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"SEARCH FOR ENZYME INHIBITORS IN CERRADO PLANTS AS STRATEGY FOR THE DISCOVERY OF PROTOTYPE DRUGS AGAINST NEGLECTED TROPICAL DISEASES"

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Thesis submitted in fulfilment of the requirements for the Degree of DOCTOR OF SCIENCE, specific area: ORGANIC CHEMISTRY.

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Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning.
[Albert Einstein]

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"If I have seen further it is by standing on the shoulders of giants."

[Isaac Newton]

ABBREVIATIONS

acetone-d6	Deuterated acetone
AMC	7-Amino-4-methylcoumarin
ARG	Arginase
Asn	Asparagine
Asp	Aspartic acid
Brij	Polyoxyethylene surfactant
Bz-nKRR-H	Benzoyl-norleucine-Lys-Arg-Arg-aldehyde
Cat L	Cathepsin L
Cat B	Cathepsin B
CHAPES	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHES	2-(Cyclohexylamino)ethanesulfonic acid
CPA	Cysteine proteases L from *Leishmania* spp.
CPB	Cysteine proteases L from *Leishmania* spp.
CPC	Cysteine proteases B from *Leishmania* spp.
CPs	Cathepsins
^{13}C NMR	Carbon 13 Nuclear Magnetic Resonance
1D	One dimension
2D	Two dimensions
DEAE	Diethylaminoethyl-sepharose
DENV	Dengue virus
DEPT	Distortionless enhancement by polarization transfer
DHF	Dengue hemorrhagic fever
DMAP	4-(Dimethylamino) pyridine
DMSO	Dimethyl sulfoxide
DSS	Dengue shock syndrome
DTT	Dithiothreitol

EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ES	Enzyme-Substrate
ESI	Enzyme-Substrate-Inhibitor
EtOAc	Ethyl acetate
EtOH	Ethanol
EGCG	Epigallocatechin-3-gallate
Gly	Glycine
Gln	Glutamine
HMBC	Heteronuclear multiple quantum correlation
^{1}H NMR	Nuclear Magnetic Resonance of hydrogen
HSQC	Heteronuclear single quantum correlation
IC_{50}	Half maximal inhibitory concentration
Ile	Isoleusine
K_i^{app}	Apparent Affinity Constant
K_D	Dissociation Constant
K_i	Affinity constant
K_{is}	Affinity constant of enzyme-inhibitor
K_{ii}	Affinity constant of inhibitor with enzyme-substrate complex
K_M	Michaelis–Menten constant
Lys	Lysine
MeOH	Methanol
MeOH-d_4	Deuterated methanol
MHz	Mega hertz
MS	Mass spectrometry
MST	Microscale Thermophoresis
Ni-NTA	Nickel-Nitrilotriacetic Acid

NOS	Nitric Oxide Synthase
iNOS	Inducible Nitric Oxide Synthase
NS3	Serine Protease of the Dengue Virus
NS2B	Cofactor of the NS3 Serine Protease
NPs	Natural Products
NTDs	Neglected Tropical Diseases
NT647-NHS	Nano Temper–amines (fluorescent dye)
OAc	Acetyl group
$OD_{600\ nm}$	Optical density at a wavelength of 600 nm
Or	Ordinate Intercept
PAs	Polyamines
pH	Potential of Hydrogen
r-CPB2.8	Recombinant cathepsin L from *L. mexicana*
rha	Rhamnoside
Sl	Slope
S	Substrate
SOB	Super Optimal Broth
TH_1	T helper cell type 1
TH_2	T helper cell type 2
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
TNF	Tumor necrosis factor
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
UV	Ultraviolet
v	Velocity
V_{max}	Maximum velocity
V_{max}^{app}	Apparent Maximum velocity
Z-Phe-Arg-AMC	Carboxybenzyl-Phe-Arg-7-amino-4-methylcoumarin

λ_{em}	Emission Wavelength
λ_{ex}	Excitation Wavelength

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RESUMO

BUSCA DE INIBIDORES DE ENZIMAS EM PLANTAS DO CERRADO COMO ESTRATÉGIA DE INVESTIGAÇÃO DE PROTÓTIPOS A FÁRMACOS DE DOENÇAS NEGLIGENCIADAS.

A primeira etapa deste trabalho de pesquisa envolveu uma triagem de extratos de plantas do cerrado paulista através de ensaios frente à arginase (ARG) de *Leishmania amazonensis*. Os resultados de inibição enzimática da ARG apresentados pelos extratos, levaram ao fracionamento biodirecionado dos extratos de acetato de etila (folhas e galhos) de *Byrsonima coccolobifolia* Kunth. (Malpighiaceae). Alguns compostos bioativos foram isolados (flavonóides, ácido galoilquínicos, uma lignana, uma fenatrenona e uma ortoquinona) o que indicou que estes compostos seriam os responsáveis pela diminuição da atividade enzimática da ARG observada nos extratos de *B. coccolobifolia*. Os flavonoides e os ácidos galoilquínicos apresentaram modo de inibição do tipo não-competitivo da ARG, com atividade inibitórias significativas (IC_{50} = 0,13 à 4,8 µM) e com forte afinidade (valores de K_i variando entre 0,10 à 3,80 µM). Os estudos de relação entrutura atividade (SAR) revelaram que o grupo catecol dos flavonóides, tanto como a unidade galoila das estruturas dos ácidos galoilquínicos, forneceram importantes características em relação as interações ARG–inibidor.

Na segunda parte deste trabalho, experimentos subsequentes foram realizados para investigar os produtos naturais frente a proteases, as quais possuem funções importantes na patogenicidade de virus e parasitas, tais como: as serino proteases NS2B-NS3 dos vírus da dengue serotipos 2 e 3 (dengue), a rodesaína de *Trypanosoma brucei rhodesiense* (tripanossomíase africana), a cruzaina de *Trypanosoma cruzi* (doença de chagas), a catepsina L de *Leishmania mexicana* (leishmaniose) e a falcipaina-2 de *Plasmodium falciparum* (malária). Através destes estudos foram descobertos alguns inibidores das proteases NS2B-

NS3 dos vírus da dengue (tipo 2 e 3) e da catepsina L (r-CPB2.8) de *L. mexicana*.

Os flavonóides agatisflavona, miricetina, e quercetina (K_i = 11,1; 4,7; e 20,7 µM; respectivamente) mostraram inibição do tipo não-competitiva frente à NS2B-NS3. Também foram realizados, um experimento de termoforese em microescala (MST) (ensaio livre de substrato) e um estudo *in silico*, de forma a obter maiores informações sobre as interações biomoleculares envolvidas.

Os metabólitos secundários, agatisflavona e ácido 3-oxo-urs-12-en-28-óico, foram determinados serem inibidores do tipo não-competitivo parcial da catepsina L do tipo r-CPB2.8, e a tetrahidrorobustaflavona como um inibidor incompetitivo desta catepsina L (r-CPB2.8), com valores de K_i na faixa de micromolar (K_i = 0,14−1,26 µM).

ABSTRACT

SEARCH FOR ENZYME INHIBITORS IN CERRADO PLANTS AS STRATEGY FOR THE DISCOVERY OF PROTOTYPE DRUGS AGAINST NEGLECTED TROPICAL DISEASES.

The early phase of this work involved a screening of plant extracts of São Paulo cerrado using assays with arginase (ARG) from *Leishmania amazonensis*. The results of enzymatic inhibition of ARG by the extracts led to the bioactivity-guided fractionation of the ethyl acetate extracts (leaves and stems) of *Byrsonima coccolobifolia* Kunth. (Malpighiaceae). Thereafter, several bioactive compounds were isolated (flavonoids, galloylquinic acids, a lignan, a phenathrenone and an orthoquinone) indicating that these compounds may are the responsible for the decrease in ARG activity observed in *B. coccolobifolia* extracts. The flavonoids and galloylquinic acids showed to be noncompetitive inhibitors of ARG with significant inhibitory activities (IC_{50} = 0.13 to 4.8 µM) and strong affinity (K_i values ranging from 0.10 to 3.8 µM). Additionally, structure activity relationship study (SAR) revealed that catechol group in flavonoids, likewise as galloyl unit in galloylquinic acids structures yielded important features for ARG–inhibitor interactions.

In the second part of this work, subsequent experiments were performed to investigate the natural products against proteases. These enzymes play pivotal roles in the pathogenicity of virus and parasites, such as: serine proteases NS2B-NS3 from dengue virus serotype 2 and 3 (dengue fever), rhodesain from *Trypanosoma brucei rhodesiense* (African trypanosomiasis), cruzain from *Trypanosoma cruzi* (chagas disease), cathepsin L from *Leishmania mexicana* (leishmaniasis), and falcipain-2 from *Plasmodium falciparum* (malaria). Through these studies were discovered inhibitors of NS2B-NS3 proteases of dengue virus and inhibitors of the *L. mexicana* cathepsin L-like r-CPB2.8.

The flavonoids agathisflavone, myricetin, and quercetin (K_i = 11, 4.7, and 20.7 μM, respectively) showed noncompetitive inhibition type on NS2B-NS3. In addition, a substrate-free microscale thermophoresis (MST) experiment and an *in silico* study were carried out regarding more informations of biomolecular interactions.

The secondary metabolites, agathisflavone and 3-oxo-urs-12-en-28-oic acid, were determined as partially noncompetitive inhibitors of cathepsin L-like r-CPB2.8, and tetrahydrorobustaflavone as an uncompetitive inhibitor of cathepsin L-like rCPB2.8, with K_i values in the low micromolar range (K_i = 0.14−1.26 μM).

SUMMARY

1 - INTRODUCTION

Neglected Tropical Diseases (NTDs) are a group of diseases associated with socioeconomic and environmental conditions affecting poor populations. Although NTDs are almost half of the cases of disease in developing countries, there is no much investment on research from governments and the pharmaceutical industry in this area. The NTDs are caused by four different pathogens: protozoa, bacteria, virus, helminth, conferring 17 diseases (e.g., chagas, sleeping sickness, leishmaniases, dengue, rabies, among others). According to the World Health Organization (WHO) these NTDs are endemic in 149 countries and affect more than 1.4 billion people (WHO, 2013).

Among the NTDs, leishmaniasis is a zoonotic disease caused by more than 20 species of *Leishmania* protozoa, which are transmitted to human by the sandflies bites of *Lutzomyia* spp and *Phlebotomus* genus (SCHLEIN, 1993; WHO, 2010). These different species of *Leishmania* can develop three forms of the disease, which are known as cutaneous (skin ulcers), mucocutaneous (mouth and nose) and visceral leishmaniasis (organs such as liver, spleen, and bone marrow), if untreated, the visceral leishmaniasis can lead to death. Leishmaniasis affect around 1.3 million people with 20000 to 30000 deaths yearly, where in Brazil were carried out 12.5 million of deaths in the time frame of 2000 to 2011, and mortality is increasing in spite of ongoing control strategies (Figure 1) (SCHLEIN, 1993; DESJEUX, 2001; WHO, 2010).

Dengue is another NTD caused by different serotypes of virus (DENV 1, 2, 3, 4, 5) and is transmitted to humans by *Aedes aegypti* and *Aedes albopictus* mosquitos (HARRIS, et al., 1998; DAS et al., 2008; FATIMA et al., 2011; NORMILE, 2013). Nowadays is one of the WHO concerns, since the incidence of dengue has increased 30-folds over the last 50 years, estimating around 50-100 million of infections in world´s population annually (Figure 2)

(WHO, 2009). Brazil is the country with one of the highest incidence of dengue in the world, with 1.4 million of cases of infection only in 2013 (Ministerio da Saúde, 2014; PESSANHA et al., 2014).

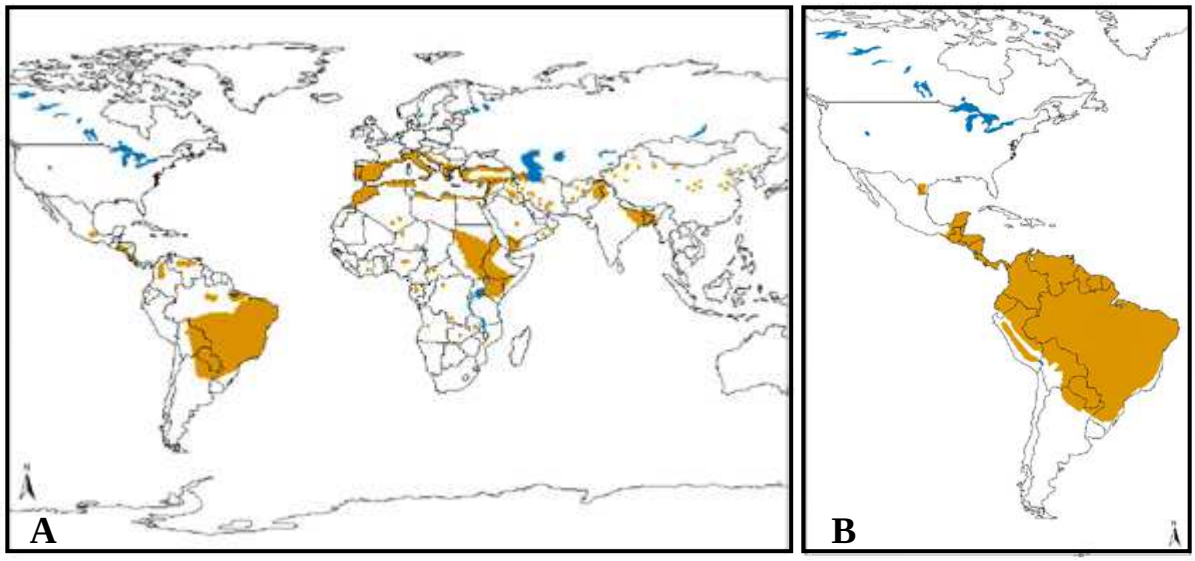


Figure 1. (A) Geographical distribution of visceral leishmaniasis in the world; (B) cutaneous and mucocutaneous leishmaniasis in the Americas, 2010. (http://www.who.int/leishmaniasis/leishmaniasis_maps/en/)

