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**ARTICLE** *in* JOURNAL OF MEDICINAL PLANT RESEARCH · APRIL 2015

Impact Factor: 0.88 · DOI: 10.5897/JMPR2014.5641



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Vol. 9(13), pp. 426-434, 3 April, 2015
DOI: 10.5897/JMPR2014.5641
Article Number: A42115152263
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

*Full Length Research Paper*

# Morelloflavone and its semisynthetic derivatives as potential novel inhibitors of cysteine and serine proteases

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Received 9 October, 2014; Accepted 11 March, 2015

**This article reports the three biflavonoids isolated from the fruit pericarp of *Garcinia brasiliensis* Mart. (Clusiaceae): Morelloflavone-4```-glycoside (compound 1), (±)-Fukugiside (compound 2), and Morelloflavone (compound 3). Structural modifications by acylation and alkylation reactions were performed on the natural biflavonoid (±) morelloflavone to obtain three semisynthetic compounds: Morelloflavone-7,4`,7``,3```,4```-penta–*O*–acetyl (compound 4), Morelloflavone-7,4`,7``,3```,4```-penta-*O*-methyl (compound 5), and Morelloflavone-7,4`,7``,3```,4```-penta-*O*-butanoyl (compound 6). The inhibitory effects of these naturally isolated biflavonoid-type compounds and three semisynthetic derivatives on the activity of the cysteine proteases papain and cruzain, and on the serine protease trypsin were investigated. The potential inhibitory $IC_{50}$ of natural bioflavonoids compounds 1, 2, and 3 were 11.0 ± 3.0, 23.0 ± 4.0, and 10.5 ± 0.3 µM, respectively, for papain; 0.86 ± 0.12, 106 ± 7, and 3.8 ± 0.1, 50 ± 2, 119.5 ± 5, and 9.6 ± 1.0 µM, respectively, for cruzain. On the other hand, the semisynthetic biflavonoids compounds 4, 5, and 6 were more efficient in the inhibition of enzyme activity with $IC_{50}$ values 0.60 ± 0.02 µM (papain) for biflavonoids compound 4, 1.64 ± 0.11 µM (trypsin) for biflavonoids compound 5, and 8.1 ± 0.6 µM (cruzain) for biflavonoids compound 6. Compound 4 is more active owing to the carbonyl group in the structure; perhaps, this modification could favor a higher nucleophilic attack by serine and cysteine proteases. However, the semisynthetic compound 5 ($IC_{50}$ = 15.4 ± 0.7 µM for papain), which has no carbonyl group in structure, was less active in the inhibition. Interestingly, structure–activity relationships (SARs) were confirmed by flexible docking simulations. Likewise, the potential usefulness of natural compound 1 as an antioxidant compound was strengthened by our results concerning the antiproteolytic activity.**

**Key words:** *Garcinia brasiliensis*, biflavonoids, proteases, antiproteolytic activity.

## INTRODUCTION

The Guttiferae family, also known as Clusiaceae, belongs to the angiosperm phylogeny group and is characterized by the conspicuous presence of latex in most of their species. This family consists of 47 genera such as *Vismia, Garcinia, Clusia, Cratoxylum, Harungana, Mesua, Hypericum* and *Kielmeyera,* and more than 1000 species that are grouped in six subfamilies widely spread all over Brazil (Piccinelli et al., 2005; Derogis et al., 2008). The *Garcinia brasiliensis* species is native to the Amazon and is cultivated throughout Brazilian territory where it is known as "bacupari." It is a tree of medium size that blooms from August to September. It has yellow fruit with a white and edible mucilaginous pulp used in folk medicine as a wound healing agent and for peptic ulcer, urinary, and tumor disease treatments (Corrêa, 1978; Santa-Cecília et al., 2011).

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases). In this system, enzymes are given an Enzyme Commission number (EC). However, proteases do not comply easily with the general system of enzyme nomenclature due to their broad structural and functional diversity. Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Rawlings and Barett, 1995). Proteases or peptidases are enzymes that catalyze reactions on peptide chains, hydrolyzing them into short fragments. Endopeptidases split the peptide bond between amino acid residues placed within the ribbon, while exopeptidases split the residues at the polymeric backbone end. These enzymes can be further joined according to the reactant groups that are present in the catalytic site, as serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic acid (EC 3.4.23), metalo (EC 3.4.24) and threonine (EC 3.4.25) proteases. Papain (EC 3.4.22.2), a plant cysteine protease isolated from *Carica papaya* latex, preferentially cleaves peptide chains on either Arg and Lys residues or hydrophobic Phe residues (Brocklehurst, 1987; Martins et al., 2009). Trypsin (E.C. 3.4.21.4) has specificity for peptides containing Arg and Lys residues, and its catalytic triad is composed of the amino acids serine, histidine and aspartate, which is similar to other serine proteases (Martins et al., 2009; Beynon, 1989). Each amino acid of the triad has a specific role in the peptide bond cleavage of the substrates. The carboxylate group of the aspartic acid forms a hydrogen bond to the amine nitrogen atom of the histidine residue, contributing to increase the electronegativity of the imine nitrogen within the same heterocyclic side chain. In this way, the free electron pair of this last imidazolyl nitrogen atom is devoted to accepting the hydrogen from the serine hydroxyl group, thereby enhancing the nucleophilic attack by this serine residue on the carbonyl carbon atom of the peptide bond that is properly oriented into the enzymatic active site.

