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# Dietary flavonoids fisetin, luteolin and their derived compounds inhibit arginase, a central enzyme in *Leishmania* (*Leishmania*) *amazonensis* infection

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ABSTRACT

Fisetin, quercetin, luteolin and 7,8-hydroxyflavone show high activity in *Leishmania* cultures and present low toxicity to mammalian cells. In this work, the structural aspects of 13 flavonoids were analyzed for their inhibition of the arginase enzyme from *Leishmania* (*Leishmania*) *amazonensis*. A higher potency of arginase inhibition was observed with fisetin, which was four and ten times greater than that of quercetin and luteolin, respectively. These data show that the hydroxyl group at position 3 contributed significantly to the inhibitory activity of arginase, while the hydroxyl group at position 5 did not. The absence of the catechol group on apigenin drastically decreased arginase inhibition. Additionally, the docking of compounds showed that the inhibitors interact with amino acids involved in the $Mn^{+2} - Mn^{+2}$ metal bridge formation at the catalytic site. Due to the low $IC_{50}$ values of these flavonoids, they may be used as a food supplement in leishmaniasis treatment.



## 1. Introduction

Leishmaniasis affects 12 million people in the world and is associated with malnutrition, weakness of the immune system and other factors related to a lack of resources. The disease primarily affects people who live in poor housing conditions with limited access to food (WHO, 2012). A balanced diet enriched with fruits and vegetables can prevent cancer and cardiovascular diseases and stimulates the immune system. In addition to their antioxidant activities, bioactive food components, such as polyphenols, can promote a healthy life (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993). Several flavonoids, such as luteolin, quercetin and quercitrin, which are abundant dietary flavones, are active against some species of *Leishmania* (Mittra et al., 2000; Muzitano et al., 2006; Sen, Mukhopadhyay, Ray, & Biswas, 2008; Tasdemir et al., 2006).

Quercetin and derived flavonoids are active by oral administration in experimental cutaneous and visceral leishmaniasis infections produced *in vivo* (Gomes, Muzitano, Costa, & Rossi-Bergmann, 2010; Muzitano et al., 2009).

We have recently shown that quercetin, quercitrin and isoquercitrin are potent inhibitors of *Leishmania* (*Leishmania*) *amazonensis* arginase (ARG-L) (da Silva, Maquiaveli, & Magalhaes, 2012a). Luteolin and quercetin promote k-DNA linearization mediated by topoisomerase II, decrease DNA synthesis, arrest the cell cycle and promote apoptosis of parasites (Mittra et al., 2000). Flavonoid dimers have been developed as potent antileishmanial agents (Wong, Chan, Chan, & Chow, 2012) and can reverse multidrug resistance in *Leishmania*.

New therapeutic targets have been considered to treat neglected diseases. For diseases caused by trypanosomatids, such as Chagas disease, African sleeping sickness and leishmaniasis, the exploration of the polyamine (PA) enzyme pathway has been important in drug development (Colotti & Ilari, 2011). PAs are valuable targets for antiparasitic chemotherapy because they play an

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essential role in the proliferation, differentiation and synthesis of macromolecules and the antioxidant mechanism in *Leishmania* (Birkholtz et al., 2011; Colotti & Ilari, 2011). The PA spermidine is the substrate for the synthesis of trypanothione (N1, N8-bis (glutationil) spermidine) in *Leishmania*. Trypanothione promotes the removal of reactive oxygen species (Fairlamb & Cerami, 1992) and reactive nitrogen species (Bocedi et al., 2010), thus protecting the parasite from oxidative stress and endogenous reactive species produced by the host's defence system.

The ARG-L hydrolyses L-arginine into L-ornithine and urea in the first step of PA biosynthesis. Double knockout of the ARG-L gene in *L.* (*L.*) *donovani* showed that arginase plays a central role in polyamine synthesis (Roberts et al., 2004). In *L.* (*L.*) major, double knockout of the ARG-L gene showed that the parasite becomes auxotrophic for PAs (Reguera, Balaña-Fouce, Showalter, Hickerson, & Beverley, 2009). ARG-L participates in a complex balance that determines the fate of L-arginine, and its subcellular localization in glycosomes may be essential for the physiological rhythm of the parasite (da Silva, Zampieri, Muxel, Beverley, & Floeter-Winter, 2012b).

In mammals, there are two arginases: the hepatic arginase (ARG-1) and the extra-hepatic arginase (ARG-2). ARG-1 can be induced in macrophages under the $TH_2$ lymphocyte response (Wanderley & Barcinski, 2010). The increase of ARG-1 leads to the consumption of L-arginine, which is also a substrate of nitric oxide synthase. Nitric oxide synthase converts L-arginine to citrulline and nitric oxide (NO). As a consequence of increased ARG-1 activity, there is a decrease in the NO production that enables *Leishmania* to survive inside the macrophage. The inhibition of ARG-1 by endogenous NOHA ($N^{\omega}$-hydroxy-L-arginine) diminishes the proliferation of *Leishmania* into the macrophage (Iniesta, Gómez-Nieto, & Corraliza, 2001).

This study examines the biochemical interaction between ARG-L and flavonoids. Additionally, a docking simulation of the interaction between inhibitors and the structural model of the ARG-L allows visualization of the interactions of dietary flavonoids within the catalytic site of the enzyme.

