non-linear (Michaelis-Menten) and linear (Lineweaver-Burk) analyses to obtain K_i according to (Martinez-Gonzalez et al., 2019). The non-linear analysis was performed at GraphPad Prism v. 6.0 using Equation 1.

\[
V_{o} = \frac{V_{max} \times [S]^{h}}{K_{M} + [S]^{h}} \quad (1)
\]

The linear analysis was performed with Equation 2 as follows:

\[
\frac{1}{V_{o}} = \frac{1}{V_{max}} + \frac{K_{M}}{V_{max} \times [S]^{h}} \quad (2)
\]

where h was the Hill coefficient value determined by the non-linear curve fitting of kinetic time course in absence and presence of flavonoids.

K_i and K_i’ (dissociation constant for free enzyme and enzyme-substrate complex, respectively) values for a mixed-type inhibition were obtained from for mixed-type inhibition) Equations 3 and 4 (Tipton, 1996).

\[
K_i' = \frac{K_{M} \times \left( 1 + \frac{[I]}{K_i} \right)}{V_{max}} \quad (3)
\]

\[
V_{max}' = \frac{V_{max}}{1 + \frac{[I]}{K_i}} \quad (4)
\]

2.4. Enzyme-flavonoid interaction measured by intrinsic fluorescence spectroscopy

The quenching effect of the flavonoids (HES, LUT, QUE, CAT and RUT) on trypsin intrinsic fluorescence intensity was assayed as previously described with some modifications (Li et al., 2014; Zeng et al., 2015). The trypsin (0.2 mg/mL) intrinsic fluorescence (Trp) intensity changes were performed in an ISS-PC1™ spectrophotometer (Horiba Scientific®, Japan). Phosphate buffer (20 mM, pH 7.0) was employed for enzymatic and flavonoids solution. Flavonoid solutions (0–100 µM) were added, and fluorescence intensity changes were plotted against the flavonoids concentrations and fitted to Equation 5 as follows:

\[
\Delta F = \frac{B_{max} \times [I]}{K_{D} + [I]} \quad (5)
\]

where ΔF is the change in fluorescence intensity at 340 nm; B_max is the maximum ΔF; and K_D corresponds to the dissociation constant.

The fluorescence quenching parameters were calculated from the linear Stern-Volmer Equation 6 (Lakowicz, 1999) as follows:
where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of the quencher (herein refers to flavonoids), respectively. \( k_q, \tau_0 \) and \( K_{sv} \) are the bimolecular quenching constant, the lifetime of the fluorescence in the absence of the quencher, and the Stern-Volmer quenching constant, respectively. Whereas \( [Q] \) is the concentration of the quencher. \( \tau_0 \) value is equal to 1.90 ns (Li et al., 2014).

A modification (Equation 7) of Stern-Volmer equation (Equation 6) was used to estimate the apparent values of the associative binding constant \( (K_a) \) of the enzyme-flavonoid complex, and the number of binding sites per protein \( (n) \) (Lakowicz, 1999).

\[
\log \frac{F_0-F}{F} = \log K_v + n \log [Q]
\]

2.5. Binding of flavonoids to enzyme by extrinsic fluorescence of ANS

The fluorescence intensity changes of trypsin-ANS complex were recorded according to (Sun et al., 2017) with some modifications. The assay was carried on in absence and presence of flavonoids (HES, LUT, QUE, CAT and RUT). The final concentrations of trypsin and ANS solutions, both dissolved in phosphate buffer (20 mM, pH 7.0), were 0.05 mg/mL and 150 µM, respectively. Sample were incubated 15 min at 37 °C, in the presence and absence of flavonoids (1–300 µM), and fluorescence measured at 380 nm excitation wavelength, and the fluorescence emission was recorded from 400 to 700 nm (monitored at 519 nm). The extrinsic fluorescence measurements were performed in a Shimadzu RF-5301 spectrofluorometer (USA) into a 1 cm path-length quartz cuvette. All samples were assayed by triplicate. The flavonoids did not exhibit FRET at that excitation wavelength. Values of apparent dissociation constant \( (K_{sv}) \) were calculated from Equation 5.