Cruzain is a member of a large family of closely-related isoforms found in the parasite *Trypanosoma cruzi*. It is involved in intracellular replication and differentiation, and is essential at all stages of the parasite's life cycle (Campetella et al., 1992; Lima et al., 1994). Since currently available therapeutics are both ineffective against the acute phase of the disease and are too toxic prolonged administration, efforts have been made to identify novel therapeutic targets, among which the proteolytic apparatus of *T. cruzi* is a possible candidate (McKerrow et al., 2009).

In this work, the bioactivity of three natural and three semisynthetic biflavonoid compounds were reported as part of our continuous study of *G. brasiliensis* (Gontijo et al., 2012a, b). The potential enzymatic inhibitory effects of the natural and semisynthetic compounds were evaluated against papain, trypsin, and cruzain. In addition, flexible molecular docking simulations with papain, trypsin, and cruzain were performed.

## MATERIALS AND METHODS

### Plant materials

Fruit pericarps of *G. brasiliensis* Mart. were collected at the campus of the Federal University of Viçosa-MG, Brazil, in February (summer) 2009. Botanical identification was performed in the Horto Botânico of the Federal University of Viçosa by Dr. João Augusto Alves Meira Neto. A voucher specimen (number VIC2604) was deposited at the Herbarium of the Federal University of Viçosa.

### General considerations

Reagents were used as received from commercial suppliers or dried by standard methods and recondensed or distilled before use. The reactions were extracted with $CH_2Cl_2$. The organic phase was separated and dried over $Na_2SO_4$. If necessary the residue was purified by column chromatography on silica, eluting with $CH_2Cl_2$ (100%). NMR spectra were measured on a multinuclear FT-NMR spectrometer (Bruker AVANCE DRX-400 or Bruker AVANCE DPX-200 MHz and DRX-500 MHz). $^1H$ and $^{13}C$ chemical shifts are $\delta$ values and given in ppm relative to $Me_4Si$. Coupling constants refer to H–H ($^1H$ NMR) or ($^{13}C$ NMR) unless stated otherwise. Molecular masses where determined by MALDI-TOF mass spectrometry (LCMS-2020EV) equipped with an ESI probe (Shimadzu), which was connected to the circuit after the UFLC SPD-20A UV detector.

Analyses were detected at 220 and 272 nm. Melting points were

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determined in sealed capillaries and uncorrected. Elemental analysis was performed using a TruSPEC (CHN-S) Analyzer by Leco Instruments, as described by Gontijo et al. (2012b). Protein Preparation Wizard protocol, Schrödinger Suite (Schrödinger Suite, 2009a).

The natural compounds were obtained from the ethyl acetate (EtOAc) extract of the fruit pericarp of *G. brasiliensis;* they were purified either by column chromatography on silica, eluting with hexane/ethyl acetate (60:40) or by Sephadex LH-20 eluting with MeOH. The isolated biflavonoids were identified as morelloflavone-4```-glycoside (compound 1) (Gontijo et al., 2012a), (±)-Fukugiside (compound 2) (Elfita et al., 2009) and morelloflavone (compound 3) (Li et al., 2002).

The three semisynthetic derivatives of morelloflavone (compound 3) (Figure 1) (compounds 4, 5 and 6) were obtained from acylation and alkylation reactions.

### Enzymes

The enzymes cruzain and rCPB2.8 (recombinant cysteine protease type B) were generously supplied by Dr. Luiz Juliano (Department of Biophysics, Federal University of São Paulo, Brazil), trypsin and papain were from Merck (Darmstadt, Germany), TLCK (N-alpha-tosyl-L-lysinyl-chloromethylketone), E-64 (1-[[(Ltrans-epoxysuccinyl)-L-leucyl]amino]-4-guanidinobutane), and fluorogenic substrate Z-FR-MCA (carbobenzyloxy-Phe-Arg-(7-amino-4-methylcoumarin) were obtained from Sigma-Aldrich Sigma (St. Louis, USA). Substrate hydrolyses were monitored in a spectrofluorometer F2500 Hitachi, and the enzymatic molar concentrations were estimated by titration according to kinetic parameters (Martins et al., 2009).

### Inhibition assays

Compounds 1 to 6 were tested for their inhibitory potential on papain, cruzain, and trypsin by spectrofluorometric method. Enzyme inhibition was expressed as the compound concentrations causing a 50% decrease in enzyme activity ($IC_{50}$ values). $IC_{50}$ values were calculated by time-course, dose-response curves using inhibitors at different concentrations, and the data were analyzed in Grafit 5.0 software using Equation 1.

$$y = \frac{Range}{1 + \left( \frac{x}{IC_{50}} \right)^{s}} \quad (1)$$

In Equation 1, Range is the fitted uninhibited value, *y* is the enzyme activity, *x* is the inhibitor concentration, and *s* is a slope factor. The equation assumes that *y* reduces with increasing *x*.

Inhibition reversibility assays for enzymes were carried out in 100 mM sodium acetate buffer, pH 5.5, 20% glycerol (to stabilize the enzymes), 5 mM EDTA, 5 mM dithiothreitol (DTT) (as cysteine protease papain and cruzain activator), and the enzyme solutions pre-activated for 5 min, at 37°C. The trypsin assays was carried out in 100 mM Trizma base HCl buffer, pH 7.5, 10% glycerol (to stabilize the enzyme). The substrate Z-FR-MCA was used as fluorogenic probe, and the hydrolysis of the substrate was monitored in a spectrofluorometer Hitachi F2500 at wavelength $\lambda_{Ex}$ = 360 nm (excitation) and $\lambda_{Em}$ = 480 nm (emission); the reference inhibitors used were E-64 for papain and cruzain, and TLCK for trypsin. The Z-FR-MCA concentrations were 18.5 µM at cruzain ($K_M$ = 1.8 µM), 7.4 µM at papain ($K_M$ = 0.7 µM) and 10.9 µM at trypsin ($K_M$ = 1.1 µM). The substrate concentrations were almost 10-fold over the $K_M$ for all enzymes in the study.