## 2. Materials and methods

### 2.1. Materials

Quercetin, isoquercitrin, quercitrin, luteolin, orientin, isoorientin, fisetin, galangin, kaempferol, 7,8-dihydroxyflavone, apigenin, vitexin, isovitexin, $MnSO_4$, L-arginine, CelLytic B, MOPS (4-morpholinepropanesulfonic acid), CHES (2-(cyclohexylamino)ethanesulfonic acid), PMSF (phenyl-methyl-sulfonyl fluoride), yeast extract and tryptone were purchased from Sigma–Aldrich. Reagents for urea analysis were purchased from Quibasa (Belo Horizonte, MG, Brazil).

### 2.2. Expression and purification of arginase

Recombinant ARG-L was expressed without a histidine tail and purified as described previously (da Silva et al., 2012b). Rat liver arginase (ARG-1) was prepared by lysing 5 g of liver cells in 100 ml of buffer containing 100 mM Tris and 1 mM EDTA, using a blender. The homogenate was centrifuged at 5000*g*, and pigments in the supernatant were removed by liquid chromatography (open column) using 5 ml of Sepharose Chelating resin (GE Healthcare) charged with $Ni^{+2}$. The resulting arginase solution was fully activated by heat at 60 °C in the presence of 10 mM of $MnCl_2$ (Kanyo, Scolnick, Ash, & Christianson, 1996). Following activation, the solution was centrifuged at 20,000*g*, and the supernatant was used to test arginase inhibition.

### 2.3. Inhibitor screening and determination of $IC_{50}$

Screening of compounds for their ability to inhibit arginase from *L.* (*L.*) *amazonensis* was performed using 125 μM concentrations of each compound at pH 9.5 with 50 mM CHES buffer and 50 mM L-arginine (pH 9.5). The samples were incubated in a water bath at 37 °C for 15 min. Quantification of urea was performed by enzymatic colorimetric Berthelot assay (Fawcett & Scott, 1960), using commercial reagents. Briefly, the catalytic activity of the arginase reactions was stopped by transferring 10 μl of reaction mixture into 750 μl of reagent A (20 mM phosphate buffer, pH 7, containing 60 mM salicylate, 1 mM sodium nitroprusside and >500 IU of urease). This mixture was incubated at 37 °C for 5 min. Next, 750 μl of reagent B (sodium hypochlorite 10 mM and NaOH 150 mM) were added, and then the samples were incubated at 37 °C for 10 min (Fawcett et al., 1960). Absorbance measurements were taken at 600 nm using a Hitachi 2810U spectrophotometer. The positive and negative controls were performed under the same conditions in the absence of inhibitor. The experiments were performed in duplicate in at least three independent experiments.

Determination of the concentration that inhibits 50% of the catalytic activity of the enzyme was carried out by varying the inhibitor concentration with a 1:10 dilution factor. The experiments were performed in duplicate in at least three independent experiments until we obtained a coefficient of non-linear regression $R^2 \geqslant 0.95$. The different concentrations of the inhibitors were obtained by serial dilution of the compound in water or a suitable solvent. The reactions were performed at pH 9.5 using 50 mM CHES buffer in the presence of 50 mM substrate L-arginine (pH 9.5). The samples were incubated in a water bath at 37 °C for 15 min, and the urea formed was analyzed as described above. We used a mathematical sigmoidal (log $IC_{50}$) model to determine the $IC_{50}$, using Origin 8.0 software.

### 2.4. Determination of the constants $K_i$, $K_i'$ and mechanism of inhibition

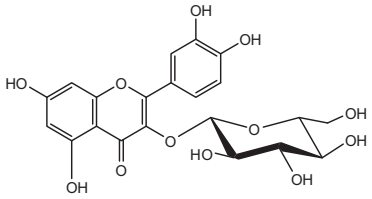
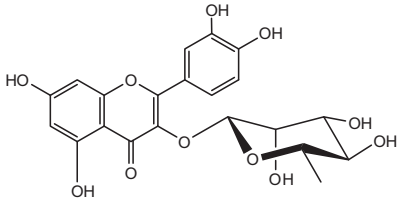
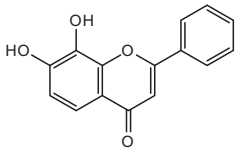
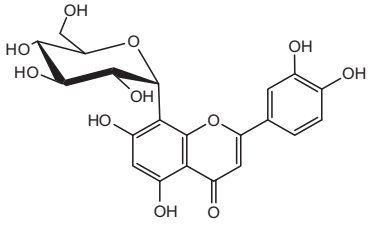
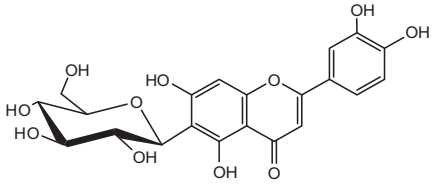
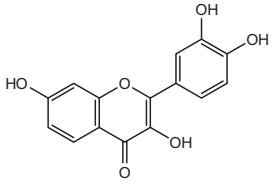
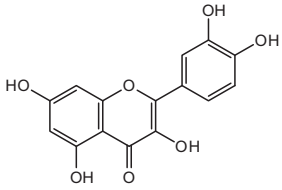
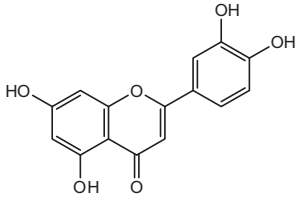
All reactions were performed in 50 mM CHES buffer, pH 9.5, containing variable concentrations of the substrate L-arginine (12.5, 25, 50 and 100 mM) at pH 9.5. Inhibitors were used at three different concentrations close to the $IC_{50}$. The different substrate and inhibitor concentrations were obtained by serial dilution. A mixture, M1, containing L-arginine (pH 9.5) at double the desirable concentration, and a second mixture, M2, containing the enzyme (2000 units) diluted in 125 mM CHES buffer (pH 9.5), were prepared. The reaction was prepared by mixing 50 μl of M1, 10 μl of inhibitor and 40 μl of M2. The addition of M2 was synchronized every 15 s, followed by immediate incubation in a water bath for 15 min at 37 °C. The urea produced was analyzed as described above. All reactions were performed in duplicate in a minimum of three independent experiments.