2.6. Binding of flavonoids to trypsin by circular dichroism

Circular dichroism spectra (CD) of trypsin in absence and presence of flavonoids (HES, LUT, QUE, CAT and RUT) were recorded according to (Zhang et al., 2013) with some modifications. The assay was performed on a JASCO® J-815 spectropolarimeter (Japan). Final concentration of enzymatic solution dissolved in phosphate buffer (20 mM, pH 7.0) was 1 µM, while flavonoids final concentrations were 1–100 µM. Measurements were performed in the near-UV region (300–450 nm), into a 1 cm path-length quartz cuvette. Ellipticity was recorded at a speed of 100 nm/min, 0.2 nm resolution, 11 accumulations, and 1.0 nm bandwidth. A control experiment with solvent at same volume was carried on and subtracted from the raw spectra. All samples were assayed by triplicate. Values of apparent dissociation constant \( (K_{sv}) \) were calculated from Equation 5.

2.7. Trypsin-flavonoids interactions by molecular docking

The analyses of possible interactions between trypsin and flavonoids (HES, LUT, QUE, CAT and RUT) were carried on according to literature with some modifications (Martinez-Gonzalez et al., 2019; Zeng et al., 2015). The three-dimension structure of trypsin was obtained from Protein Data Bank (code 1S18) and used as template. Flavonoid structures were generated and minimized using PyMOL software v. 1.3 (Schrodinger® USA). Automated molecular docking studies of the flavonoid and the trypsin were performed with AutoDock Vina using the interphase installed in USCF-Chimera v. 4 (Regents of the University of California, USA) run with the default parameters and a search volume of approximate the same size as enzyme. The three-dimensional structure of the enzyme was considered rigid, and the ligands structures were considered flexible during the performance. According to the scores and binding energy value (herein refers to ΔG value), the best pose for each flavonoid was obtained and analyzed. The best pose was also chosen by its root mean square deviation value (RMSD). Non-covalent interactions, such as Hydrogen bonding, were determined from analysis of the two atoms involved in it, their spatial positions, and distance between them.

2.8. Statistical analysis

All the experimental assays were run in triplicates. Results are expressed as mean values ± standard deviation. Analysis of variance and Fisher’s least significant difference analysis were performed by SPSS v. 20 software (IBM® USA) for the determination of statistically significant differences between treatments with a level of significance of 0.05.

3. Results and discussion

3.1. Inhibition of trypsln activity

The inhibitory capacity of flavonoids against trypsin activity are shown in Table 1. Kinetic parameters were calculated by linear (data not shown) and non-linear analyzes at three flavonoid concentrations, and inhibition pattern studies. \( IC_{50} \) values were also calculated, where LUT showed the lowest value (37.60 ± 0.50 µM), followed by QUE and HES (45.20 ± 1.00, 48.10 ± 1.80 and 60.50 ± 2.40 µM, respectively). The flavone LUT was observed to have the highest inhibition, while the flavan-3-ol CAT did not show any trypsin inhibition. When the inhibitory activity of QUE and RUT where compared, no effect of glycosylation was observed. The activity of RUT against trypsin has not been previously reported, but the activity of flavonoids such as QUE and apigenin has been compared (Li et al., 2014). Greater inhibitory activity was related to a larger number of hydroxyl groups (three) in the B and C rings of QUE, compared to the number of these groups in apigenin (one). Hydroxyl groups at the C3 and C4 positions of QUE were indicated as essential for trypsin inhibition (Li et al., 2014).

LUT and QUE showed the highest inhibitory capacity against trypsin. Apparently, the hydroxylation at the C3 position decreases the inhibitory activity of QUE with respect to LUT. The presence of this group in QUE could hinder the planar C ring structure for inhibition, as observed for \( \alpha \)-amylase and lipase activity (Martinez-Gonzalez, et al., 2019, 2020), and a yeast \( \alpha \)-glucosidase (Tadera et al., 2006). The substitution of a hydroxyl for carbohydrate in C3 for RUT could be related to its higher IC50 value compared to QUE. This substitution causes an increase in the polarity and molecular size of the flavonoid, and a steric hindrance is generated for its interaction with the protein (Gonzalez et al., 2015).