### Computational docking

In the docking studies, the 1.8 Å resolution structure files for trypsin, papain and cruzain were downloaded from the Brookhaven Protein Data Bank (PDB code 2RA3, 1PE6 and 1F2C, respectively) (Assis et al., 2012). This structure was prepared using the Protein Preparation Wizard protocol implemented in the Schrödinger Suite (2009a). After the protein preparation step, the docking procedure was carried out using the Induced Fit Protocol (IFD) Suite (Schrödinger Suite, 2009b).

The top 20 poses for each test ligand (with regard to the GlideScore) from the initial softened-potential docking step, were retained to sample the protein plasticity using the prime program from the Schrödinger, LLC suite. In stage 2, a conformational search and minimization were carried out on residues having at least one atom within 5.0 Å of distance to the ligand for each of the 20 ligand-protein complexes. In stage 3, the 20 induced conformations created for each inhibitor were ranked by the total prime energy (molecular mechanics plus solvation) of the complexes. Those within 30 kcal $mol^{-1}$ of the minimum energy structure were subjected to re-docking with the ligands and scored using glide. Glide XP (extra precision) was used for all re-docking calculations. The binding affinity of each complex was established through the GlideScore, which approximates the Gibbs free energy. The more negative the value of the GlideScore, the more favorable the binding (Jacobson et al., 2004). Maestro 9.0 was used as the graphical user interface (GUI) for all of Schrödinger's computational programs (Maestro, 2009).

### Data analysis

Statistical analysis was performed using Grafit 5.0 software. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Values of $p < 0.05$ were considered statistically significant. Data are expressed as mean ± standard deviation (SD) unless otherwise specified.

## RESULTS AND DISCUSSION

The natural biflavonoid compounds 1 to 3, semisynthetic compounds 4 to 6 and the reference compounds TLCK and E64 were assayed against the cysteine proteases papain and cruzain and the serine protease trypsin to determine the potential inhibitory effects as quantified in terms of $IC_{50}$ values (Table 1). The results show that all compounds tested inhibit the assayed proteases with different degrees of selectivity. The derivative compounds 4 and 5 were the most potent inhibitors to papain ($IC_{50}$ = 0.60 ± 0.02 µM) and trypsin ($IC_{50}$ = 1.64 ± 0.11 µM), respectively.

From the results (Table 1), compound 4 has a 35-fold higher affinity to papain than to trypsin. On the other hand, compound 5 has a 9-fold higher affinity to trypsin compared to papain. The results therefore showed that only compounds 4 and 5 exhibit a substantial degree of selectivity between the cysteine and serine proteases. However, these same compounds (4 and 5) did not efficiently inhibit the cysteine protease cruzain, where we

**Table 1.** Quantitative *in vitro* inhibitory effects of natural and semisynthetic biflavonoid derivatives on the cysteine proteases cruzain and papain and the serine peptidase trypsin.

| Compound | $IC_{50}$ value[a] (μM) | | |
|---|---|---|---|
| | Papain | Trypsin | Cruzain |
| 1 | 11.00 ± 3.00 | 0.86 ± 0.15 | 50 ± 2 |
| 2 | 23.00 ± 4.00 | 106 ± 7.00 | 119 ± 5 |
| 3 | 10.5 ± 0.3 | 3.8 ± 0.1 | 9.6 ± 1 |
| 4 | 0.60 ± 0.02 | 21.1 ± 1.3 | 537 ± 63 |
| 5 | 15.4 ± 0.7 | 1.64 ± 0.11 | 235 ± 38 |
| 6 | 15.7 ± 0.5 | 19.8 ± 1.3 | 8.1 ± 0.6 |
| TLCK[c] | - | 11.8 ± 0.6 | - |
| E64[c] | 0.0013 ± 0.0001 | - | 0.0068 ± 0.0002 |

[a]Each $IC_{50}$ value represents the mean ± standard deviation of triplicate determined by the software Grafit 5.0. [b]ND: $IC_{50}$ value not established by the sensitive method employed. [c]The compounds E-64 and TLCK were used as reference inhibitors of the corresponding proteases where they have been assayed.

found the $IC_{50}$ values of 537 ± 93 and 235 ± 38 μM, respectively. Comparing the inhibitory effects of both natural and semisynthetic biflavonoid compounds against the trypanossomatid cysteine proteases rCPB2.8 and rCPB3 from *L. mexicana* (Gontijo et al., 2012b) and cruzain from *T. cruzi*, it is clear that the biflavonoids studied here also showed species selectivity. The derivative compounds 4 and 5 are 801- and 335-fold more potent inhibitors for rCPB2.8 and rCPB3, respectively, than for cruzain (Data not shown, Table 1) (Gontijo et al., 2012b).

As shown in Table 1, the inhibitory potential ($IC_{50}$) of compound 1 was approximately 123 and 4.4-fold higher than compounds 2 and 3, respectively, against trypsin. Compound 1 also displayed 2.1 and 2.4-fold higher inhibition against papain and cruzain than compound 2, respectively. Compared to compound 1, compound 3 showed a similar inhibitory effect against papain, but was more active against cruzain (5.2-fold). Excluding the natural glycoside compound 2, compounds 1 and 3 and the semisynthetic compounds 4, 5 and 6 showed $IC_{50}$ values ranging from 0.6 to 15.7 μM against papain, and from 0.86 to 21.1 μM against trypsin.