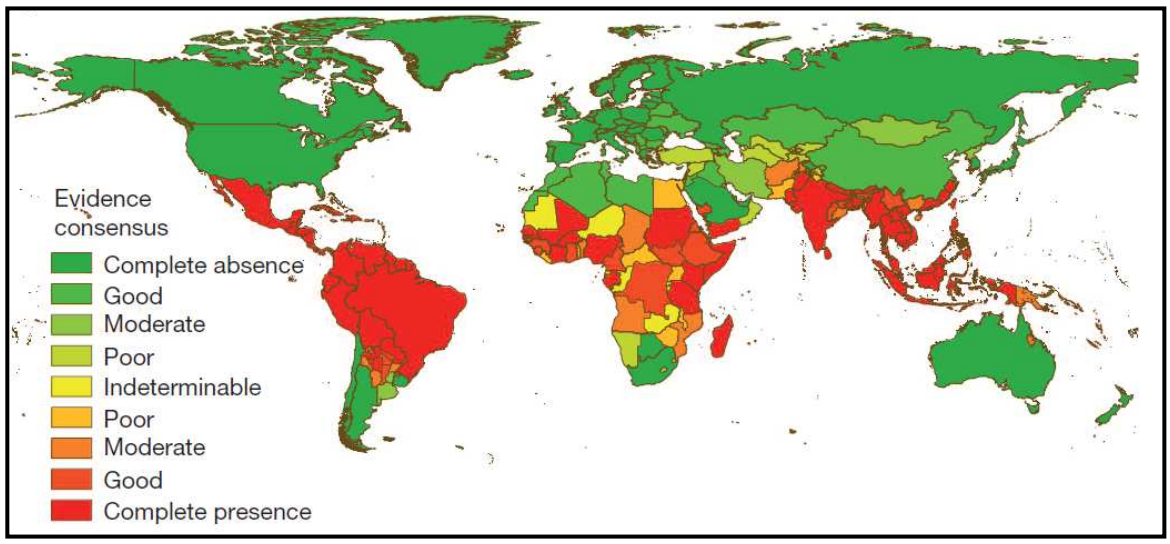


Figure 2. Areas at risk of dengue transmission and with dengue incidence, 2010. In green is complete absence going through to red, a complete presence of dengue infection (BRADY et al., 2012; BHATT et al., 2013).

The different species of *Leishmania* and the diversity of serotypes of dengue virus make challenging the development of vaccines to prevent these infections. There are no available antiviral drugs againt dengue so far, therefore the treatment of this illness is just supportive (WHO, 2009; WHO, 2010, BHATT et al., 2013). Currently some chemotherapeutic drugs are used against leishmaniasis such as pentavalent antimonials (stibogluconate of sodium – Pentostam®, *N*-methylglucantime – Glucantime®), pentamidine –Pentacarinato®, amphotericin B – Fungizone®, paramomycin and miltefosin, but those agents have showed parasite resistance, severe side effects and high cost (AMATO et al., 2000; SANTOS et al., 2008; GÒMEZ et al., 2014).

In the search for new drugs against NTDs some strategies can be used: HTS (high-throughput screening) by *in vitro* assay targeting the microorganism of interest; HTS targeting the microorganism enzymes, through enzymatic assays screening; *in silico* study for inhibitor-structure optimization (BAJORATH, 2002).

An enzyme used as molecular target, usually is a protein involved in essential biochemical pathways for the pathogens (protozoa, bacteria, virus, helminth) development (VERLINDE et al., 2001). The enzyme inhibitors have been used as pharmacological intervention becoming almost half of the drugs in clinical use presently (HOPKINS and GROOM, 2002; COPELAND, 2005; ROBERTSON, 2005). Among the enzymes, the proteases are estimated to be around 5–10% of all pharmaceutical targets demonstrated by marketed drugs (DRAG and SALVESEN, 2010).

Proteases are proteolytic enzymes that catalyse the hydrolysis of the peptide bonds of proteins, and efforts have been made to develop protease inhibitors (PIs) as drugs. There are several molecules as PIs in development, such as inhibitors of cathepsin K, a cysteine protease associated with osteoporosis illness. It was found four potential cathepsin K inhibitors that are in clinical trials, such as AAE581 (balicatib) passed Phase II; SB-462795

developed by GlaxoSmithKline (Phase II) (Figure 3) (PALMER et al., 2005; TURK, 2006). Additionally, many licensed drug-like protease inhibitors are available for clinical use, such as: tipranavir, saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir and darunavir, used for the treatment of HIV infection (Figure 3) (DE CLERCQ et al., 2009).

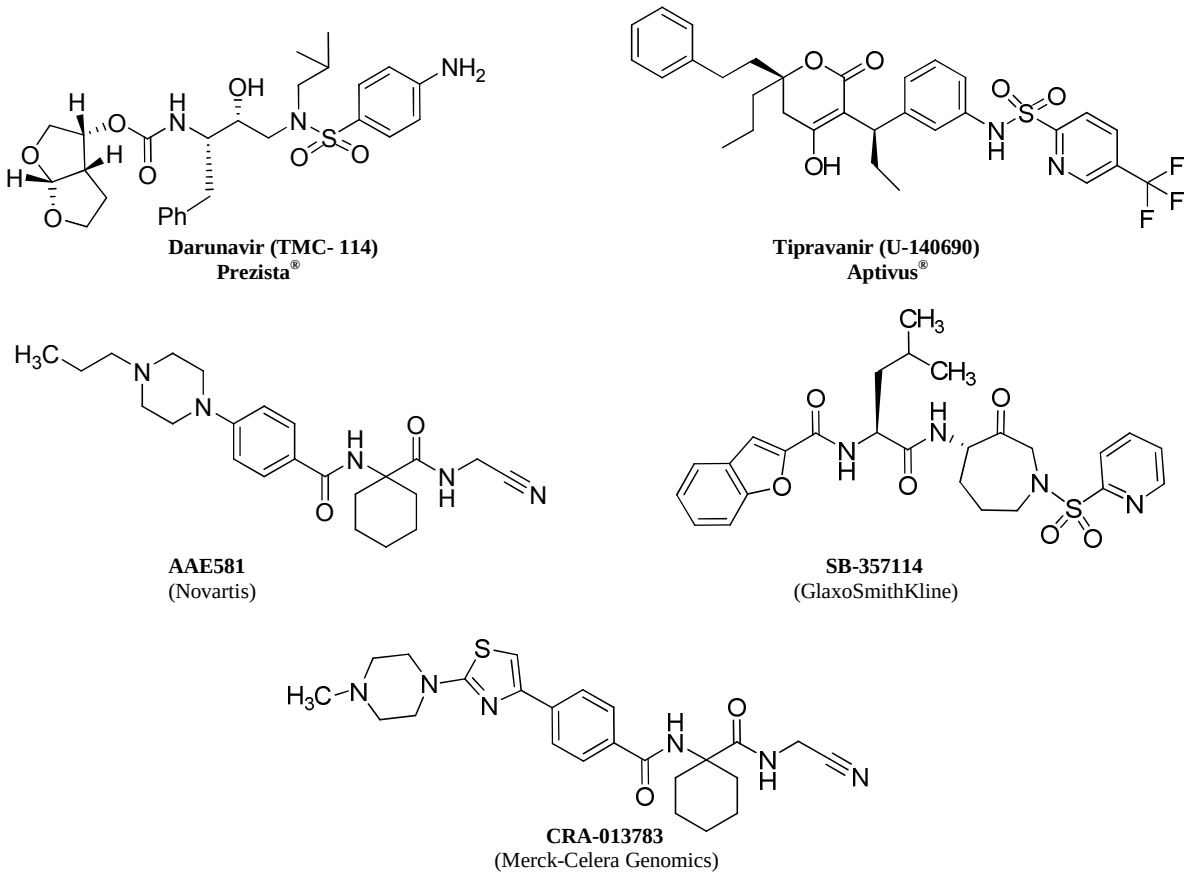


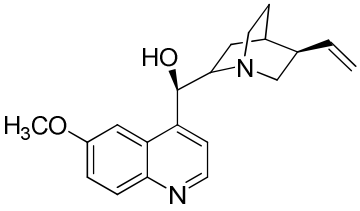
Figure 3. Inhibitors of HIV protease (darunavir and tipranavir) and of the protease cathepsin K associated to osteoporosis disease (AAE581, SB357114, CRA-013783) (PALMER et al., 2005; TURK, 2006; DE CLERCQ et al., 2009).

The discovery of bioactive molecules can be performed experimentally by screening of natural products or synthetic compounds. Natural Products (NPs) biodiversity is a rich source of investigation by academic community and pharmaceutical industry. Currently, many drugs are NPs or NPs derived (CLARDY and WALSH, 2004; GANESAN, 2008). The study of the chemistry of NP (plants, bacteria, fungi and marine organisms) as

source on searching for antiviral and antiprotozoal drugs have been carried out for centuries and still is an increasing interest in modern science (MENESES et al., 2009; MISHRA and TIWARI, 2011; KADIR et al., 2013).

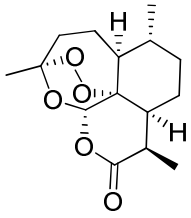
There are several available drugs for parasitic and viral diseases that came from plants as following: quinine a alkaloid from *Cinchona calisaya* Wedd (Rubiaceae) (ANDRADE-NETO et al., 2003; REYBURN et al., 2009) and a sesquiterpene lactone artemisinin (qinghaosu) from *Artemisia annua* L. (Qinghao) (Asteraceae) used against malaria (KLAYMAN, 1985; BROWN, 2012); podophyllotoxin, a toxin lignan from the roots and rhizomes of *Podophyllum* species, used against condyloma acuminata (human papilloma virus) (Figure 4) (VON KROGH, 1991; GORDALIZA et al., 2004).

Cinchona calisaya



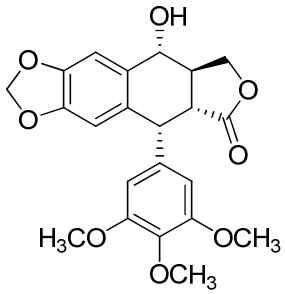
quinine

Artemisia annua



artemisinin

Podophyllum peltatum



podophyllotoxin

Figure 4. Quinine from *Cinchona calisaya* Wedd (Rubiaceae); artemisinin from *Artemisia annua* L. (Asteraceae) and podophyllotoxin from *Podophyllum peltatum* L. (Mayapple) (pictures of plants by FOREST and KIM STARR, 2005; PIERINI, 2009; ANDERSON, 2002).

The Cerrado (savannah) is the second largest biome in Brazil (23.9% of the total Brazil area) with a high chemical diversity, thereby a huge

source of new bioactive secondary metabolites (IBGE, 2004; BOLZANI et al., 2012). Few plants from this biome have been explored so far, there are only 71 species studied for bioactivity, wich number consists into 0.60% of the plant species in the Cerrado (NOVAES et al., 2013). Most of examples include antifungal, insecticidal, antibacterial, antiviral and antiprotozoal activities (GUILHON-SIMPLICIO and PEREIRA, 2011; NOVAES et al., 2013).

In view of the potential of Brazilian Cerrado, this research included several species of plants from São Paulo Cerrado: *Byrsonima coccolobifolia* Kunth., *Aegiphila lhotzkiana* Cham., *Ouratea spectabilis* (Mart.) Engl., *Bauhinia holophylla* Steud., *Anadenanthera falcata* (Benth.) Speg, *Tocoyena formosa* (Cham & Schltdl.) K. Schum., *Cochlospermum regium* (Schrank) Pilg. and *Qualea grandiflora* Mart, chosen based on chemotaxonomic and ethnopharmacological literature. These plants were screened using arginase, a protease from *Leishmania amazonensis*, as investigative tool for leishmanicidal prototypes in drug design. The plant that showed the best enzyme inhibition was *Byrsonima coccolobifolia* Kunth. (Malpighiaceae) (Figure 5) which was studied further.

Malpighiaceae is a family of trees, shrubs, and vines of flowering plants distributed in the tropical and subtropical forests and savannahs, containing around 1300 species in 77 genera, where the *Byrsonima* genus is one of the biggest one of 150 species (DAVIS and ANDERSON, 2010).

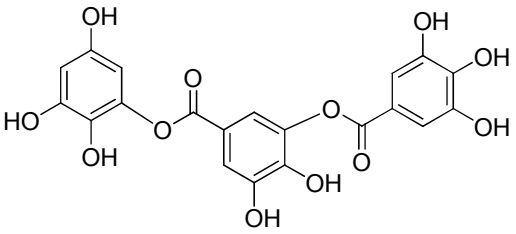
Several species of this family are used for medicinal purposes by the population. The phytochemicals of Malpighiaceae consist in carbolinic alkaloids, indole bases, phenylpropanoids, flavonoids, triterpenoids and polysaccharides (GUILHON-SIMPLICIO and PEREIRA, 2011).

A   B

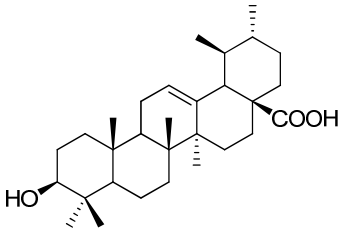
Figure 5. (A) *Byrsonima coccolobifolia* Kunth. ; (B) Voucher specimens (8367) of *Byrsonima coccolobifolia* Kunth. deposited at the Herbarium of the Botany Laboratory (HUFSCar) at UFSCar. The picture of the plant (A) was taken by MERCADANTE, 2012.