The apparent catalytic parameters \( (V_{app}^{\max } and K_{app}^{\max }) \) are also
reported in Table 1. All flavonoids, except CAT (which did not inhibit trypsin) showed mixed inhibition type. Flavonol such as CAT have been reported as ligands with low inhibitory activity, probably because they only interact with enzymes in the presence of substrate, as uncompetitive inhibitors (Tadera et al., 2006). The mixed inhibition constants values, $K_i$ and $K_i'$ (Table 1) indicate that all flavonoids, (except CAT), behaves mainly as competitive inhibitors, showing greater preference for the free enzyme than for the enzyme-substrate (ES) complex. This same mixed inhibition pattern for trypsin, has been observed for phenolic compounds such as tannic acid (Xiao et al., 2015). More studies are required on the inhibition pattern of phenolic compounds, since non-competitive inhibitions have also been reported (Wu et al., 2013). Phenolic compounds that inhibit trypsin activity can interact with the same substrate binding site. This agrees with the lack of significant differences between LUT values, compared to the rest of the flavonoids. Trypsin fluorescence quenching has been reported for QUE and other flavonoids such as apigenin (Li et al., 2014). HES showed a large bathochromic effect (Figure 2a, from 329 to 350 nm). This bathochromic effect may be due to a change in the polarity of the environment surrounding the Trp in the presence of HES, which may leave them exposed, and could be related to a deployment of the polypeptide chain (Wu et al., 2013; Wu et al., 2011). The bathochromic shift is also related to an unfolding of the protein structure that covers the indole group of Trp (Lakowicz, 1999).

The FI values were adjusted by non-linear (Equation 5) and linear (Equations 6 and 7) analysis to calculate the $K_{sv}$, $K_{q}$, $k_{sv}$, $n$ and $K_q$ values, and results are shown in Table 2. Only for $k_q$, $K_q$, $K_{sv}$, and $K_q$, the differences between LUT values, compared to the rest of the flavonoids were remarkable. CAT presented lower trypsin fluorescence quenching effect, in agreement with its null inhibitory activity. In the case of $K_{sv}$ value (Table 2) LUT and QUE showed the lowest values ($p<0.05$). Both flavonoids have the highest affinity for the enzyme, which agrees with their highest $K_q$ values, and with their high enzymatic inhibitory activity. QUE results are in agreement with those previously published, where authors attributed its high affinity to the ability of the hydroxyl groups in B ring to interact with tryptophan moieties (Li et al., 2014) similar to those observed for catechol structures (Gonzales et al., 2015).

LUT had significantly higher $K_{sv}$ and $K_q$ values compare to the other flavonoids (Table 2). Higher $K_{sv}$ values correspond to thermodynamically more spontaneous enzyme-ligand interactions,
Figure 2. Quenching of intrinsic trypsin fluorescence in the presence of different concentrations of flavonoids (3.0–100.0 µM). (a) HES, (b) LUT, (c) QUE, (d) CAT, and (e) RUT. Fluorescence was recorded after 60 min of incubation with the flavonoid, at an excitation wavelength of 290 nm. Data represent the average of three experimental replicates.

Table 2. Fluorescence quenching parameters \(K_{app}, K_s, k_q, n\) of trypsin in the presence of flavonoids

| Flavonoid | \(K_{app}\) (µM) | \(K_s\) \((10^{-1} \text{ mM}^{-1})\) | \(k_q\) \((10^{-12} \text{ mM}^{-1} \text{ s}^{-1})\) | \(n\) | \(K_a\) \((10^{-1} \text{ mM}^{-1})\) |
|-----------|-----------------|-----------------|-----------------|-------|-----------------|
| HES       | 38.00 ± 4.02\text{a} | 0.96 ± 0.05\text{b} | 0.10 ± 0.01\text{c} | 0.70 ± 0.15\text{b} | 0.60 ± 0.00\text{d} |
| LUT       | 16.10 ± 3.00\text{c} | 1.87 ± 0.10\text{a} | 9.86 ± 1.05\text{a} | 0.90 ± 0.01\text{a} | 1.60 ± 0.22\text{a} |
| QUE       | 17.50 ± 1.50\text{c} | 1.22 ± 0.30\text{b} | 6.43 ± 0.40\text{b} | 0.80 ± 0.20\text{b} | 0.90 ± 0.14\text{b} |
| CAT       | n.d.              | 0.00 ± 0.00\text{d} | 0.00 ± 0.00\text{d} | 0.00 ± 0.00\text{d} | 0.00 ± 0.00\text{d} |
| RUT       | 31.00 ± 2.67\text{b} | 0.51 ± 0.00\text{c} | 6.61 ± 0.15\text{b} | 0.60 ± 0.03\text{c} | 0.70 ± 0.01\text{c} |