As observed in Table 1, cruzain was poorly inhibited by both the natural and semisynthetic compounds. However, the biflavonoid compounds 3 (natural) and 6 (semisynthetic) were significantly effective in the inhibition of cruzain with $IC_{50}$ values of 9.6 ± 1.0 and 8.1 ± 0.6 μM, respectively. The derivative compounds 4 and 5 showed different degrees of inhibition of proteases, although the compound 4 bound preferentially to papain ($IC_{50}$ = 0.60 ± 0.02 μM) making it a potential antiproteolytic. It is important to highlight that the classical reference irreversible inhibitor of serine proteases, TLCK, was 7-fold less effective at trypsin inhibition ($IC_{50}$ = 11.8 ± 1.3 μM) than compound 5. On the other hand, the cysteine protease irreversible inhibitor E64 is 4614-fold and 78970-fold more effective in the inhibition of papain and cruzain, respectively. All three tested enzymes are known as trypsin-like hydrolases due to $S_1$ specificity for both Arg and Lys basic residues, cleaving the C–N bond of substrate Z-FR-MCA between the Arg amino acid and MCA group (Assis et al., 2012).

At this point, the following interesting structure–activity relationship could be proposed: the capacity to inhibit the enzymatic activity of papain decreases according to the number of carbons in the $R_1$=$R_2$=$R_3$=$R_4$=$R_5$ ligands (acetyl, methyl or butanoyl ligands) attached at the biflavonoid core; the absence of carbonyl group in compound 5 increases the potential inhibitory effect over trypsin by almost 13-fold.

The docking simulations of compounds with enzymes (Figure 2) showed very similar modes of binding. The amino acid sequences of papain and cruzain are 38% homologous, and their active sites are nearly identical (Assis et al., 2012; Melo et al., 2001; PDB ID, 1996). Papain and trypsin were selected because they are commercially available in large quantities, and their structures are known. The hydrophobic interactions and hydrogen bonds for compound 3 in papain, trypsin and cruzain were related previously (Martins et al., 2007, 2009; Assis et al., 2012; Sasaki et al., 1986, 1990).

According to the molecular docking simulations, papain establishes hydrophobic interactions with ring A and with rings A/B by the residues of the $S_2$ subsite with compound 1 (Figure 2A). One the other hand, hydrophobic interactions are established with ring C and ring A with residues of the $S_2$ subsite with compound 2 (Figure 2B).

Compounds 4, 5 and 6 are semisynthetic molecules derived from natural compound 3 (Figures 2C, 3C and 4C) with modifications in the five hydroxyl groups as shown in Figure 1. The acetyl, methyl and butanoyl groups of ring C from compounds 4 (Figure 2D), 5

**Table 2.** Values of established intramolecular hydrogen bonds of natural and semisynthetic biflavonoid derivatives on cysteine proteases cruzain and papain and serine peptidase trypsin.

| Enzyme | H-Bonds | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | # 1 | Amino acid | Å | # 2 | Amino acid | Å | # 4 | Amino acid | Å | # 5 | Amino acid | Å | # 6 | Amino acid | Å |
| | 2A(O34) | VAL133(H) | 2.21 | 2B(H73) | GLY66(O) | 2.33 | 2D(O47) | TRP69(HE1) | 1.76 | 2E(O34) | TYR67(HH) | 1.81 | 2F(H93) | TRP69(HE1) | 2.15 |
| | 2A(H82) | GLY66(O) | 2.30 | - | - | - | 2D(O52) | LYS156(HZ1) | 2.15 | 2E(O36) | ALA160(H) | 2.23 | 2F(O52) | LYS156(HZ1) | 2.03 |
| Papain | 2A(H72) | SER205(O) | 2.14 | - | - | - | 2D(O54) | LYS156(HZ1) | 2.06 | - | - | - | - | - | - |
| | 2A(H59) | SER131(O) | 2.57 | - | - | - | 2D(H70) | GLY23(O) | 1.99 | - | - | - | - | - | - |
| | 2A(H79) | ASP158(O) | 1.91 | - | - | - | 2D(O56) | ALA160(H) | 2.11 | - | - | - | - | - | - |
| | 3A(H74) | TRP151(O1P) | 1.67 | 3B(H80) | SER190(HG) | 1.84 | 3D(O52) | HIS57(HD1) | 2.13 | 3E(H61) | SER214(O) | 1.79 | 3F(H80) | SER214(O) | 1.96 |
| | 3A(H79) | TRP215(O) | 1.70 | 3B(O55) | CYS191(N) | 2.86 | 3D(O54) | SER195(HG) | 2.13 | 3E(O40) | GLY216(H) | 2.12 | - | - | - |
| | 3A(H78) | ASP189(OD2) | 2.06 | 3B(H79) | CYS191(O) | 1.96 | 3D(H65) | CYS220(H) | 2.09 | - | - | - | - | - | - |
| Cruzain | 3A(H77) | GLY216(O) | 1.88 | 3B(H76) | GLN192(OE1) | 2.11 | - | - | - | - | - | - | - | - | - |
| | 3A(O36) | ARG96(HH21) | 1.97 | 3B(H66) | GLN192(OE1) | 2.32 | - | - | - | - | - | - | - | - | - |
| | - | - | - | 3B(H82) | TRP215(O) | 1.94 | - | - | - | - | - | - | - | - | - |
| | - | - | - | 3B(H75) | LYS97(O) | 1.79 | - | - | - | - | - | - | - | - | - |
| | 4A(O37) | GLN19(HE22) | 2.00 | 4B(H49) | GLY66(O) | 1.99 | 4D(H70) | GLY23(O) | 1.98 | 4E(O37) | GLN19(HE22) | 2.23 | 4F(O35) | GLN19(HE22) | 2.04 |
| | 4A(H72) | GLN21(O) | 1.85 | 4B(H74) | LEU157(O) | 1.87 | 4D(O54) | GLN156(HE21) | 1.99 | - | - | - | 4F(H108) | ALA136(HB3) | 2.13 |
| | 4A(H74) | GLY20(O) | 1.88 | 4B(O48) | ASP158(O) | 2.96 | 4D(O52) | GLN156(HE22) | 2.27 | - | - | - | - | - | - |
| Trypsin | 4A(H78) | GLY66(H) | 1.77 | 4B(H73) | ASP158(O) | 2.05 | - | - | - | - | - | - | - | - | - |
| | 4A(H82) | LEU157(O) | 1.96 | 4B(H79) | GLU205(OE1) | 2.29 | - | - | - | - | - | - | - | - | - |
| | 4A(H71) | ASP158(O) | 1.74 | 4B(H82) | GLU205(OE1) | 1.62 | - | - | - | - | - | - | - | - | - |
| | 4A(O47) | ASP158(HA) | 2.55 | - | - | - | - | - | - | - | - | - | - | - | - |