The constant $K_i$ was determined for inhibitors that showed mechanisms of mixed or competitive inhibition, whereas $K_i'$ was determined for inhibitors that showed uncompetitive or mixed inhibition (Cornish-Bowden, 1974). Each constant was determined by calculating $x$ for the intersecting points between two lines obtained by linear regression. For non-competitive inhibition, $y = 0$ was used for the equation to find the values of the constants $K_i$ and $K_i'$.

### 2.5. Data analysis

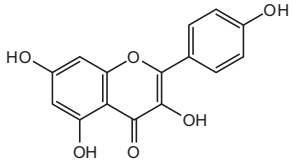
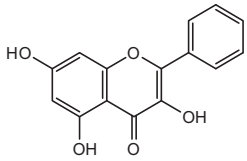
Statistical analysis was performed by ANOVA and *post hoc* Tukey's tests, using Origin 8.0. For all tests, differences of $p < 0.05$ were considered significant. Linear regressions were obtained using MS Excel 2010.

**Table 1**
Dissociation constants and the mode of arginase inhibition by natural compounds.

| Ligand | Structure | $^{1}IC_{50}$ | $K_i$ (μM) | $K_i$' | Mode of inhibition |
|---|---|---|---|---|---|
| Isoquercitrin[a] |  | 3,8* | 6.9 ± 0.3 | 6.9 ± 0.3 | Noncompetitive |
| Quercitrin[a] |  | 10* | 7.2 ± 0.9 | 7.2 ± 0.9 | Noncompetitive |
| DHF[a] |  | 12 ± 1 | 7.4 ± 0.4 | 7.4 ± 0.4 | Noncompetitive |
| Orientin[b] |  | 16 ± 2 | – | 24 ± 2 | Uncompetitive |
| Isoorientin[b] |  | 9 ± 1 | – | 22 ± 2 | Uncompetitive |
| Fisetin[c] |  | 1,4 ± 0,3 | 1.9 ± 0.5 | 4.2 ± 0.2 | Mixed |
| Quercetin[d] |  | 4,3* | 8 ± 1 | 12 ± 1 | Mixed |
| Luteolin[e] |  | 9 ± 1 | 8 ± 1 | 18 ± 2 | Mixed |

(*continued on next page*)

**Table 1** (*continued*)

| Ligand | Structure | $^{1}IC_{50}$ | $K_i$ (μM) | $K_i$' | Mode of inhibition |
|---|---|---|---|---|---|
| Kaempferol[f] |  | ~50 | nd** | nd | nd |
| Galangin[g] |  | ~100 | nd | nd | nd |

[*] da Silva, Maquiaveli, and Magalhaes, (2012a).
[**] nd = Not determined, DHF = 7,8-dihydroxyflavone.
[1] Data are expressed as the median ± SEM. $K_i'$ differs for a compound without a common letter ($p < 0.05$).

### 2.6. Molecular modeling

The target compounds (Table 1) were modeled *in silico*, and energy minimization was performed over 1000 steps, using the steepest descent method, Gasteiger–Hückel charges, a dielectric constant of 80, and the Tripos force field. The structures were further optimized by the conjugated gradient method.

The target enzyme used in this work was a previously constructed comparative model of ARG-L (da Silva, Castilho, Pioker, Silva, & Floeter-Winter, 2002). The docking simulations (Dias & de Azevedo, 2008) were carried out by applying the MolDock algorithm (Thomsen & Christensen, 2006), which was implemented in the Molegro Virtual Docker software (Molegro Virtual Docker/4.0 in, 2009).

## 3. Results

### 3.1. Arginase inhibition by flavonoids

A group of 13 flavonoids (Table 1) was selected to determine a structure–activity relationship using ARG-L as the drug target. The compounds were screened at 125 μM concentrations in the presence of 50 mM substrate L-arginine at pH 9.5, the optimal pH of the enzyme. Under these conditions, only three compounds, apigenin, isovitexin and vitexin, inhibited less than 50% of the enzyme activity. Galangin and quercitrin achieved 50–70% inhibition, whereas isoquercitrin, isoorientin and orientin achieved 70–75% inhibition. The best inhibitors were fisetin (87%), luteolin (83%), quercetin (83%) and 7,8-dihydroxyflavone (80%). Using the same conditions, these compounds did not significantly inhibit ARG-1 from the rat, which was used as a model for the mammalian enzyme. At a concentration of 1 mM, all of the tested compounds inhibited ARG-1 by <50%. Based on results from this study, the flavonoids showed specific inhibition of ARG-L, and did not act through the ARG-1 route.