Data are the mean value ± standard deviation of triplicate analysis. Different letters in the same column indicate statistically significant values (Fisher’s least significant difference analysis, \(p \leq 0.05\)) respect to control. n.d. means not determined.
and to higher affinity (Zhang et al., 2013). After LUT, QUE and HES, showed similar results, followed by RUT. HES presented $K_w$ values like those of QUE, but their affinity ($K_a$ and $K_D$) was smaller. The $k_q$ values for all flavonoids indicated a static quenching mechanism, that is, there is a formation of a non-fluorescent enzyme-flavonoid complex.

The calculated $n$ values (Table 2) for all flavonoids indicate approximately one binding site per trypsin, in agreement with the calculated Hill coefficient value for catalytic enzyme activity assays. QUE and HES ligands did not show significant differences between them for the value of $n$. Greater flexibility of the B ring in flavonoids, such as HES, is associated with less interaction with enzymes (Lo Piparo et al., 2008). HES interacted with the enzyme in a manner comparable to QUE, but this interaction could be affected by its flexibility. In the case of RUT, the presence of glycosylation prevents interaction with the enzyme (Costamagna et al., 2016) comparable to that of LUT.

3.3. Enzyme-flavonoid binding by extrinsic fluorescence spectroscopy

The change on fluorescence emission of the ANS-trypsin complex was analyzed in the presence of flavonoids, after eliminating the

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**Figure 3.** Fluorescence spectra of trypsin-ANS complex in the absence and presence of different concentrations (up to 300 µM) of flavonoids: (a) HES, (b) LUT, (c) QUE, (d) CAT, and (e) RUT. Insert shows plot of flavonoid concentration (mM) versus corrected fluorescence intensity change $DF$. Samples were excited at a wavelength of 290 nm. Yellow arrows indicate the trend of fluorescence change as the concentration of flavonoids increases.
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Figure 4. Circular dichroism absorption spectra (CD) of trypsin in the presence of different concentrations (10–100 µM) flavonoids: (a) HES, (b) LUT, (c) QUE, (d) CAT, and (e) RUT. Absorption wavelength (λ), and degrees of ellipticity, are in nanometers (nm), and in milligrade ellipticity (mdeg), respectively. Yellow arrows indicate changes in the spectrum (increase or decrease), as the concentration of flavonoids increases.