#Compound number; Å is extension of hydrogen bond in Angström.

(Figure 2E) and 6 (Figure 2F), respectively, are establishing hydrophobic interactions with the $S_2$ subsite of papain.

The hydroxyl groups from ring A of compound 1 are responsible for forming three hydrogen bounds (Table 2) with papain, and the OH- from the glycosyl group are forming another two H-bonds, and ring C is establishing a π-π stacking interaction between $Trp^{69}$ from the $S_2$ subsite (Figure 2A). Compound 2 are forming H-bonds between OH- from ring A with $Glu^{66}$, and π-π stacking interaction between $S_2$ subsite with rings A`/B` (Figure 2A). Hydrogen bonds (Table 2) are established between compound 4 (Figure 2D) with $Trp^{69}$, $Ala^{160}$, $Lys^{156}$, and $Gly^{23}$; compound 5 (Figure 2E) with $Tyr^{67}$ and $Ala^{160}$; and compound 6 (Figure 2F) with $Lys^{156}$ and $Trp^{69}$.

Observing the molecular modeling of trypsin (Figure 3), we verified hydrophobic interactions between compound 1 (Figure 3A) at rings A`/B` with residues of $S_1$`, ring C` with residues of the $S_2$ subsite, and the glycosyl group with residues of amino acids that stabilize the $S_1$ subsite; on the other hand, compound 2 (Figure 3B) established hydrophobic interactions of the glycosyl group with amino acids of the $S_2$ subsite and $Gly^{193}$/$Ser^{195}$, which are responsible for forming the oxyanion hole of trypsin.

For interaction of compounds 4, 5 and 6 (derivatives from compound 3) with the serine protease trypsin, we verified hydrophobic interactions of acetyl groups of compound 4 (Figure 3D) at residues of $S_1$, $S_1$´, and $S_3$/$S_4$ subsites; a methyl group of compound 5 (Figure3E) with residues of $S_1$ subsite; and butanoyl groups of compound 6 (Figure 3F) with residues of $S_1$ and $S_3$/$S_4$