The interaction of fisetin with ARG-1 was less stable than that with ARG-L, confirming the selectivity of fisetin for the parasite enzyme. The energy value found for the interaction between fisetin and ARG-1 was −62.5 kcal/mol, compared to −85.8 kcal/mol with the parasite ARG-L. Fisetin docking (Fig. 3) shows a rotation of 180° in the position of interactions with ARG-1 and ARG-L. There is an inversion of fisetin interaction with the distinct enzyme when it looks for Ser150 and Asp245 in ARG-L, and equivalent amino acids Ser137 and Asp234 in ARG-1: the catechol group from fisetin donates a hydrogen bond (H-bond) to Ser150 in ARG-L, while, in ARG-1, the hydroxyl group at position 7 on the flavone group donates an H-bond to Ser137, which is the position equivalent to Ser150 in ARG-L. This inversion allows for a close hydrophobic interaction of His154 and His139 with the double ring of the flavone group of fisetin, and enhances the stability of this inhibitor with ARG-L.

### 3.2. Mechanism of arginase inhibition by flavonoids

The constants $K_i$ and $K_i'$ refer to the equilibrium established between the enzyme ($E$) and substrate ($S$) in the presence of an inhibitor ($I$). The inhibition constant $K_i$ refers to the dissociation constant of the complex EI, while $K_i'$ refers to the dissociation of the EIS (Cornish-Bowden, 1974). Eight compounds, with an $IC_{50}$ of less than 20 μM, were selected for analysis of the mechanism of enzyme inhibition.

The aglycone compounds, such as quercetin, luteolin and fisetin, exhibited mixed inhibition, while the glycoside flavonoids, such as orientin and isoorientin, showed uncompetitive inhibition. The compounds quercitrin, isoquercitrin and 7,8-dihydroxyflavone showed non-competitive inhibition. Table 1 summarizes the kinetic data obtained with the Dixon and Cornish-Bowden plots that were used to calculate the constants $K_i$ and $K_i'$ (Fig. 1). The mixed inhibition mechanism was set to the intersection point of the straight curve that occurred in the second quadrant bounded by the $X$ and $Y$ axes (counterclockwise) for the Dixon plot ($K_i = -x$) and the third quadrant for the Cornish-Bowden plot ($K_i' = -x$). The mechanism of uncompetitive inhibition was determined at the intersection of the Cornish–Bowden plot occurring in the second quadrant and the intersection of the Dixon plot occurring at $y = -\infty (K_i >>> K_i')$.

### 3.3. Structure–activity relationship (SAR) of compounds

Fisetin (3,3′,4′,7-tetrahydroxyflavone) was four times more potent than quercetin (3,3′,4′,5–7-pentahydroxyflavone), which indicates that the hydroxyl at position 5 may not be necessary to inhibit arginase. Moreover, quercetin, which has a hydroxyl at position 3, is twice as potent as luteolin (3′,4′,5–7-tetrahydroxyflavone).

Direct comparison of fisetin with luteolin indicates that the hydroxyl at position 3 of fisetin provides an inhibition ten times greater than that when the hydroxyl is at position 5 in luteolin. Surprisingly, tetrahydroxyflavone fisetin was expected to have the optimal number and a better distribution of hydroxyls, but we found that 7,8-dihydroxyflavone ($IC_{50}$ = 12 μM) presented an $IC_{50}$ close to the $IC_{50}$ of luteolin (tetrahydroxyflavone).