Byrsonima plants are known as “muricis”, and the species of this genus have been used in folk medicine for the treatment of stomach disorders, antidiarrheal, skin infections, gastritis and many others. Previous studies of *Byrsonima* species have showed the biological activities: antibacterial, antifungal, antimycobacterial, antimutagenic, antiprotozoa (e.g. antileishmanial, trypanocidal), antioxidant, anti-inflammatory, molluscicide and antiviral (GUILHON-SIMPLICIO and PEREIRA, 2011; CECÍLIO et al., 2012).

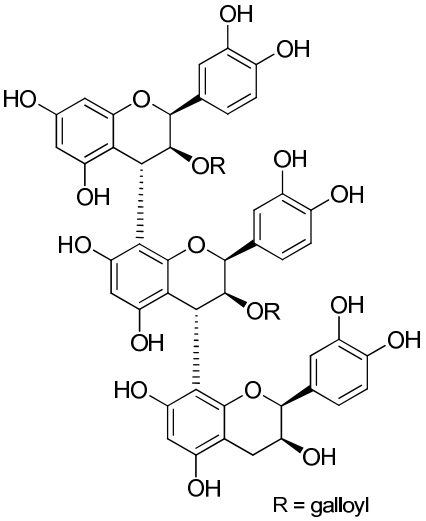
Flavonoids, flavanones, biflavonoids, proanthocyanidins, triterpenes, gallic acids and derivatives, quinic acid and derivatives are the most common classes of secondary metabolites found in different species of *Byrsonima* genus (Figure 6) (GUILHON-SIMPLICIO and PEREIRA, 2011). *B. coccolobifolia* Kunth. (Malpighiaceae) have showed in previous studies antibacterial and molluscicidal activities (SANNOMIYA et al., 2005). In addition, a few compounds (flavonoids, gallic acid and a xantone) have been isolated from the leaves previouslly (Figure 7) (LORENZI et al., 2006; LORENZI et al., 2007).



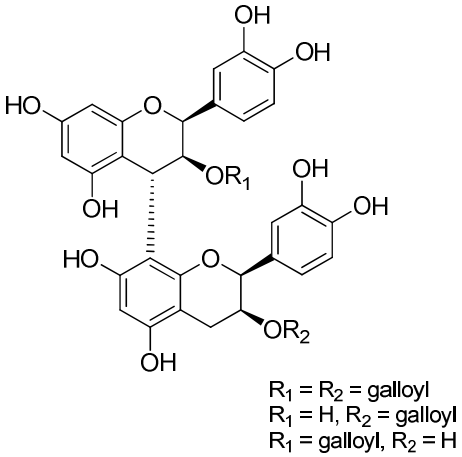
methyl m-trigallate
leaves of *B. bucidaefolia*
(CASTILLO-AVILA et al., 2009)



ursolic acid
leaves of *B. verbascifolia*
(GUILHON-SIMPLICIO and PEREIRA, 2011)

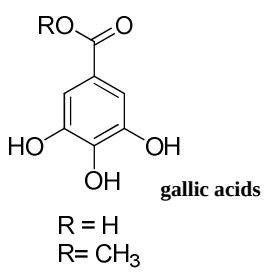


Proanthocyanidins
stems of *B. crassifolia*
(GEISS et al., 1995)

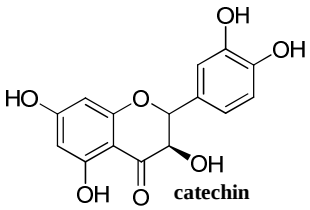


Proanthocyanidins
stems of *B. crassifolia*
(GEISS et al., 1995)

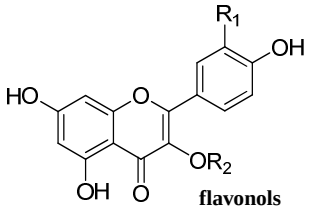
Figure 6. Secondary metabolites isolated from *Byrsonima* species



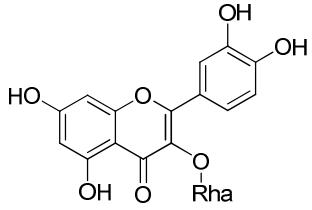
gallic acids



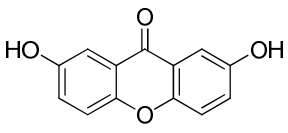
catechin



flavonols



quercitrin



2,7-dihydroxy xanthone

Figure 7. Secondary metabolites isolated from *B. coccolobifolia* (LORENZI et al., 2006; LORENZI et al., 2007).

In this context, this search for new protease inhibitors in cerrado plants was performed in two parts, the first part it was carried out in the chemistry department, Federal University of São Carlos in Brazil, and the second part was carried out in the Institute of Pharmacy and Biochemistry, Johannes Gutenberg Universität Mainz in Germany.

2 - OBJECTIVES

This project aimed to contribute with the phytochemical study and evaluation of extracts and secondary metabolites isolated from cerrado plants against proteases that are associated to development of Neglected Tropical Diseases.

2.1 - Part I

Screening of crude extracts of plants from São Paulo cerrado using arginase (ARG) from *Leishmania amazonensis* as molecular target.

To perform a bioactivity-guided fractionation of the crude extract that shows significant inhibitory activity on ARG to isolate the active compounds.

To determine the mechanism of action for the most active pure compounds against ARG.

2.2 - Part II

To investigate the natural products as enzyme inhibitors using the proteases associated with pathologies such as: dengue (dengue virus serine proteases from dengue virus serotype 2 and 3), African trypanosomiasis (rhodesain from *T. b. rhodesiense*), chagas disease (cruzain from *T. cruzi*), leishmaniasis (r-CPB2.8 from *L. mexicana*), malaria (falcipain-2 from *P. falciparum*) and mammalian cathepsins B and L for selectivity.

3 - PAPERS

3.1 - PAPER 1 – Journal of Natural Products

JOURNAL OF NATURAL PRODUCTS

Note

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Isolation of Arginase Inhibitors from the Bioactivity-Guided Fractionation of *Byrsonima coccolobifolia* Leaves and Stems

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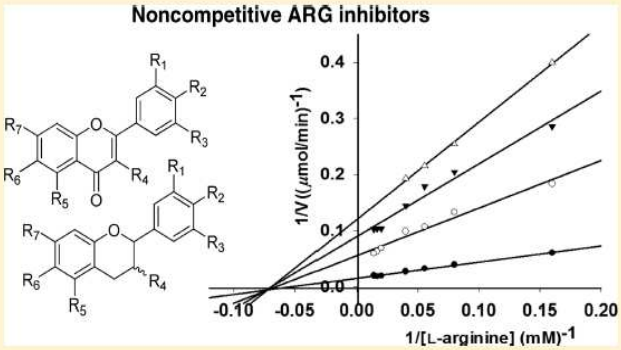
Supporting Information

ABSTRACT: *Byrsonima coccolobifolia* leaf and stem extracts were studied in the search for possible leishmanicidal compounds using arginase (ARG) from *Leishmania amazonensis* as a molecular target. Flavonoids **1b**, **1e**–**1g**, **2a**, **2b**, and **2d**–**2f** showed significant inhibitory activity, with IC_{50} values ranging from 0.9 to 4.8 μM. The kinetics of the most active compounds were determined. Flavonoids **1e**, **1f**, **2a**, **2b**, and **2e** were characterized as noncompetitive inhibitors of ARG with dissociation constants (K_i) ranging from 0.24 to 3.8 μM, demonstrating strong affinity. Structure–activity relationship studies revealed some similarities in the structural features of flavonoids related to ARG activity.



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Introduction

Affecting millions of people per year, leishmaniasis is one of the most neglected tropical diseases and an urgent problem in global public health according to the World Health Organization. To overcome obstacles such as adverse reactions and parasite resistance to available drugs against leishmaniasis, a number of plants have been tested in the search for new frontline drugs.[1,2]

Brazilian cerrado plants, the biodiversity of which is still largely unknown, represent a rich source of new lead compounds.[3] Species such as *Byrsonima coccolobifolia* Kunth. (Malpighiaceae) and *Byrsonima crassa* have been used in folk medicine for the treatment of stomach disorders and gastric ulcers.[4,5] Chemical study of extracts from *B. crassa* with an antiulcerogenic effect has led to the isolation of flavonoids and biflavonoids.[5] Furthermore, crude extracts from the leaves and stems of *Byrsonima crassifolia* and *Byrsonima bucidaefolia* were shown to exhibit good inhibition of the growth of promastigotes of *Leishmania mexicana*. Flavonoids were the principal phytochemicals isolated from *Byrsonima*,[4] which indicates that this genus could contribute to the search for new leishmanicidal compounds because flavonoids have demonstrated antileishmanial activity.[6,7] Recently, some of these phenols were found to be inhibitors of arginase (ARG) from *Leishmania amazonensis* and arginase (ARG-II) from mammals,[8−10] which has motivated the further study of this class of plant secondary metabolites.

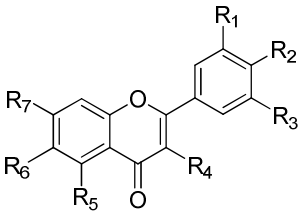
Arginase is a metalloenzyme from *L. amazonensis*[11,12] that catalyzes the hydrolysis of L-arginine to L-ornithine and urea, the first reaction in the metabolic pathway of polyamines (PAs), which are essential molecules in most living organisms, including *Leishmania* parasites.[13] Additionally, ARG is associated with the production of nitric oxide (NO) molecules, high concentrations of which could kill the parasites.[14] The relationships of ARG with such molecules (PAs and NO) with distinct properties are explained by the usage of the same substrate, L-arginine, for the enzymatic activities in two biosynthetic pathways.[14]

Studies of mutant *L. mexicana* and *Leishmania major* parasites lacking ARG confirmed that the ARG pathway is essential for parasite viability and infectivity.[13,15,16] Importantly, the X-ray crystal structure of arginase from *L. mexicana* was solved in recent work, which differs only in two amino acids from *L. amazonensis*, making them 99.4% identical.[13,17] The knowledge of

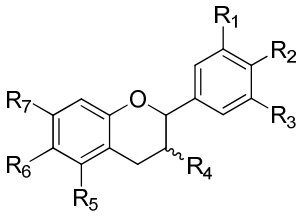
leishmanial ARG structure has revealed important features for inhibitor interactions, thus making ARG an attractive target for leishmanicidal drug design.[17]

In a search for new leishmanicidal compounds, extracts from several Brazilian cerrado plants were evaluated against ARG. The crude extracts from the leaves and stems of *B. coccolobifolia* showed the most promising results, with inhibition higher than 60.0% against ARG at a concentration of 31.25 μg/mL. Ethyl acetate extracts from the leaves and stems of *B. coccolobifolia* showed 64% and 65% inhibition of ARG, respectively.

Table 1. Structures and IC_{50} Values of Several Flavonoids as Inhibitors of Arginase



1



2

Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	IC_{50} (μM)[b]
1a[a]	OH	OH	H	*O*-rha α	OH	H	OH	12.2 ± 1.8
1b	OH	OH	H	glc β	OH	H	OH	2.0 ± 0.1
1c	OH	OCH_3	H	*O*-rha α	OH	H	OH	88.1 ± 9.1
1d	OH	OCH_3	OH	*O*-rha α	OH	H	OH	223.5 ± 15.9
1e	OH	OH	OH	*O*-rha α	OH	H	OH	2.4 ± 0.2
1f	OH	OH	OH	OH	OH	H	OH	2.1 ± 0.2
1g	OH	OH	H	OH	OH	H	OH	4.0 ± 0.5
1h	H	OH	H	OH	OH	H	OH	55.0 ± 4.0
1i	OAc	OAc	H	OAc	OAc	H	OAc	120.8 ± 12.1
1j	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	127.9 ± 9.8
2a	OH	OH	H	OH α	OH	H	OH	1.7 ± 0.1
2b	OH	OH	H	OH β	OH	H	OH	1.6 ± 0.2
2c	H	OH	H	*O*-rha α	OH	H	OH	35.1 ± 3.2
2d	OH	OH	H	OAc α	OH	H	OH	3.7 ± 0.3
2e	OH	OH	H	OAc α	OAc	H	OAc	0.9 ± 0.1
2f	OAc	OAc	H	OAc α	OAc	H	OAc	4.8 ± 0.5

[a]Quercitrin was used as a positive control. [b]The values represent means of individual experiments ± SD.

Phytochemical study of these two EtOAc extracts led to the active known compounds, including flavonoids (**1a**, **1b**, **2a**, **2b**),[8,9,18−20] (+)-syringaresinol (**3**),[21] and trigonostemone (**4**).[22] Compound **1a** was previously isolated from a methanolic extract from the leaves of *B. coccolobifolia*,[23] and compounds **1b**, **2a**, and **2b** have been reported from other species in the genus *Byrsonima*.[4] Furthermore, this is the first report of compounds **3** and **4** in *Byrsonima*.