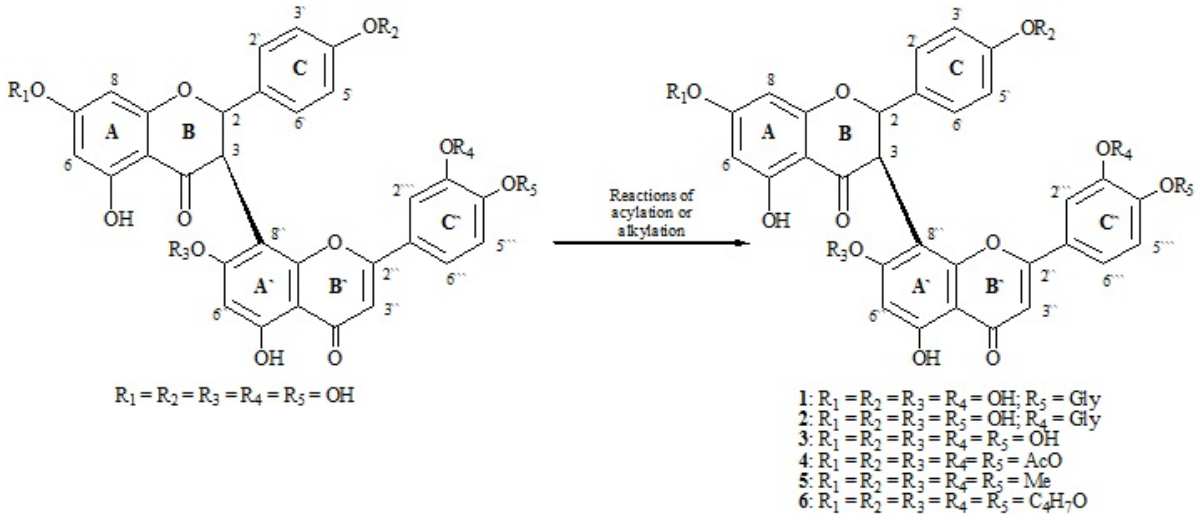


**Figure 1.** Structures of the natural and semisynthetic biflavonoid compounds **1 to 6**.

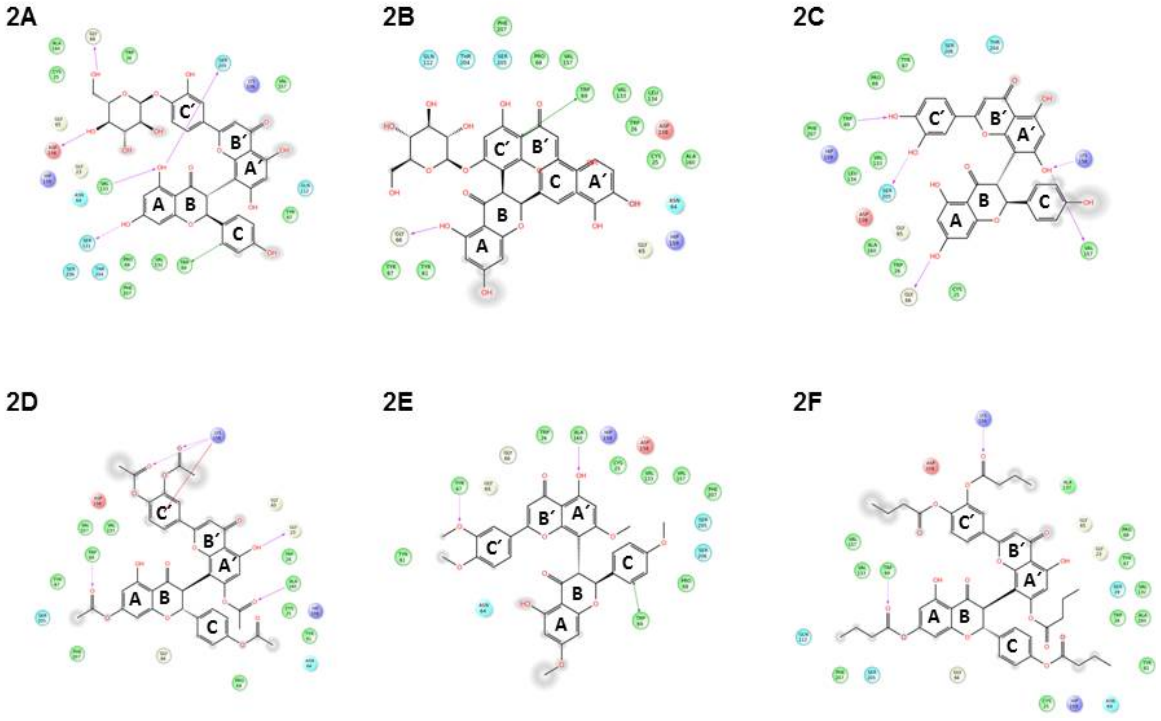


**Figure 2.** Molecular docking of compounds **1 to 6** with papain. The protease structures (1.8Å resolutions) were obtained from the Brookhaven Protein Data Bank. The PDB code was 2RA3 for papain (PDB ID, 1996). A) Morelloflavone-4```-glicoside (**1**)/papain; B) (±)-Fukugiside (**2**)/papain; C) Morelloflavone (**3**)/papain; D) Morelloflavone-7,4`,7``,3```,4```-penta–*O*–acetyl (**4**)/papain; E) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-methyl (**5**)/papain; F) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-butanoyl (**6**)/papain.

subsites.

It is important to note that some amino acid residues of trypsin can occupy two subsites, as can be observed for Gln[192] (member of $S_1$ and $S_1$` subsites) and for residues of $S_3$ and $S_4$ subsites like Leu[99], Trp[215], Val[213], Ser[217].

Moreover, residues of trypsin established hydrogen bonds (Table 2) between compound 1 (Figure 3A) and residues Arg[96], Trp[151], Asp[189], Trp[215], and Gly[216]. On the other hand, compound 2 is establishing OH-bonds (Figure 3B) with residues Lys[97], Ser[190], Cys[191], Gln[192],