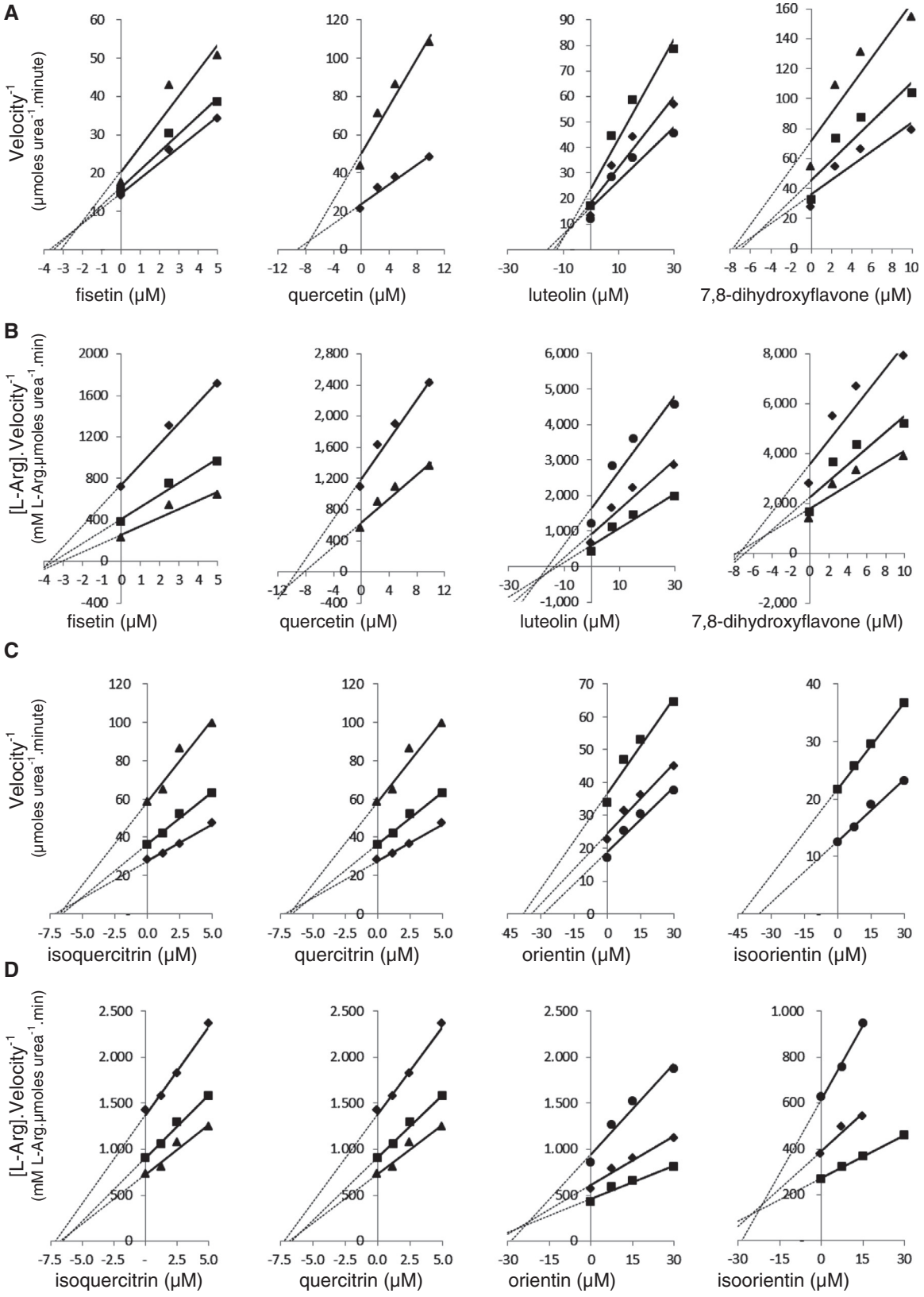



**Fig. 1.** The mechanism of arginase inhibition by eight flavonoids. The $K_i$ constants were measured using Dixon plots (A, C), and $K_i'$ constants were determined by a Cornish–Bowden plot (B, D). Panel A and B: The aglicones fisetin, quercetin and luteolin are mixed inhibitors ($K_i \neq K_i'$), and 7,8-dihydroxyflavone is a noncompetitive inhibitor ($K_i = K_i'$). Panel C and D: quercitrin and isoquercitrin (glucosides derived from quercetin) are noncompetitive inhibitors, whereas orientin and isoorientin (glucosides derived from luteolin) are uncompetitive inhibitors of ARG-L ($K_i >>> K_i'$). The concentration of L-arginine used: 100 mM (●), 50 mM (♦), 25 mM (■) and 12.5 mM (▲). The inhibitor concentration was varied, ranging from 1.25 to 30 μM.

**Table 2**
Energy docking results.

| Ligand | $E_{docking}$ (kcal/mol) | $E_{hb}$ (kcal/mol) | $E_{vdW}$ (kcal/mol) | $E_{Steric}$ (kcal/mol) | MW (u) | Torsions |
|---|---|---|---|---|---|---|
| Isoquercetrin | −144.40 | −24.00 | −44.31 | −173.96 | 464.38 | 4 |
| Quercetrin | −110.50 | −22.95 | −29.79 | −139.97 | 448.38 | 3 |
| Orientin | −98.21 | −19.02 | −7.49 | −139.71 | 448.38 | 3 |
| Isoorientin | −87.14 | −19.45 | −27.32 | −109.73 | 448.38 | 3 |
| Fisetin | −85.77 | −6.42 | −34.41 | −108.91 | 286.24 | 1 |
| Quercetin | −85.26 | −11.80 | −35.00 | −101.98 | 302.24 | 1 |
| Luteolin | −84.42 | −19.69 | −18.46 | −105.50 | 286.24 | 1 |
| Kaempferol | −81.62 | −11.79 | −31.86 | −98.79 | 286.24 | 1 |
| 7,8-Dihydroxyflavone | −80.96 | −3.76 | −27.38 | −111.10 | 254.24 | 1 |
| Galangin | −77.20 | −5.42 | −31.98 | −101.53 | 270.24 | 1 |