Isoquercitrin (**1b**), (+)-catechin (**2a**), and (−)-epicatechin (**2b**) showed potent inhibitory activities against ARG, with IC_{50} values ranging from 1.6 to 2.0 μM (Table 1). Quercitrin (**1a**), (+)-syringaresinol (**3**), and trigonostemone (**4**) exhibited moderate inhibitory activity, with IC_{50} values of 12.2 ± 1.8, 13.7 ± 1.5, and 16.6 ± 1.6 μM, respectively. The IC_{50} values of the compounds quercitrin (**1a**), isoquercitrin (**1b**), quercetin (**1g**), and kaempferol (**1h**) were reported previously (10, 3.8, 4.3, and ∼50 μM, respectively).[9,24] Flavonoids **1a** and **1b** were characterized as noncompetitive inhibitors (K_i = 7.20 and 6.90 μM, respectively), and **1g** was characterized as a mixed inhibitor.[24]

In an effort to establish a relationship of the chemical structures of flavonoids with potent ARG inhibitory activity and also to describe the type of inhibition for the potent inhibitors, other flavonoids were also investigated (**1c**−**j** and **2c**−**f**). Catechin and quercetin were acetylated (**1i**, **2d**−**f**) to analyze the effect of substituting hydroxy groups for acetyl groups in the flavonol and flavan-3-ol structural classes on ARG activity. Although this class of natural products has been characterized recently as inhibitors of recombinant ARG from *L. amazonensis*, there are still only a few studies that have explored these compounds for their inhibitory effects.[8,9,24] The present investigation, which searched for new ARG inhibitors, differs from the results of previous reports in that these active flavonoids were identified through a bioactivity-guided approach.

The comparison of quercitrin (**1a**) (IC_{50} = 12.2 ± 1.8 μM) with compound **1e** (IC_{50} = 2.4 ± 0.2 μM) demonstrates an improvement in inhibitory activity when the aromatic ring B of the flavonoid has one extra hydroxy group. The observed increase in the IC_{50} values of flavonoids with the same skeleton as quercitrin (**1a**) (12.2 ± 1.8 μM) and compound **1c** (88.1 ± 9.0 μM) can be related to the presence of a methoxy group instead of a hydroxy group as in the catechol of **1a**. The same trend is seen for the glycoflavonols **1e** (IC_{50} = 2.4 ± 0.2 μM) and **1d** (IC_{50} = 223.5 ± 15.8 μM), which have three substituents in aromatic ring B, in which the replacement of a hydroxy group with a methoxy group decreased the inhibitory activity.

The flavonol aglycones **1f** and quercetin (**1g**) both showed a substantial inhibition of ARG, with IC_{50} values of approximately 2.1 and 4.0 μM, respectively. However, compounds **1h**, which contains only one hydroxy group in aromatic ring B, **1i** (acetylated flavonol), and **1j** (methoxylated flavonol) had less potent effects. These compounds displayed lower activities (IC_{50} = 55.0, 120.8, and 127.9 μM, respectively), and again this observation can be correlated directly with the influence of the functional groups in the B ring. Previous in vitro studies on the leishmania parasite showed that leishmanicidal potential was lowered or completely lost with an increase in the number of methoxy groups in the flavonol structure.[7] Additionally, a recent study showed that galangin, a flavonoid without any substituents in the B ring, exhibited only low inhibition against ARG ($IC_{50} \approx 100$ μM).[24]

IC_{50} values in the range 0.9 to 4.8 μM (Table 1) suggest that flavan-3-ols also have important structural features that allow these substances to bind to the enzyme. The presence of hydroxy groups as substituents on the aromatic ring A and at position 3 of the pyran ring of the flavonoid skeleton has been demonstrated also to be important in relation to ARG inhibition.[24] However, the selective acetylation of (+)-catechin (**2a**) at different positions did not show much influence on the enzyme inhibition activities (**2d**−**f**) (Table 1).

In silico studies were performed previously for some flavonoids, demonstrating interactions with ARG enzyme occurring between the ring B moiety and amino acids that are involved with metal bridge Mn_A^{2+}−Mn_B^{2+} coordination in the active site.[9,24] By the present experimental approach the results supported such findings, showing that a catechol group is highly important in reducing ARG activity. Figure 1 shows Lineweaver−Burk plots for some of the most potent compounds analyzed, providing valuable information about the mechanism of inhibition. In this investigation, the kinetics of recombinant ARG were measured at pH = 9.6 and afforded a K_M value of 22.6 ± 1.7 mM (R^2 = 0.996), consistent with the data in the literature.[12]

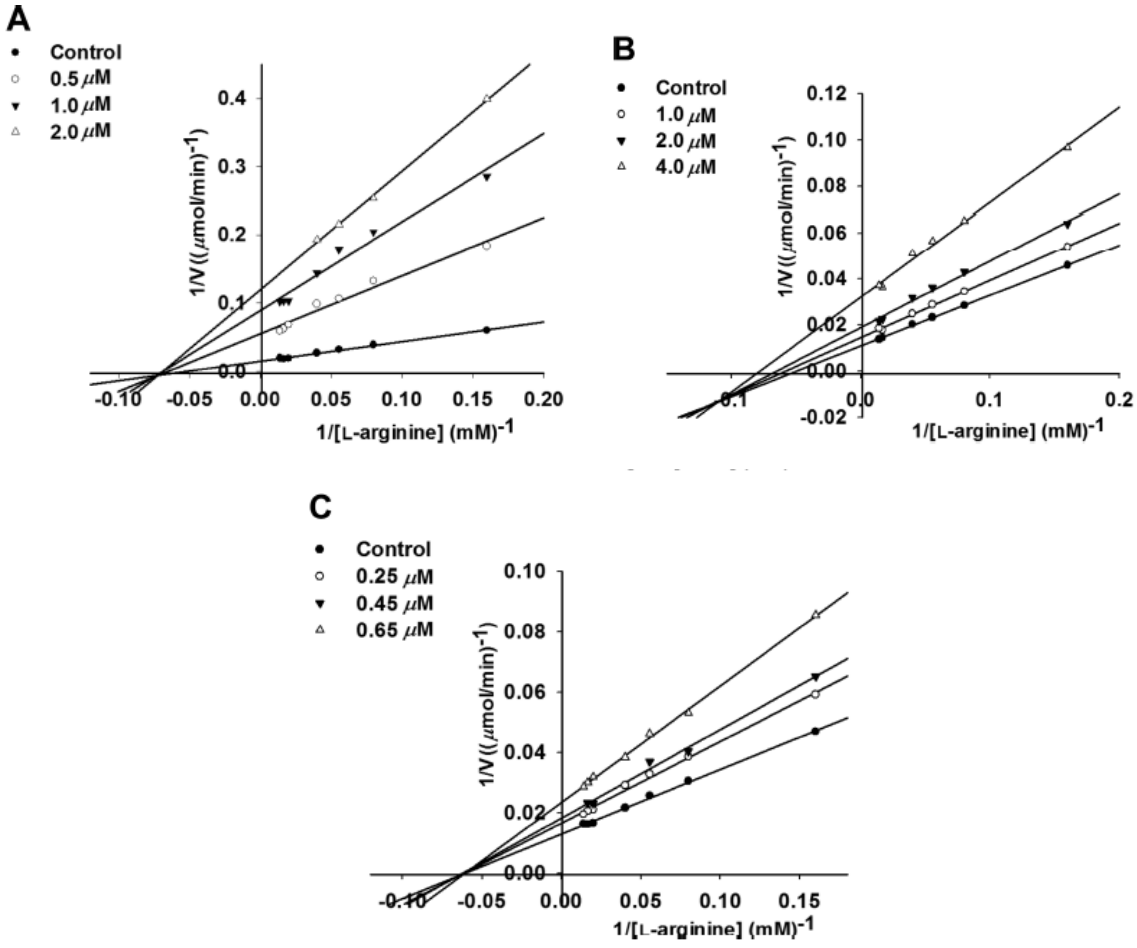


Figure 1. Lineweaver–Burk plots indicate noncompetitive ARG inhibition by (–)-epicatechin (**2b**) (A), myricetin 3-*O*-*α*-L-rhamnoside (**1e**) (B), 3,5,7-triacetylcatechin (**2e**) (C).

The double-reciprocal plots for the evaluated compounds (**1e**, **1f**, **2a**, **2b**, **2e**) showed a decrease in V_{max} values without affecting the affinity (K_M).

These results reveal that flavonoids interact with ARG in a noncompetitive manner. Kinetic analysis indicates that these flavonoid interactions can occur in the free enzyme and also in the enzyme−substrate complex at a site that is distinct from the catalytic site, thus inducing changes in the shape of the active site such that the substrate will no longer fit well.

The dissociation constant (K_i) values were obtained using Dixon reciprocal plots of the initial velocities ($1/v$) versus a varying concentration of inhibitor at a constant concentration of L-arginine (Table 2).

Table 2. K_i Values of Some of Flavonoids

	compound	K_i (μM)
1a	quercitrin[a]	7.9
1f	myricetin	1.2
2a	(+)-catechin	0.6
2b	(–)-epicatechin	0.2
2e	3,5,7-triacetylcatechin	0.9

[a]Quercitrin was used as a positive control.

Quercitrin (**1a**) was characterized previously as a noncompetitive inhibitor.[24] The double-reciprocal plot of glycoflavonol **1e** showed a noncompetitive inhibitory mechanism. It is possible to deduce that inhibitor **1e** preferentially binds to the ES complex (K_{is} = 1.9 μM); in addition, **1e** also interacts with the free enzyme (K_i = 3.8 μM), as demonstrated by the intersecting lines that converge to the left of the *y*-axis and below the *x*-axis ($\alpha < 1$) in the Lineweaver−Burk plot.

Overall, bioactive flavonoids with potent ARG inhibitory activity were identified by screening the active ethyl acetate extracts of *B. coccolobifolia* leaves and stems, thereby indicating these compounds are responsible for the significant decrease in ARG activity. The type of inhibition for the most active flavonoids (**1e**, **1f**, **2a**, **2b**, **2e**) was determined by kinetic studies showing strong

affinity and potency on ARG. Compounds with a flavan-3-ol unit represent a new subclass of noncompetitive flavonoid ARG inhibitor.

Flavonoids **1a**, **2a**, **2b**, **1f**, and **1g** were described previously as inhibitors of the growth of amastigotes of *L. donovani*.[7,25,26] Additionally, quercitrin (**1a**) and quercetin (**1g**) significantly reduced the growth of *L. amazonensis* in an in vivo murine model of cutaneous leishmaniasis.[27] The mechanism of action of quercetin in *L. amazonensis*-infected macrophages was recently related to the increase in reactive oxygen species.[28] Furthermore, the leishmanicidal activities of these flavonoids may also be due to arginase inhibition, confirming these compounds as new lead candidates in the search for leishmanicidal drugs.

Experimental Section

General Experimental Procedures

The urea concentration was detected at 600 nm on a Beckman Coulter DU 800 spectrophotometer. The 1D- and 2D-NMR determinations were carried out in acetone-d_6 and MeOH-d_4, using TMS as the internal reference, on a Bruker DRX-400 NMR spectrometer (^{1}H: 400 MHz; ^{13}C: 100 MHz). MS spectra were recorded on a Bruker Daltonics micrOTOF-Q II-ESI-TOF mass spectrometer. Isolation procedures were carried out on silica gel 60 (Merck, 230−700 mesh) and Sephadex LH-20 (Amersham Pharmacia Biotech AB). Thin-layer chromatography (TLC) on precoated aluminum silica 60 F_{254} (Merck) was used to monitor isolation. Compounds were visualized by exposure under $UV_{254/366}$ light and by spraying with sulfuric acid−vanillin solution, followed by heating. The solvents used for extract preparation and chromatographic fractionation were obtained from Vetec.

Plant Material

Leaves and stems of *B. coccolobifolia* were collected in July 2011 from the cerrado at the Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil. Voucher specimens (8367) were deposited at the Herbarium of the Botany Laboratory (HUFSCar) at UFSCar after identification by one of the authors (M.I.S.L.).

Extraction and Isolation

Ethanol extracts were prepared by maceration of air-dried and powdered plant parts. The crude extracts were obtained after drying to remove the solvents by rotatory evaporation under reduced pressure at a temperature of 40 °C. Dried extracts were submitted to enzymatic assays against ARG. The ethanol extracts of the leaves (10.0 g) and stems (30.5 g) of *B. coccolobifolia* exhibited ARG inhibitory activity. These crude extracts were subjected to liquid−liquid partitioning to produce hexane, EtOAc, and hydroalcoholic extracts. The EtOAc extracts from the leaves (2.0 g) and stems (10.0 g) showed significant inhibition of ARG. The leaf EtOAc extract was fractionated on a silica gel 60 column (18.0 × 5.5 cm; 1:9 acetone−hexane), affording 11 fractions (BF1−BF11). Among them, BF11 (1.2 g), with 90.5% ARG inhibition, was chromatographed over silica gel 60 (18.0 × 5.5 cm; 6:4 acetone− hexane), leading to four subfractions (F14I−F14IV). Active subfractions F14III (60.0 mg) and F14IV (128.0 mg), with 65.0% and 95.0% ARG inhibition, respectively, after chromatographic separations by Sephadex L-20 [(F14III: 56.0 × 2.0 cm; MeOH isocratic); (F14IV: 53.0 × 4.0 and 37.0 × 1.4 cm; MeOH isocratic)], afforded flavonoids **1a** (24.9 mg) and **1b** (2.0 mg). Bioactivity-guided fractionation was performed for the stem EtOAc extract, and fraction BC4 (4.0 g), which exhibited the highest ARG inhibition (80.0%), was obtained by elution with 1:9 MeOH−CH_2Cl_2 on silica gel 60 (50.0 × 4.5 cm), yielding 10 subfractions. Flavonols **2a** (12.0 mg) and **2b** (12.0 mg), (+)-syringaresinol (**3**)

(0.5 mg), and trigonostemone (**4**) (1.2 mg) were isolated by purification of subfraction 10 (35.0 mg) using a Sephadex LH-20 column (52.0 × 1.5 cm; MeOH isocratic) and monitoring by TLC. The isolated compounds were characterized by NMR (^{1}H and ^{13}C, DEPT-135, HSQC, and HMBC) and mass spectroscopy and compared with data published in the literature.[29−36]

Chemicals

The following natural products were previously isolated in our laboratory: 3,4,6,7,3′,4′,5′-heptamethoxyflavone (**1j**),[29] myricetin 3-*O*-*α*-rhamnoside (**1e**),[30] catechin-3-*O*-*α*-rhamnopyranoside (**2c**),[31] mearnsetin (**1c**), and tamarixetin 3-*O*-*α*-L-rhamnoside (**1d**).[32,33]

Myricetin (**1f**) was obtained by the acid hydrolysis of **1e**.[34] Quercetin (**1g**) was acetylated with the acetic anhydride/pyridine/ DMAP method to produce quercetin pentaacetate (**1i**).[35] 3,5,7,3′,4′- Pentaacetylcatechin (**2f**), 3,5,7-triacetylcatechin (**2e**), and 3-acetylcatechin (**2d**) were obtained by acetylation of (+)-catechin with the acetic anhydride/pyridine/DMAP method (**2a**).[35,36] The acetylation of (+)-catechin was monitored by TLC, and the reaction generated partially and completely acetylated compounds, which were purified by chromatography using a silica gel 60 column. All derivatives were fully characterized from their spectroscopic data.