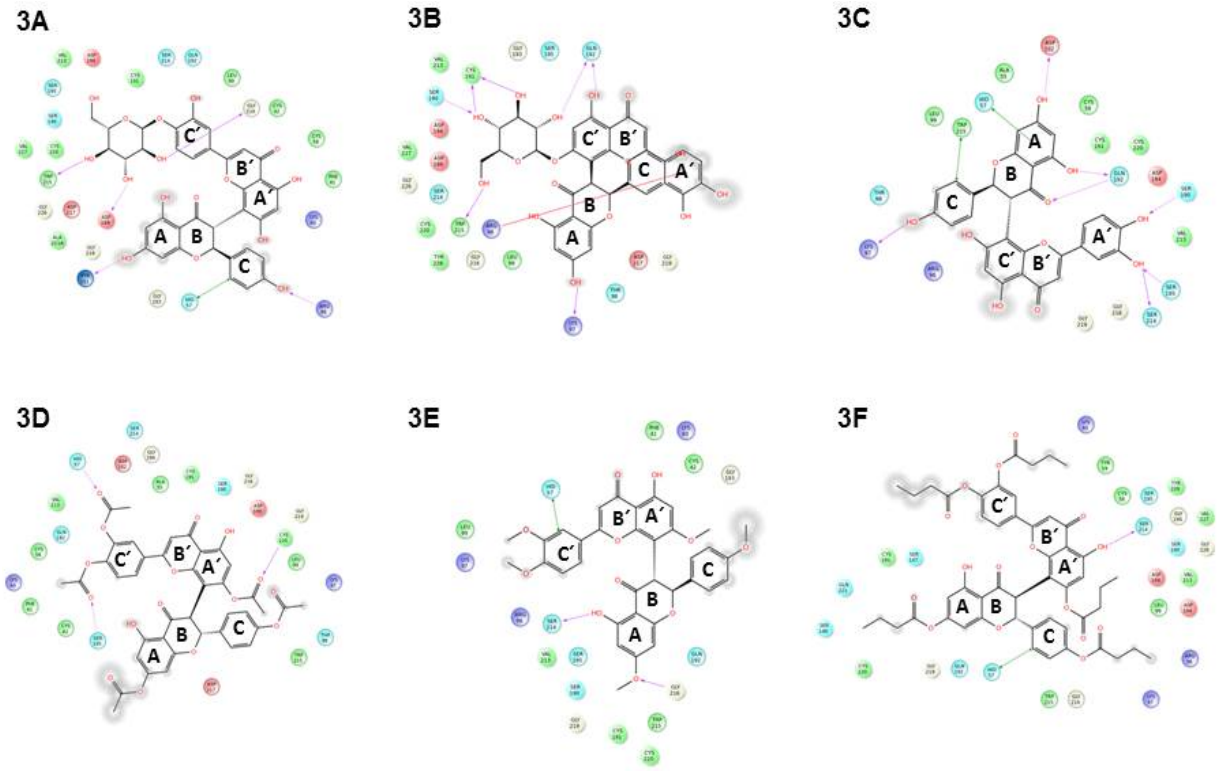


**Figure 3.** Molecular docking of compounds **1 to 6** with trypsin. The protease structures (1.8Å resolutions) were obtained from the Brookhaven Protein Data Bank. The PDB code was 1F2C for trypsin (PDB ID, 1996). A) Morelloflavone- 4```-glicoside (**1**)/trypsin; B) (±)-Fukugiside (**2**)/trypsin; C) Morelloflavone (**3**)/trypsin; D) Morelloflavone-7,4`,7``,3```,4```-penta–*O*–acetyl (**4**)/trypsin; E) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-methyl (**5**)/trypsin; F) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-butanoyl (**6**)/trypsin.

and Trp[215]. Furthermore, the substitution of hydroxyl groups of compound 3 at the acetyl (Figure 3D→ compound 4), the methyl (Figure 3E→ compound 5) or the butanoyl (Figure 3F→ compound 6) promotes a decrease in the number of hydrogen bonds with trypsin when compared with compounds 1, 2, and 3. Compound 6 also performs a π-π stacking interaction between His[57] and ring C.

In the molecular docking of cruzain with compounds 1 (Figure 4A) and 2 (Figure 4B), the glycosyl groups are establishing hydrophobic interactions with residues of the $S_2$ subsite (Trp[26], Leu[67], Met[68], Ala[133], and Gly[160] ); and compound 4 (Figure 4D) at the acetyl group with residues of the $S_2$ subsite; and compound 5 (Figure 4E) at the methyl group of C4` of ring C with Cys[22], Gly[23], Cys[25], Ala[136], Ser[139], Trp[141], and Asp[158]. Furthermore, it performs a π-π stacking interaction between ring A with Trp[177]. Compound 6 (Figure 4F) performs hydrophobic interactions at the butanoyl group of C3``` of ring C` with residues of the $S_2$ subsite.

The Trp[177] residue is widely conserved throughout the papain superfamily (McGrath 1999), and the quality of the aromatic–aromatic interactions is, in part, dependent upon the length of the inhibitor. The indole ring of this residue promotes the correct orientation of catalytic asparagine to interact with catalytic histidine by a π-NH interaction facilitating the formation of a thiolate-imidazolium ion pair during the catalysis (Brinen et al., 2000). Thus, blocking the formation of this ion pair prevents catalysis.

Cruzain protease is establishing hydrogen bonds (Table 2) with bioflavonoid compounds. Compound 1 (Figure 4A) performs H-bonds with Gln[19], Gly[20], Gln[21], Gly[66], Leu[157], and Asp[158]; compound 2 (Figure 4B), with residues Gly[66], Leu[157], Asp[158], and Glu[205]; compound 4 (Figure 4D), with Gly[23] and Gln[156A]; compound 5 (Figure 4E), one H-bond with Gln[19]; and compound 6 (Figure 4F), H-bonds with Gln[19] and Ala[136]. The increase of hydrophobicity of the compounds causes a reduction to the number of hydrogen bonds due to replacement of OH-groups by carbon chains.

## Conclusion

Three semisynthetic biflavonoid derivatives have shown potential inhibitory effects on papain and trypsin proteases; the structural modifications of compounds are