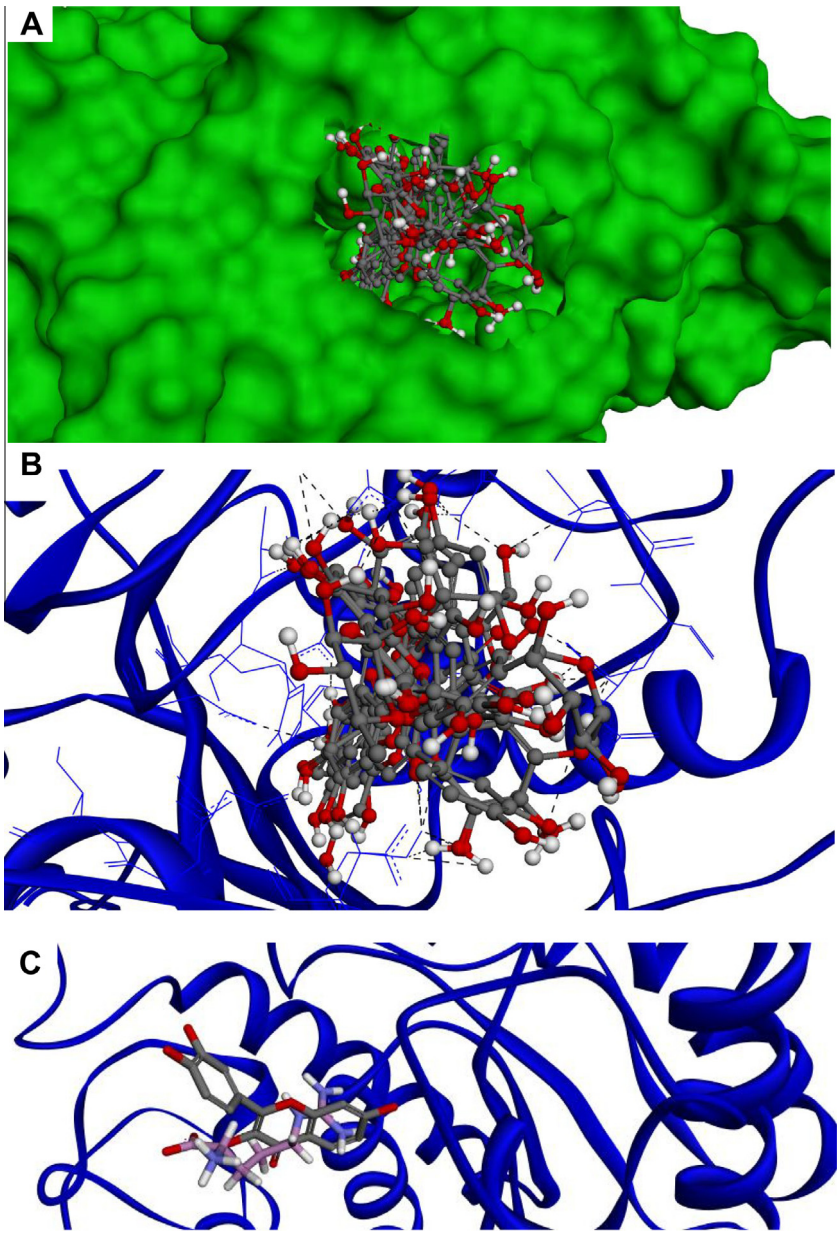



**Fig. 2.** (A) Compounds docked in the ARG-L binding site. (B) A close-up image of the docked compounds in the ARG-L binding site. (C) Fisetin (gray) docking superposition with L-arginine, in magenta (PDB: 1T5F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A key feature of the inhibitors is the presence of the catechol group because, in its absence, the compounds apigenin, vitexin and isovitexin displayed no significant inhibition. A comparative analysis of quercetin and structurally related compunds (kaempferol and galangin) showed that the catechol group is more important for inhibition than are the phenol and benzyl groups. The 7,8-dihydroxyflavone, in which the aromatic ring has no hydroxyls, contrasts with the other inhibitors. The hydroxyl at position 8 exhibited ten times the inhibition of galangin (benzyl group) and five times the inhibition of kaempferol (phenol group).

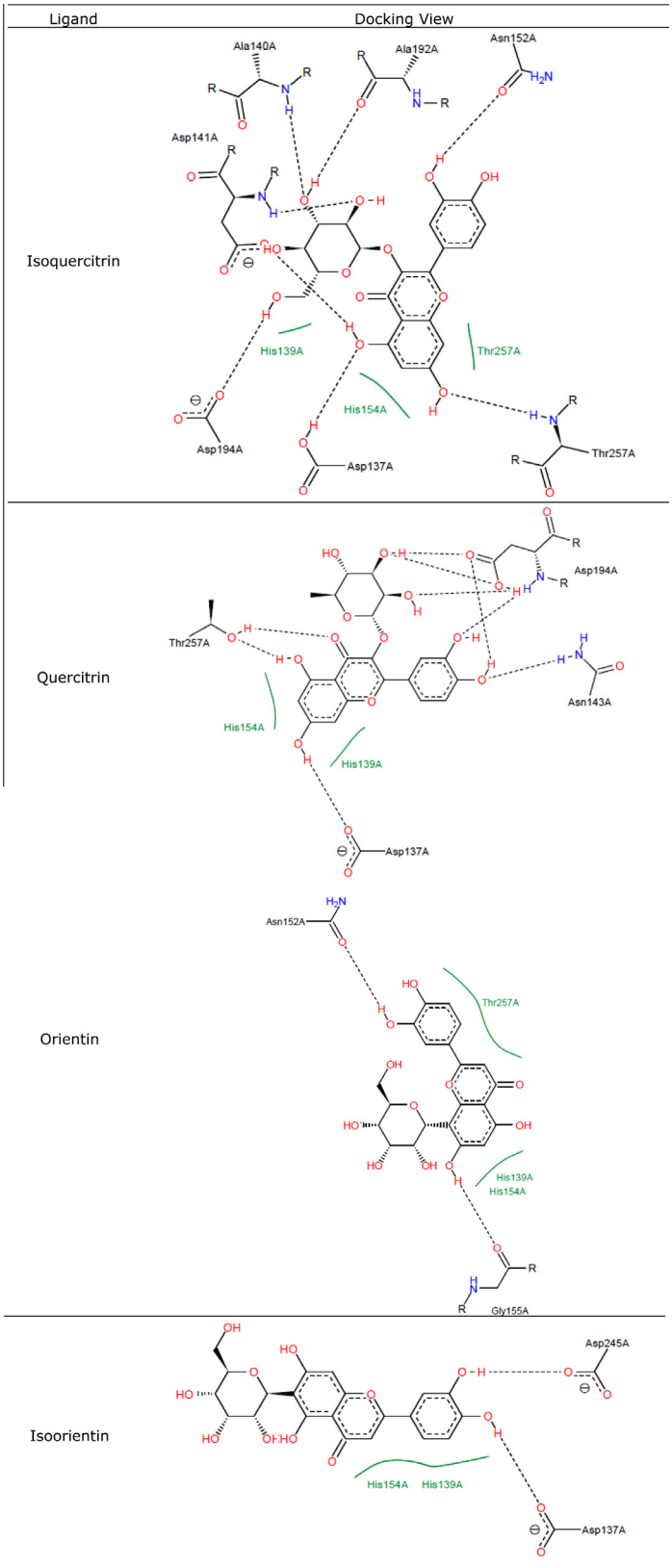



**Fig. 3.** 2D-Representation of the compound-enzyme interactions. Black dashed and full green lines represent hydrogen and hydrophobic intermolecular interactions, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### *3.4. Molecular modeling*