Sigma-Aldrich supplied quercitrin hydrate (**1a**), ≥78% (Sigma Q3001); (+)-catechin hydrate (**2a**), ≥98% (Sigma C1251); (−)-epicatechin (**2b**), ≥90% (Sigma E1753); kaempferol (**1h**), ≥97% (Sigma 60010); quercetin hydrate (**1g**), ≥95% (Aldrich 337951); and quercetin 3-*β*-D-glucoside, ≥90% (Sigma 17793). The enzymatic urea kit was purchased from Biotécnica (Varginha, MG, Brazil).

Expression and Purification of Recombinant ARG

The recombinant enzyme was expressed and purified as described previously,[6] with some modifications. Briefly, the arginase expression plasmid pRSET-Arg was transformed into *E. coli* Rosetta (DE3) pLysS cells, and the culture was grown at 37 °C in SOB medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol until the $OD_{600\ nm}$ reached 0.6−0.8. Arginase expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. Incubation was extended for an additional 16 h at 30 °C. The cells were harvested by centrifugation (12 × 1000g for 10 min at 4 °C) and stored at −80 °C. The cell pellet from 200 mL of medium was thawed at room temperature and resuspended in 30 mL of 2 mM Tris buffer pH 7.0 with 0.3% Triton X-100 (buffer 1). The cellular suspension was submitted to ultrasonic disruption (6 pulses of 1 min with intervals of 30 s, amplitude 20%), and the lysed cells were centrifuged at 12 × 1000g for 20 min at 4 °C. The supernatant was applied to a Ni-NTA Superflow column (1 mL), according to the Qiagen protocol. The column was washed with buffer 1 plus 20 mM imidazole (5 mL) and eluted with 250 mM imidazole in buffer 1 (5 mL). The fractions eluted from the column were pooled, dialyzed against buffer 1, and applied to a DEAE Sepharose Fast Flow (HiTrap, 1 mL) anionic column previously equilibrated with column buffer 1 and eluted with a NaCl gradient (0 to 1 M) at a flow rate of 1 mL/ min. The protein concentration was determined by the method of Bradford[37] using the protein assay kit (BioRad) and bovine serum albumin as standard.

Kinetic Measurements and Inhibition Assay

The activity of recombinant arginase of *L. amazonensis* was defined as the amount of enzyme that produced 1 μmol of urea in 1 min per mg of protein.[12,38] K_M and V_{max} values were determined based on the rate of the reaction for different L-arginine concentrations (6.25, 12.5, 15.0, 25.0, 50.0, 62.5, 75.0,

and 100.0 mM), as determined from Michaelis−Menten kinetic parameters and Lineweaver−Burk analysis. The samples were evaluated against the recombinant ARG at an initial concentration of 100 μM. The IC_{50} values for the active samples were determined by rate measurements for at least 10 inhibitor concentrations (1280, 1024, 512, 256, 128, 64, 16, 4.0, 1.0, and 0.25 μM). For 10 dilutions of inhibitor, mix I was prepared using 50 μL of CHES buffer solution at pH 9.6, 8 μL of arginase solution, and 292 μL of water. A 5 μL sample of each inhibitor was added to 35 μL of mix I, and the reaction mixture was incubated for 10 min at 37 °C. Then, 10 μL of L-arginine solution was added to the reaction and incubated again for 10 min at 37 °C. The final volume of the reaction mixture was 50 μL and contained 50 mM CHES buffer at pH 9.6 and 50 mM of the substrate L-arginine, at pH 9.6. The second reaction was performed using an enzymatic colorimetric assay[39] with a commercially available assay kit (Biotecnica, Brazil). To hydrolyze urea for quantification, 10 μL of the reaction mixture were added to 500 μL of reagent 1 previously prepared (50 mM phosphate buffer, pH 6.7, 60 mM salicylate, 3.2 mM sodium nitroprusside, and 30 000 IU urease) and incubated at 37 °C for 10 min. Then, 500 μL of reagent 2 (140 mM NaOCl and 150 mM NaOH) was added, and the reaction was incubated at the same temperature for a further 10 min. Additionally, control assays were performed without inhibitor as a negative control and in the presence of the known inhibitor quercitrin[9] as a positive control. The enzymatic assay was carried out in duplicate, and the urea concentration was quantified spectrophotometrically at 600 nm. The type of inhibition and K_i values were determined using the same experimental approach with three concentrations of inhibitor and a control under increasing substrate concentrations (6.25, 12.5, 18.0, 25.0, 50.0, 60.0, and 72.0 mM). The kinetics data were analyzed by Lineweaver−Burk plot analysis with the SigmaPlot 12.0 enzyme kinetics module.

Acknowledgments

The authors wish to thank Dr. L. M. Floeter-Winter from the Department of Physiology-IB USP for the clone provided. This research project received financial support from the State of São Paulo Research Foundation (FAPESP, Fundação de Amparo à Pesquisa do Estado de São Paulo) Proc. 2010-52326-9, INBEQMEdi, and the National Council for Scientific and Technological Development (CNPq, Conselho Nacional de Pesquisa e Desenvolvimento), Brazil.

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Supporting Information

^{1}H, HSQC, HMBC

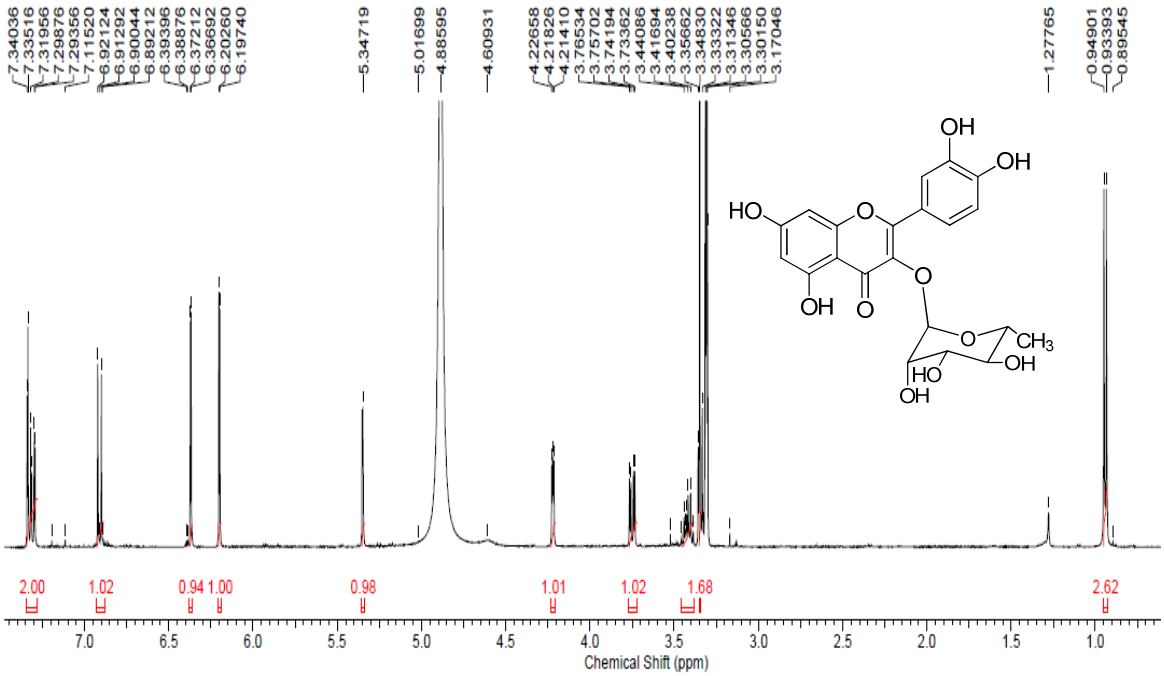
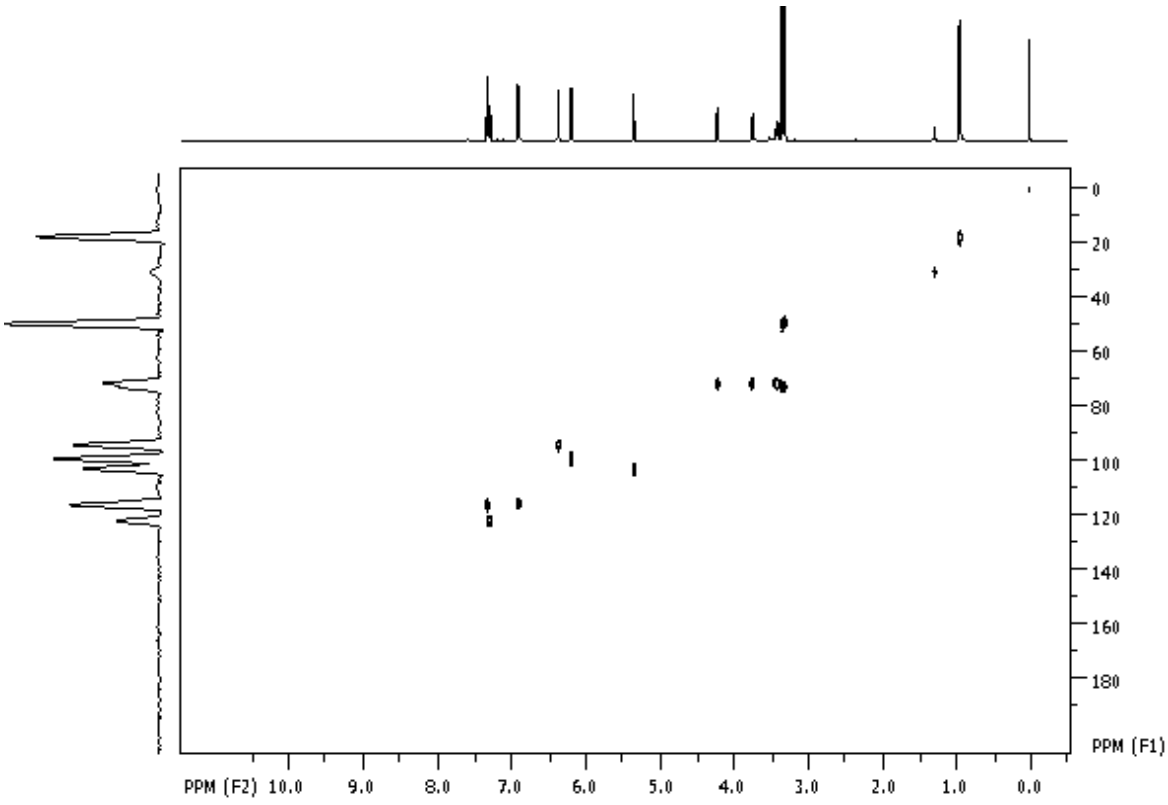


Figure S1. ^{1}H NMR of quercitrin (**1a**) acquired at 400 MHz in MeOH-d_4.



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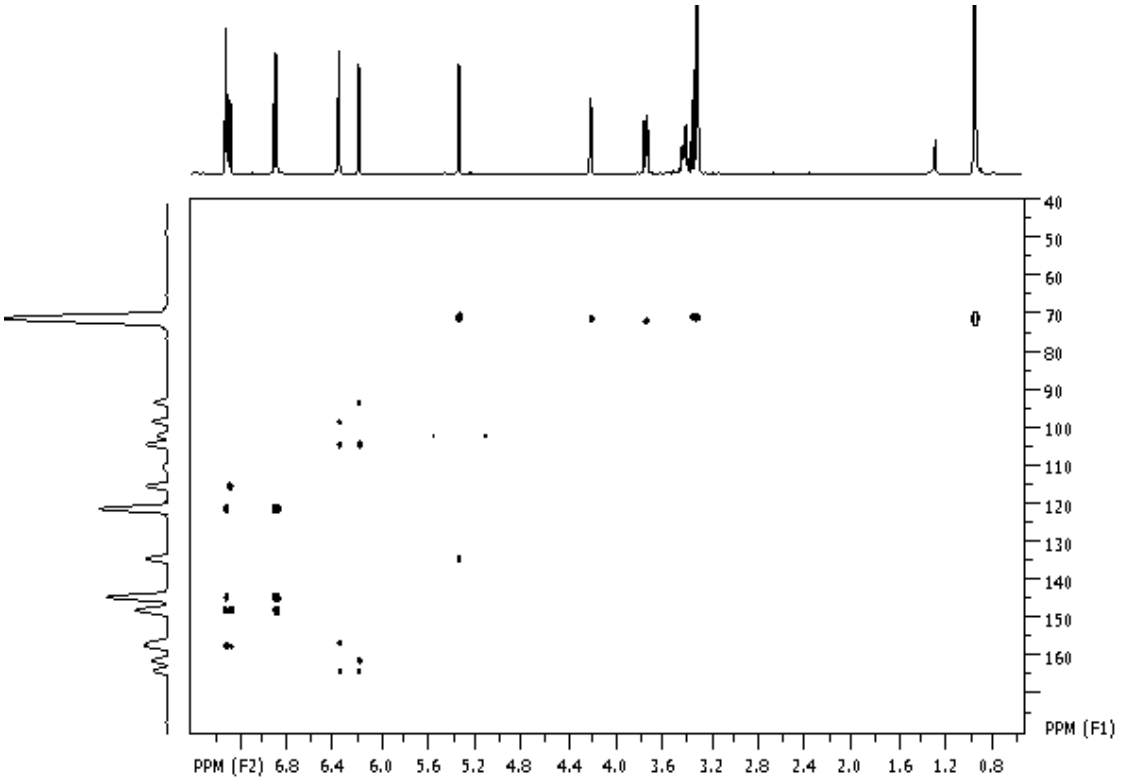

Figure S3. HMBC of quercitrin (**1a**) acquired at 400 MHz in MeOH-d_4.