effects of the internal filter (corrected FI or FIc) (Figure 3). QUE exhibited the greatest decrease in FI of the ANS-trypsin complex, followed by RUT and LUT (Figure 3c, e and b, respectively). These results indicate that flavonoids and ANS compete for the same binding site in the enzyme. HES and CAT (Figure 3a and d, respectively) showed an FI increase of the ANS-trypsin complex, indicating that both flavonoids interact with the enzyme in a different site respect to ANS. These results were similar to those reported for the interactions of the same flavonoids with the ANS-α-amylase complex (Martinez-Gonzalez et al., 2019). QUE showed the greatest effect on the stability of the enzyme-ANS complex, competing with ANS for the same binding site (Halim et al., 2017). The calculated $K_D$ (Equation 5) values for QUE, RUT, and LUT were $126.10 \pm 10.05$, $61.70 \pm 3.20$, and $30.90 \pm 2.80$ µM, respectively. The large difference on $K_D$ values observed for QUE and RUT, can be attributed to the glycosylation at C3 (RUT), in agreement with Wu et al. Wu et al., (2013). The extra hydroxyl group of QUE, compared to LUT, gives it a greater ability to interact with the enzyme. The low quenching effect of LUT over the ANS-trypsin complex, may be explained considering the flat structure of B ring, which allows it to easily interacts with its binding site, without obstructing the binding of ANS to the enzyme (Tadera et
can be related to its lower $K_{sv}$ value compared to the other flavoenzyme, and exposure of Trp moieties. The RUT lower CD results compared to the rest of the flavonoids could be responsible for this observation. The larger size (higher molecular weight) of HES, LUT, and QUE, while no inhibition was observed with CAT. HES facilitates a better interaction, and greater inhibition, with larger enzymes such as α-amylase and α-glucosidase, compared to the interaction of naringenin, which has only one hydroxyl group at C5′ of HES spatial arrangement on this site. This difference in the spatial arrangement explains its different inhibitory activity compared to LUT and QUE. It has been described that the hydroxyl at C5′ of HES fosters a better interaction, and greater inhibition, with larger enzymes such as α-amylase and α-glucosidase, compared to the interaction of naringenin, which has only one hydroxyl group at C5′ (Tadera et al., 2006). The participation of other characteristics of HES such as the methoxy group, which could prevent its adjustment in the binding site (avoiding its interaction with Gln192), and cause changes in the conformation of the enzyme, observed in the extrinsic fluorescence assays, should be evaluated. In vitro studies on digestion models, and in vivo studies, both with trypsin and other pancreatic enzymes must be done to completely elucidate the interaction mechanism of flavonoids to trypsin.

4. Conclusion

LUT showed the largest trypsin inhibitory capacity, followed by QUE, RUT and HES, while no inhibition was observed with CAT. π-stacking hydrophobic and hydrogen bonding were the main observed interactions for all flavonoids. HES, LUT and QUE (Figure 5c–e) showed hydrophobic π stacking interactions with Tyr151, in agreement with those reported for the interaction of phenolic compounds with trypsin and pepsin (Wu et al., 2013; Xiao et al., 2015). Trypsin interactions with CAT and RUT were only through hydrogen bonding, with residues such as Asn192, and Asn97, respectively. Hydrogen bonding interactions have been reported for CAT and trypsin (Cui et al., 2015).

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Figure 5. Molecular docking of trypsin and potential binding sites for the flavonoids HES, LUT, QUE, CAT, RUT, and the substrate, BAPNA. Surface representation for the full tridimensional structure of trypsin (dark gray), the amino acids of the active site are represented in yellow and the possible binding sites for the ligands (a). Flavonoids binding sites and the main amino acids residues (sticks) are shown (b), as well as the main for the flavonoids and the main specific interactions for HES (c), LUT (d), QUE (e), CAT (f), and RUT (g) are noted. The dot lines and distances (Å) with colors correspond to different interactions such as hydrogen (red), and hydrophobic binding (green).
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Table 3. Gibbs free energy (ΔG°, kCal/mol), the trypsin amino acid residues interacting with flavonoids, and the distance (Å) for the possible enzyme-flavonoid conformations

| Flavonoids | Amino acid residues lining the binding site, distance and binding energy interaction | Hydrophobic | Hydrogen Binding | ΔG° |
|------------|----------------------------------------------------------------------------------|------------|-----------------|-----|
| HES        | Ty151 (3.6; 3.8) Asp74 (2.8), Ty151 (3.2)                                           |            |                 | −6.7|
| LUT        | Ty151 (3.8; 3.7; 3.7; 3.8) Asp74 (3.1), Gln192 (2.4)                                |            |                 | −7.3|
| QUE        | Ty151 (3.9; 3.7; 4.0; 3.9) Gly193 (3.1)                                           |            |                 | −6.5|
| CAT        | Ninguna Asn34 (3.1), Arg62 (2.8), Gln64 (3.1)                                     |            |                 | −7.5|
| RUT        | Ninguna Asn97 (2.9; 3.1), Ser195 (2.8), Gln192 (2.7), Gly216 (2.7)                 |            |                 | −7.0|

Figure 5. (continued)
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