The optimum docking protocol was constructed with flexibility in the enzyme binding pocket ('induced fit'). Each compound was docked with softened potentials (steric, hydrogen bonding, and electrostatic forces) and, at this point, the enzyme residues were kept rigid at their default conformation. Then, all residue side-chains that were close enough to the compound to interact with

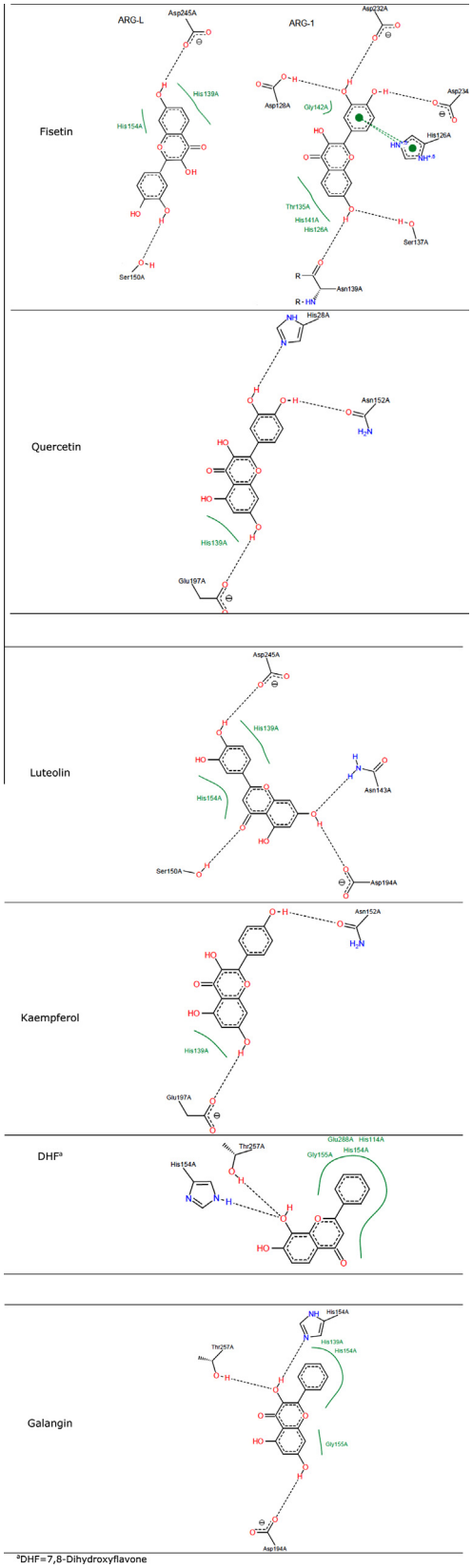



**Fig. 3** (*continued*)

it were energy-minimized. The final step was the energy minimization of the compounds. The docking scores of the interactions between the arginase from *L. amazonensis* and the target compounds, as well as the hydrogen bonding, steric, and van der Waals energy contributions and the number of possible atom–atom free torsions, are shown in Table 2.

**Table 3**
Cross-correlation matrix of the intermolecular energy terms.

| | $E_{docking}$ | $E_{hb}$ | $E_{vdW}$ | $E_{Steric}$ | MW | Torsions |
|---|---|---|---|---|---|---|
| $E_{docking}$ | 1.00 | | | | | |
| $E_{hb}$ | **0.71** | 1.00 | | | | |
| $E_{vdW}$ | 0.31 | 0.16 | 1.00 | | | |
| $E_{Steric}$ | **0.96** | 0.68 | 0.10 | 1.00 | | |
| MW | **0.74** | **0.84** | 0.10 | **0.78** | 1.00 | |
| Torsions | **0.84** | **0.81** | 0.02 | **0.88** | **0.97** | 1.00 |

Fig. 2A shows the solvent-accessible surface of arginase from *L. amazonensis*, including the docked compounds in the binding pocket. A close up of the docking interaction with the enzyme is shown in Fig. 2B and C. Fig. 3 shows a 2D-representation of the flavonoid-enzyme interactions (Schomburg, Ehrlich, Stierand, & Rarey, 2010). The intermolecular hydrogen bonds are shown as black dashed lines in both Fig. 2B and Fig. 3. These hydrogen bonds serve as "molecular anchors" for binding the compounds into the enzyme active site.

To semiquantitatively estimate the contribution of each energy component to the docking score, a cross-correlation matrix of the values shown in Table 2 was calculated (Table 3). The hydrogen bonding and steric energy components, as well as the molecular weights and numbers of free atom–atom bond torsions (entropic contribution), are related to the docking score energies. Consequently, those features should be considered carefully in the design of new lead compounds.

## 4. Discussion

Knowledge of the biology of the host-parasite relationship is central to establishing a paradigm to treat leishmaniasis. PA synthesis is a metabolic pathway that has been explored for drug development against *Trypanosoma* and *Leishmania* (Colotti & Ilari, 2011). The inhibition of PA synthesis can cause oxidative stress in parasite cells, due to a deficiency in trypanothione production (Colotti & Ilari, 2011). Arginase from *Leishmania* is the first enzyme in the PA pathway, and blocking it can lead to oxidative stress and promote infection control.