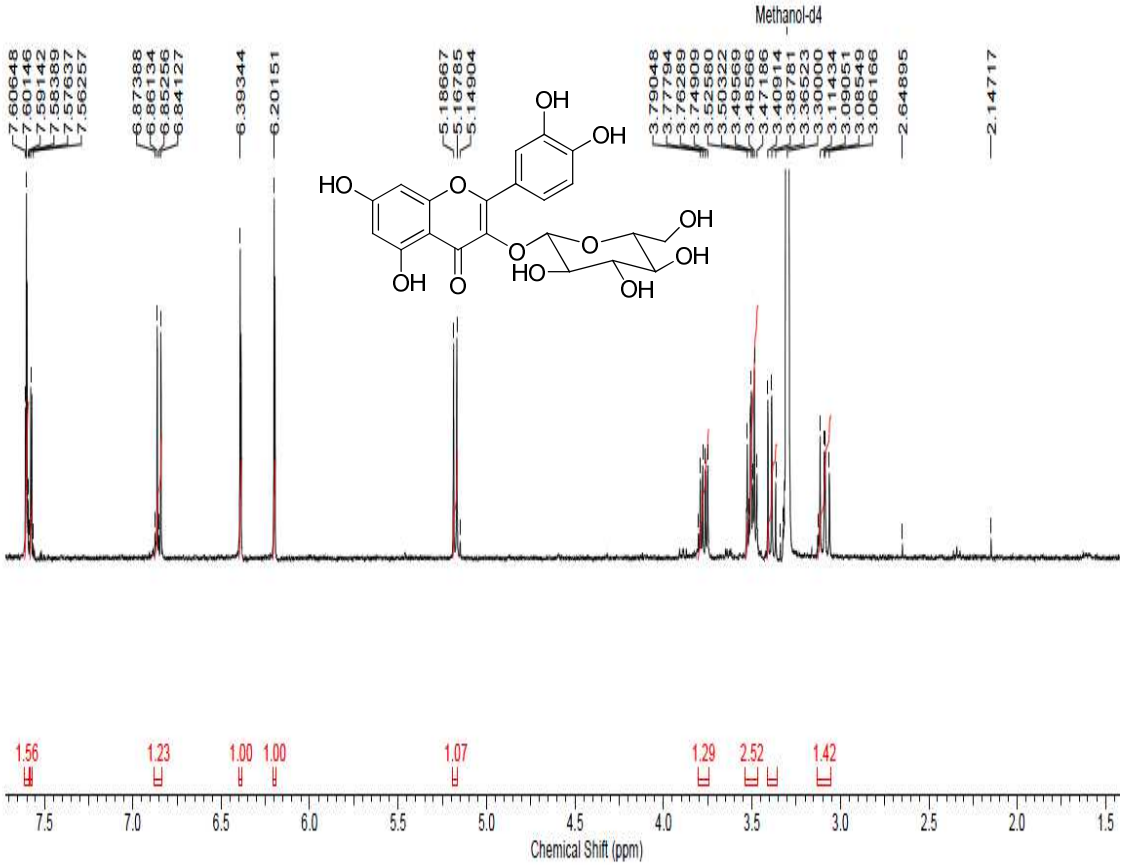

Figure S4. ^{1}H NMR of isoquercitrin (**1b**) acquired at 400 MHz in MeOH-d_4.

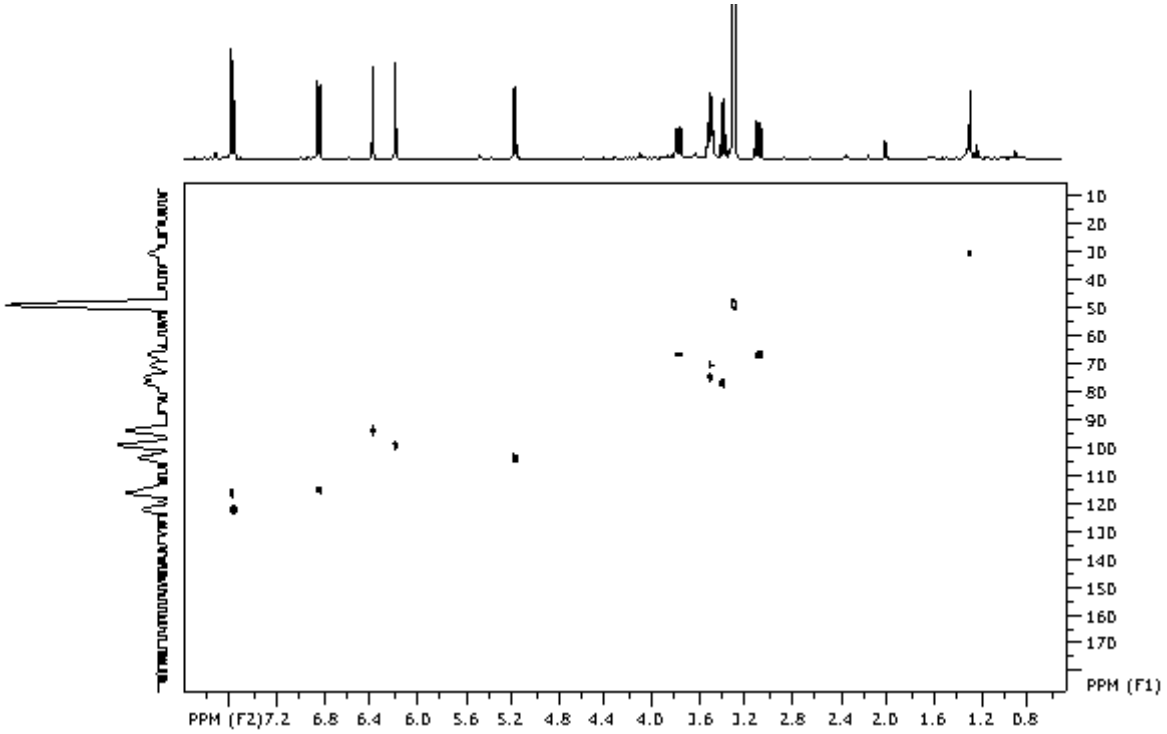

Figure S5. HSQC of isoquercitrin (**1b**) acquired at 400 MHz in MeOH-d_4.

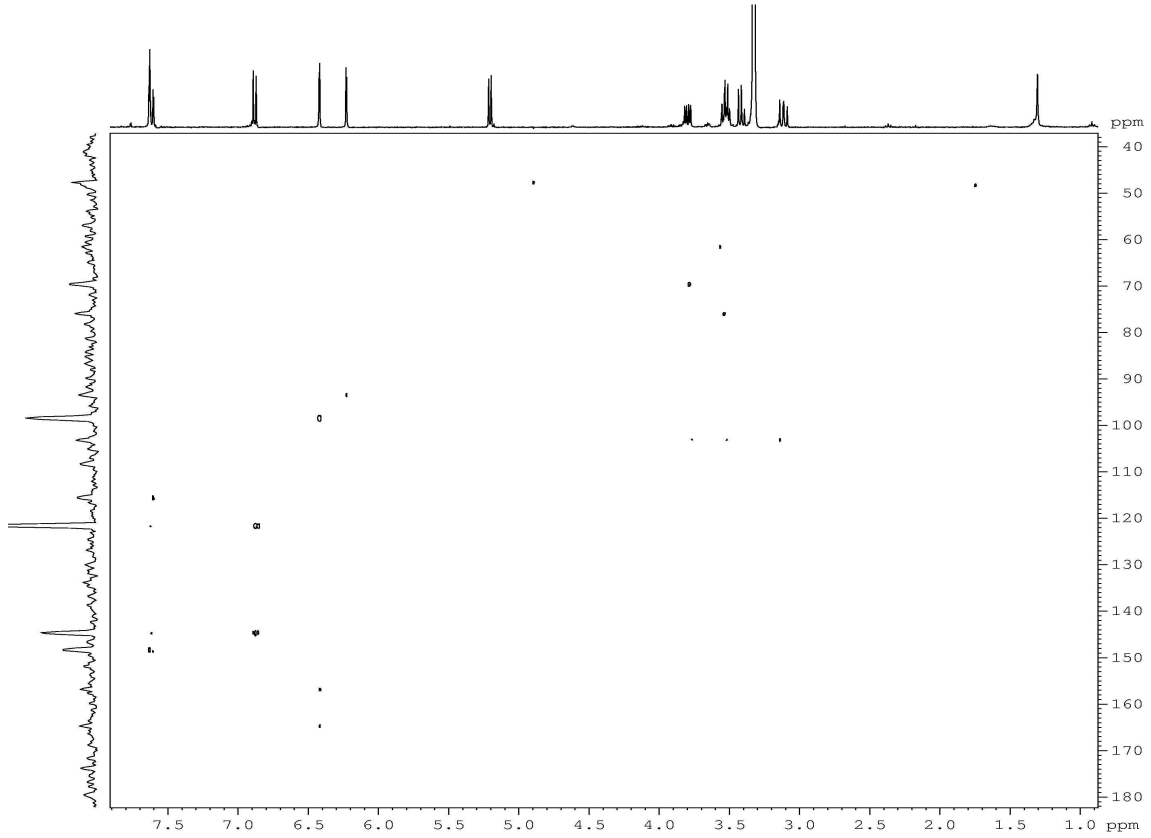

Figure S6. HMBC of isoquercitrin (**1b**) acquired at 400 MHz in MeOH-d_4.

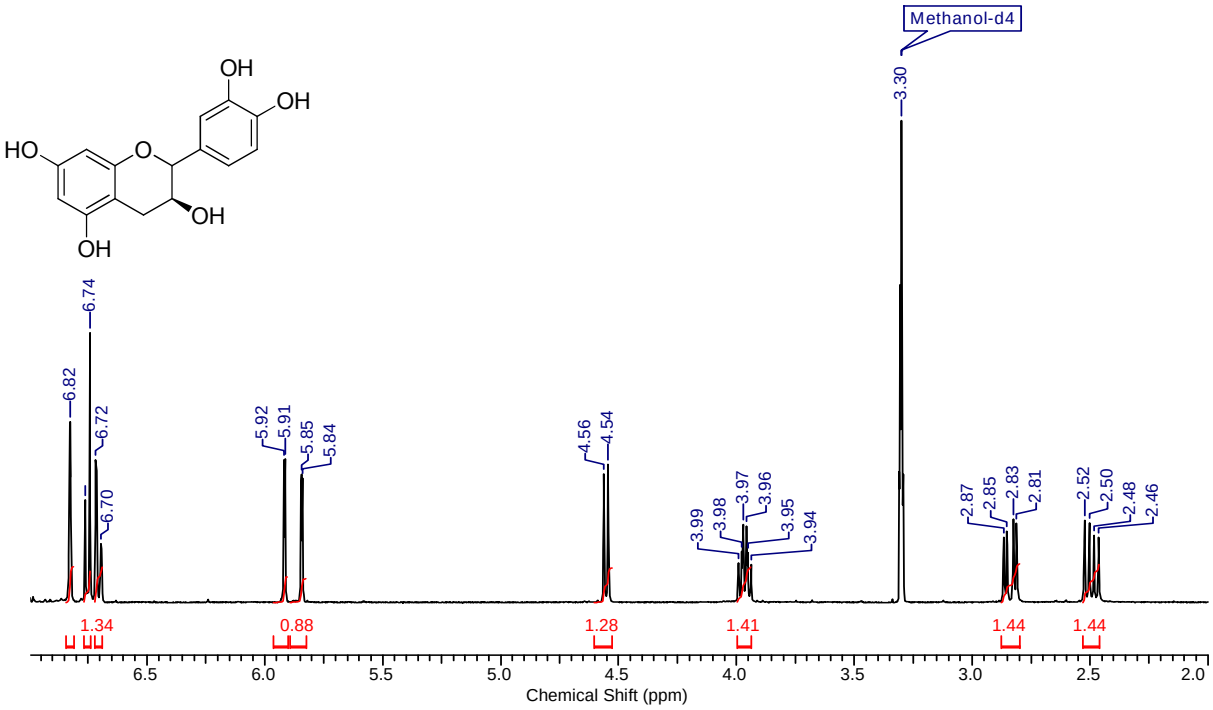

Figure S7. ^{1}H NMR spectrum of catechin (**2a**) acquired at 400 MHz in MeOH-d_4.