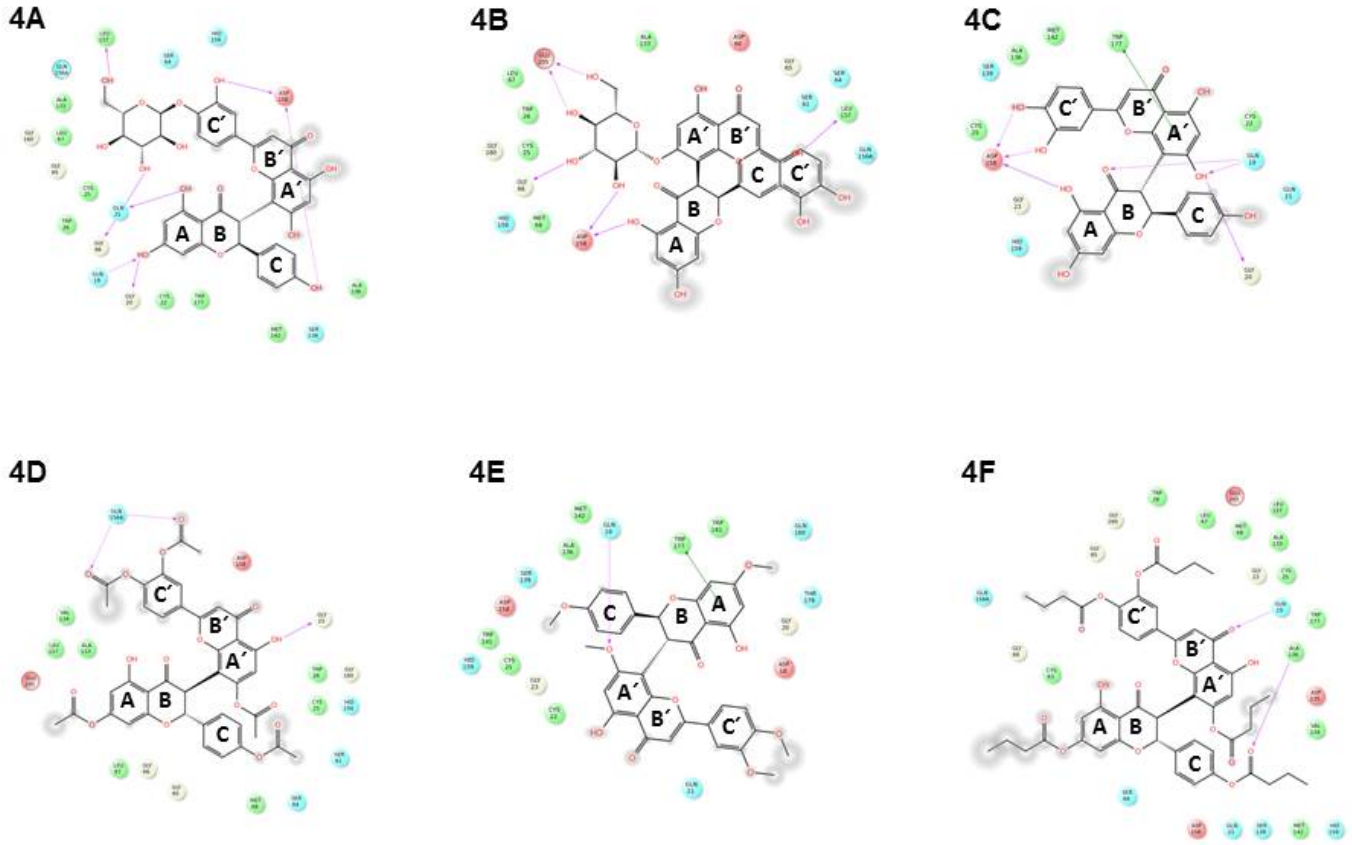


**Figure 4.** Molecular docking of compounds **1 to 6** with cruzain. The protease structures (1.8Å resolutions) were obtained from the Brookhaven Protein Data Bank. The PDB code was 1PE6 for cruzain (PDB ID, 1996). A) Morelloflavone-4```-glicoside (**1**)/cruzain; B) (±)-Fukugiside (**2**)/cruzain; C) Morelloflavone (**3**)/cruzain; D) Morelloflavone-7,4`,7``,3```,4```-penta–*O*–acetyl (**4**)/cruzain; E) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-methyl (**5**)/cruzain; F) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-butanoyl (**6**)/cruzain.

important for inhibitory activity of enzymes. The concentration of morelloflavone-7,4`,7``,3```,4```-penta-O-butanoyl (compound 6) to selectively inhibit trypsin is only 1.6-fold to that of the peptide-based classical inhibitor of this enzyme, TLCK. TLCK can potentiate such derivative as antiproteolytic drug for treatment of diseases in which this protease is involved. Therefore, the structural modifications were significantly enhanced inhibition of protease enzymes by improving the activity of natural compound 3, as observed by compounds 4 and 5 over papain and trypsin, respectively. The molecular docking models showed that the compounds mainly interact with the cysteine proteases papain and cruzain at the $S_2$ subsite; on the other hand, the interactions occur mainly at the $S_1$ subsite of the serine protease trypsin. However, parts of amino acid residues can participate in more than one subsite, making characterization of residue interaction with a specific subsite difficult. The structural characteristics and the hydroxyl group substitutions in the natural compound 3 increased the potency of some compounds *in vitro*. These semisynthetic biflavonoid derivatives are therefore potential candidates to therapeutically inhibit proteases involved in parasitic infections caused by *T. cruzi*.

## ACKNOWLEDGEMENTS

This work was supported by grants from Universal Project (CNPq, Brazil, #477876/2011-0) and Universal Projects (FAPEMIG, Brazil, #CEX-APQ-01072-08 and #CDS-BPD-00301/12). The authors are also grateful for the fellowships granted by CAPES, CNPq and FAMEMIG.

## Abbreviations:

**r-CPB2.8,** Recombinant cysteine protease type B; **EOAc,** ethyl acetate extract; **MeOH,** methanol; **Z-Phe-Arg-MCA,** carbobenzoxicarbonil-Phe-Arg-7-amino-4-methylcoumarin; **DTT,** dithiothreitol; **SARs,** structure–activity relationships; **FT-NMR,** Fourier transform nuclear magnetic resonance; **$Me_4Si$,** trimethylsilane; **TLCK,** N-alpha-tosyl-L-lysinyl-chloromethylketone; **E-64,** 1-[[(Ltrans-epoxysuccinyl)-L-leucyl]amino]-4-guanidinobutane.

## Conflict of interest

Authors have not declared any conflict of interest.

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