In a study of 105 natural compounds, the leishmanicidal activity of the flavonoids fisetin, quercetin, luteolin and 7,8-dihydroxyflavone showed high potency against the amastigotes of *L.* (*L.*) *donovani* (Tasdemir et al., 2006). These four compounds also showed potential as inhibitors of ARG-L.

Fisetin is a flavonoid present in strawberries; quercetin is abundant in onions and broccoli, and luteolin can be found in celery, green pepper, parsley and chamomile tea (Shimoi et al., 1998). In this study, we observed that fisetin is a flavonoid that possesses a high potency in arginase inhibition. Fisetin was the most potent arginase inhibitor, with four and ten times higher potency than quercetin and luteolin, respectively. Comparing the structures of these flavonoids revealed that the hydroxyl group at position 3 contributed significantly to the inhibitory activity of arginase, while the hydroxyl at position 5 did not.

In the absence of a catechol group on the galangin, arginase inhibition declined sharply, suggesting that the catechol group is important for inhibition activity. The absence of a hydroxyl group at position 3 and catechol on the apigenin inhibited only 6% of ARG-L at 125 μM. C-glycosylation on the isoorientin (luteolin-6-C-glucoside) and the orientin (luteolin-8-C-glucoside) did not enhance arginase inhibition. In contrast, the 7,8-dihydroxyflavone showed an $IC_{50}$ of 12 μM when the hydroxyl at position 3 and

the catechol group were absent. These data indicate that position 8 enhanced the inhibition activity of this compound.

The inhibition of ARG-L increased due to the hydroxylation of the phenyl group of molecules hydroxylated at positions 3, 5, and 7, such as in galangin ($IC_{50}$ 100 μM), kaempferol ($IC_{50}$ 50 μM) and quercetin ($IC_{50}$ 4.3 μM). Galangin is not hydroxylated, kaempferol is hydroxylated at position 4′ (phenol group), and quercetin is hydroxylated at positions 3′ and 4′, generating a catechol group. The inhibition activity of quercetin is not significantly altered if the hydroxyl at position 3 is conjugated with glucose to generate isoquercitrin, but it loses half of its potency if this position is conjugated with rhamnose, as in quercitrin (da Silva et al., 2012a).

Structural analysis from docking studies (Fig. 3) showed hydrogen bond (H-bond) interaction between ARG-L and the substituent at the 3-positions present in isoquercitrin (quercetin-3-*O*-β-glucoside) and quercitrin (quercetin-3-*O*-rhamnoside) that inhibit ARG-L by a noncompetitive mechanism, where an inhibitor binds to both the enzyme-substrate complex or to the free enzyme. The higher docking energies were observed just for these 2 compounds (Table 2). In C-glucosides, such as orientin (luteolin-8-C-glucoside) and isoorientin (luteolin-6-C-glucoside), the glucoside group does not show any interaction with ARG-L residues, and both are uncompetitive inhibitors which bind exclusively to the enzyme–substrate complex, resulting in an inactivated enzyme–substrate–inhibitor complex. The aglycones fisetin and luteolin show common H-bonds with residues Asp245 and Ser150, and quercetin is a unique compound that shows H-bonding with His28 and Glu197. These three aglycones showed mixed inhibition of ARG-L. In conclusion, the three mechanisms of inhibition shown here for these compounds were closely related to the absence or presence of a glucoside in the 3-O-glucoside position, where mixed and noncompetitive inhibition are observed, respectively, while the C-glucoside showed an uncompetitive inhibition.

There are now multiple targets that have been described for the leishmanicidal action of flavonoids. Quercetin inhibits ARG-L and ribonucleotide reductase (da Silva et al., 2012a; Sen et al., 2008). Quercetin induces cell death by increasing ROS (reactive oxygen species) and causing mitochondrial dysfunction in *L.* (*L.*) *amazonensis* (Fonseca-Silva, Inacio, Canto-Cavalheiro, & Almeida-Amaral, 2011). Luteolin and quercetin promote apoptosis mediated by topoisomerase II, resulting in kinetoplast DNA cleavage in *L.* (*L.*) *donovani* (Mittra et al., 2000).

The $E_{docking}$ (Table 2) results support the $IC_{50}$ (Table 1) data obtained from the aglycones and glycoside flavonoids. The only exception was that 7,8-dihydroxyflavone had an $IC_{50}$ lower than those of kaempferol and galangin. Docking suggested interactions between the flavonoids and the amino acids Asp137, Asp141, Asp243, Asp245 and His139 (ARG-L numbering) that are involved in metal bridge $Mn_A^{2+} - Mn_B^{2+}$ coordination in the active site of arginase (Kanyo et al., 1996). Another important interaction can be attributed to His154, which is a conserved amino acid involved in substrate binding (Ash, 2004). His 139 and His 154 showed hydrophobic intermolecular interactions with several flavonoids (Fig. 3).

Leishmaniasis is a complex disease; therefore, the use of specific targets in rational drug design is not the ideal approach to treat this illness (Cavalli & Bolognesi, 2009). The multiple targets of action of quercetin, luteolin and fisetin, make these compounds candidates for drug design against leishmaniasis. Future research could determine whether fisetin, luteolin and quercetin can be used as a lead or prototype drug with multiple targets for the treatment of leishmaniasis.

In conclusion, the *in vitro* and *in silico* study of these compounds can facilitate rational drug design and the development of new, safer drugs to treat leishmaniasis, using arginase as a drug target. Moreover, the low $IC_{50}$ values observed here may lead to the use of flavonoids as dietary supplements for leishmaniasis patients.

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