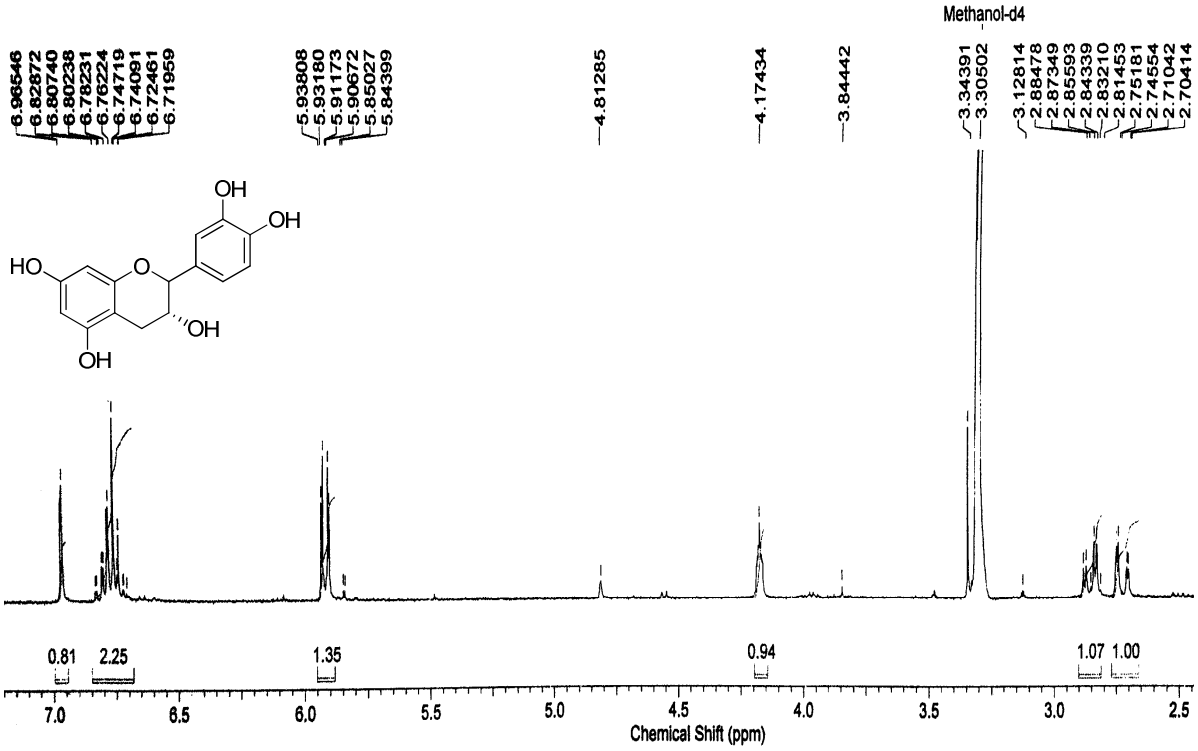

Figure S8. ^{1}H NMR of epicatechin (**2b**) acquired at 400 MHz in MeOH-d_4.

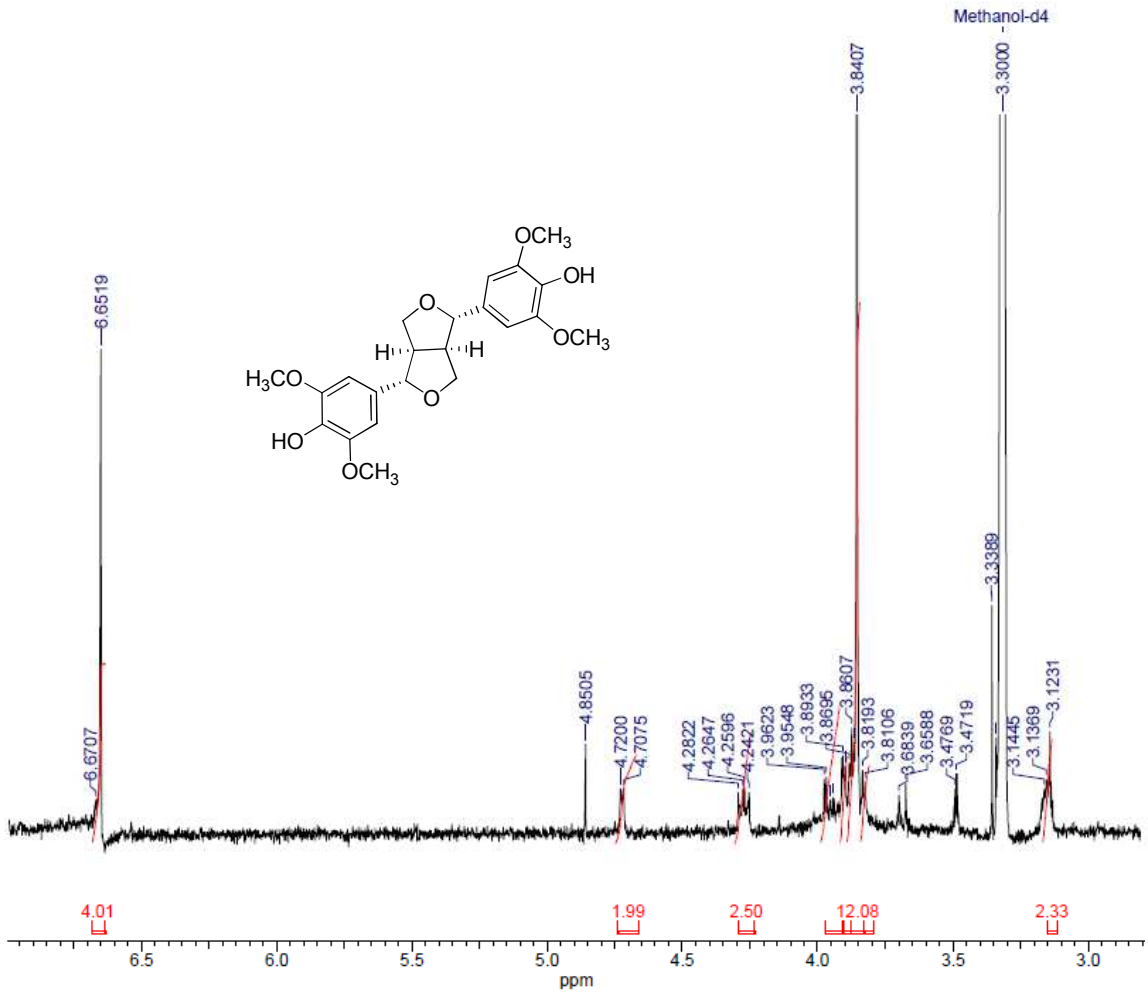

Figure S9. ^{1}H NMR of syringaresinol (**3**) acquired at 400 MHz in MeOH-d$_4$.

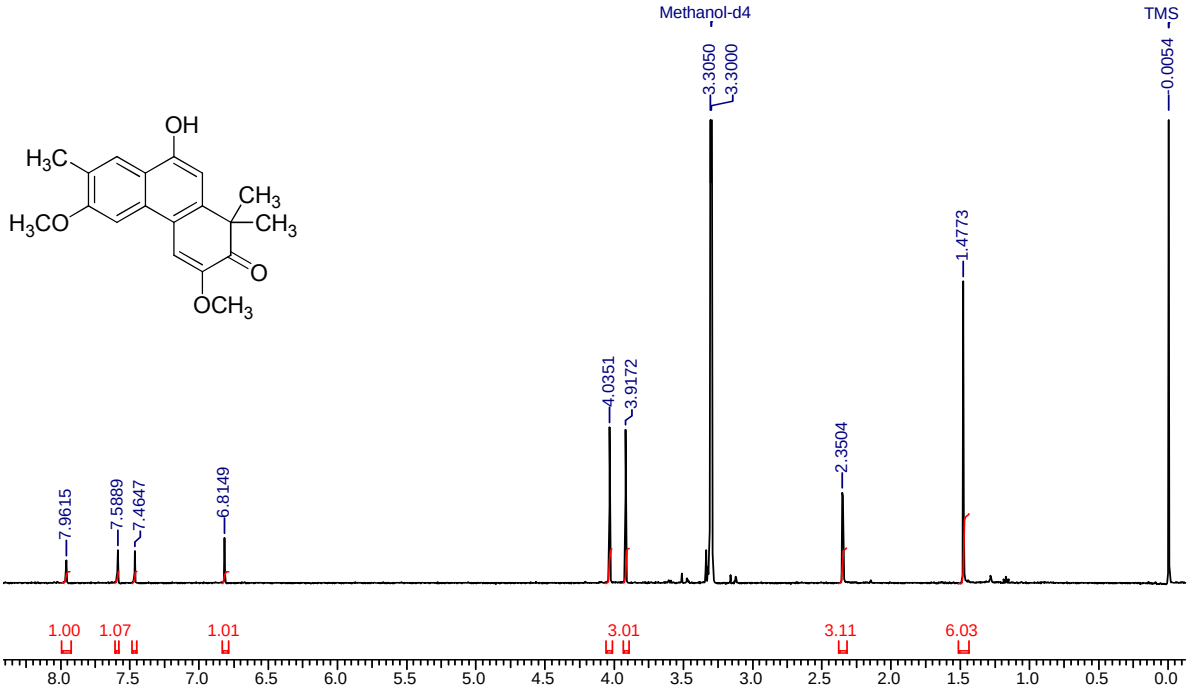

Figure S10. ^{1}H NMR of trigonostemone (**4**) acquired at 400 MHz in MeOH-d$_4$.

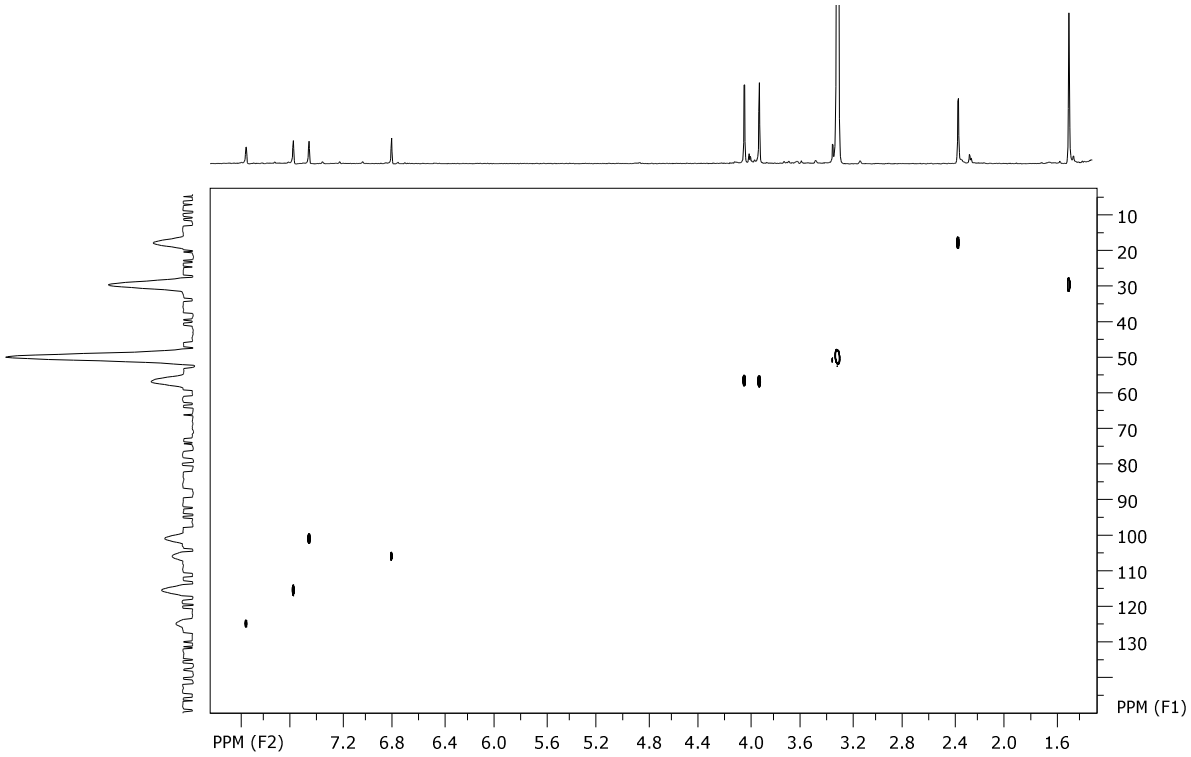

Figure S11. HSQC of trigonostemone (**4**) acquired at 400 MHz in MeOH-d_4.

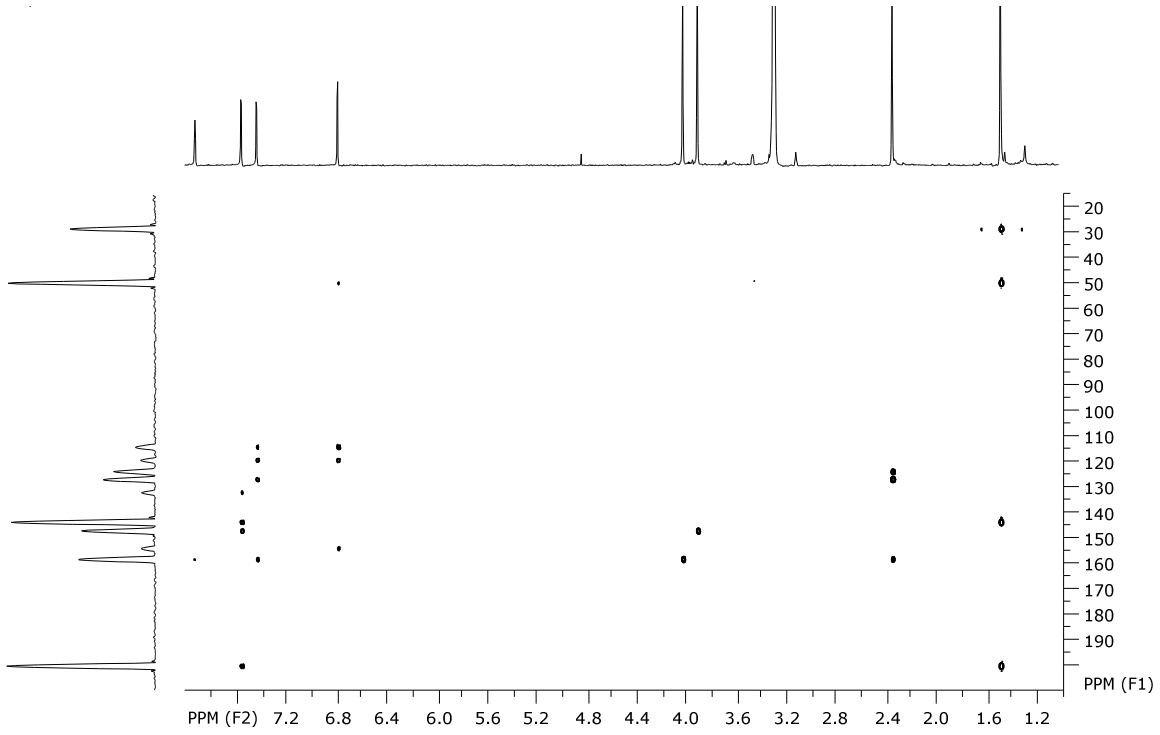

Figure S12. HMBC of trigonostemone (**4**) acquired at 400 MHz in MeOH-d_4.

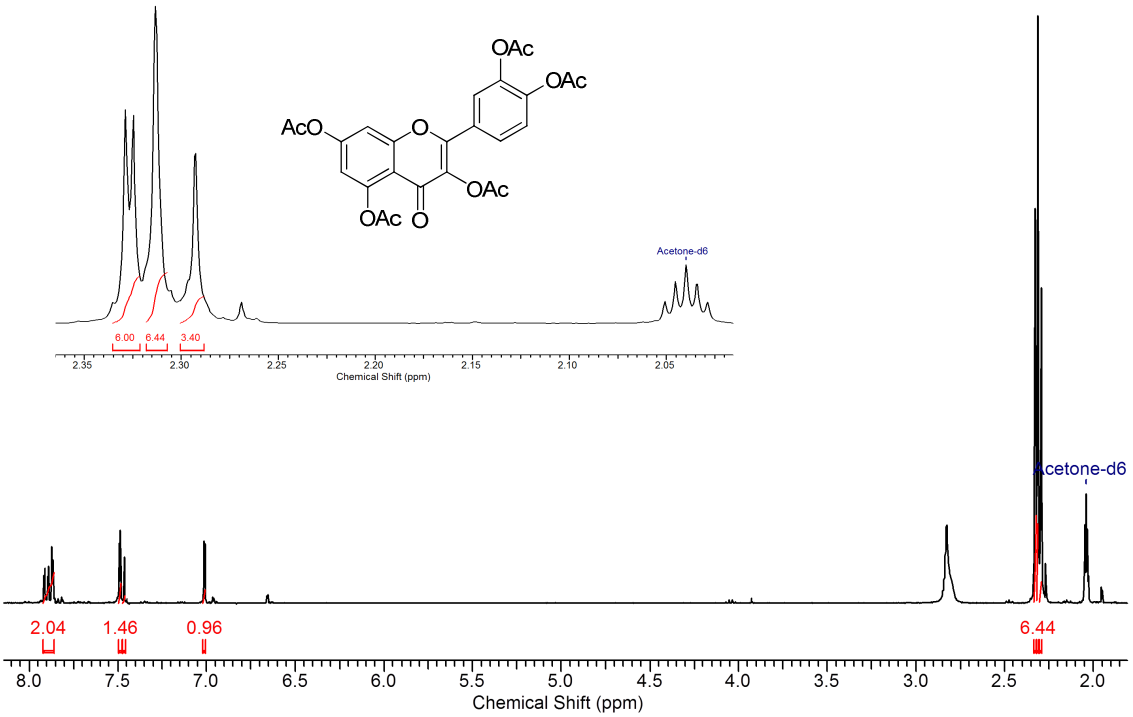

Figure S13. ^{1}H NMR of quercetin pentaacetate (**1i**) acquired at 400 MHz in acetone-d6.

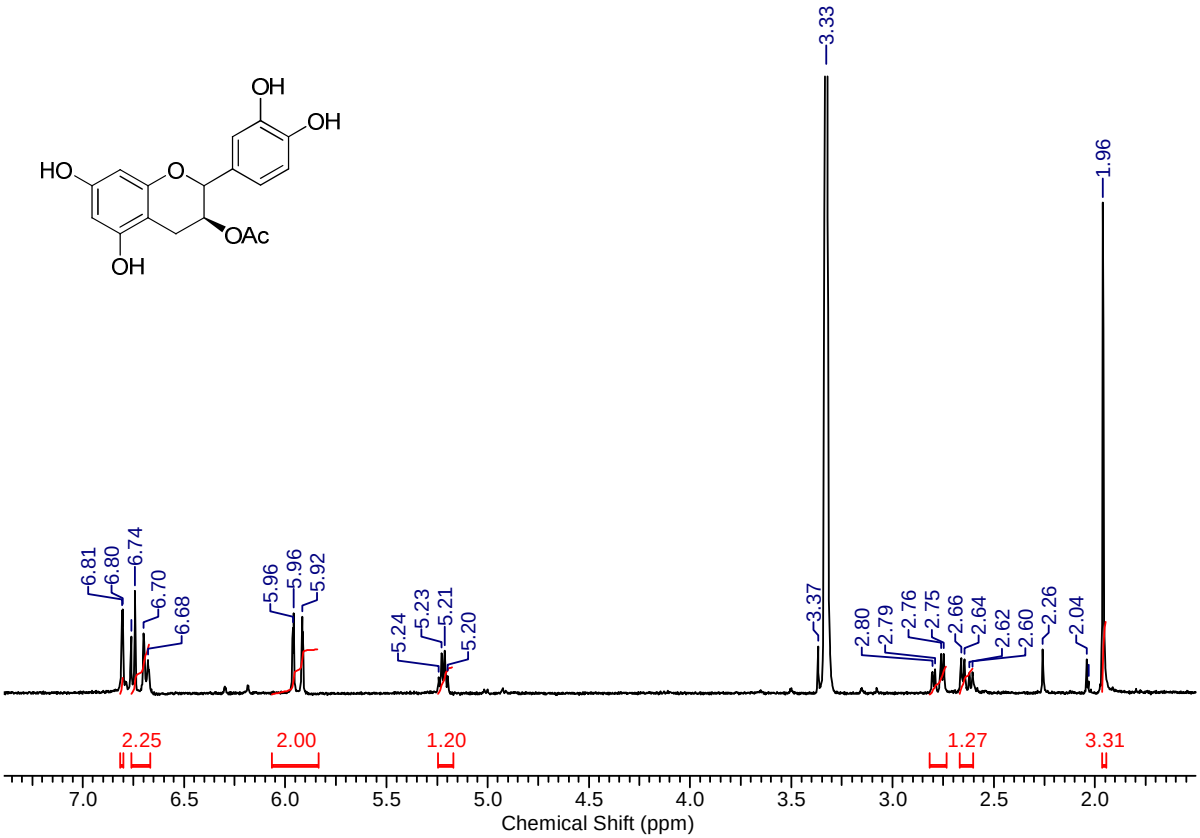

Figure S14. ^{1}H NMR of 3-acetylcatechin (**2d**) acquired at 400 MHz in MeOH-d_4.

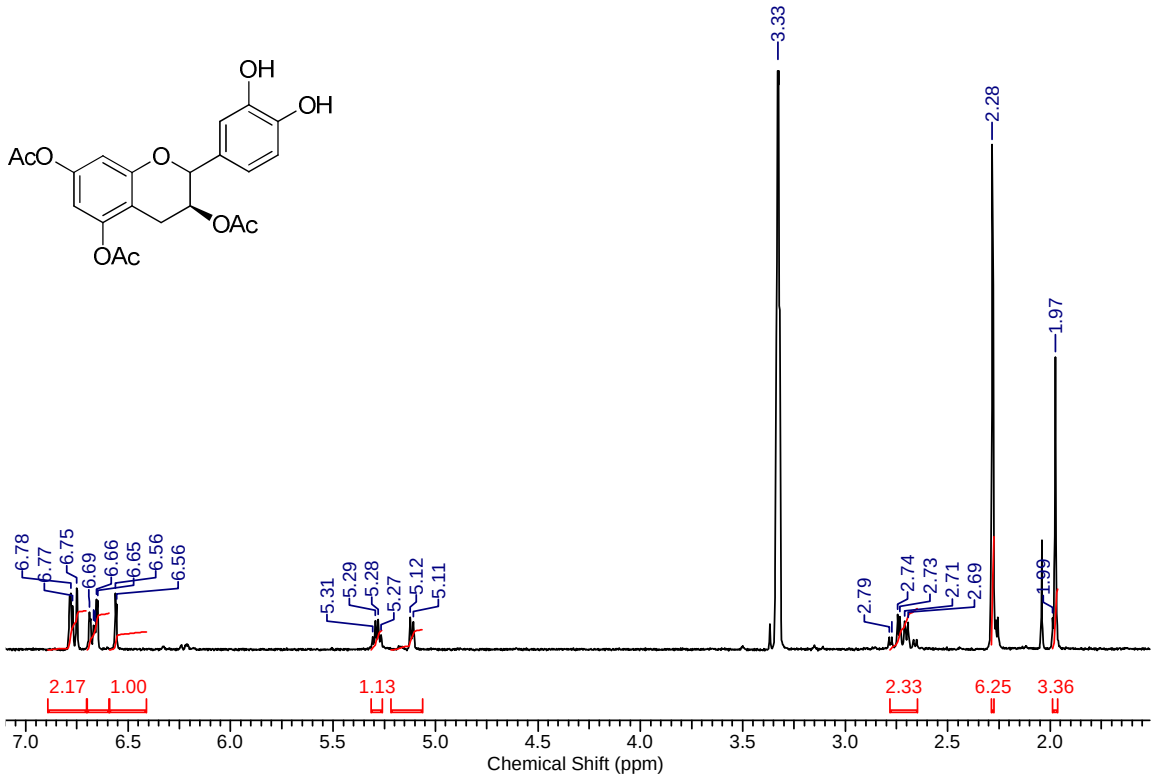

Figure S15. ^{1}H NMR of 3,5,7-triacetylcatechin (**2e**), acquired at 400 MHz in MeOH-d_4.

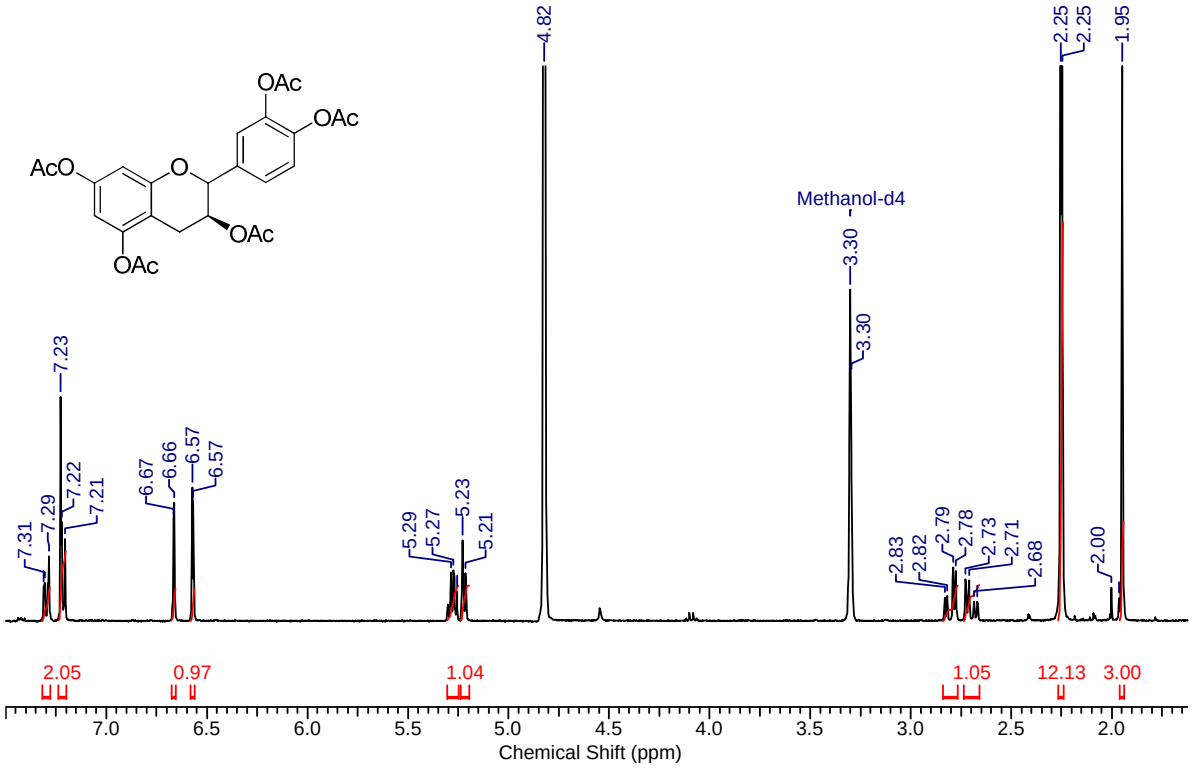

Figure S16. ^{1}H NMR of 3,5,7,3′,4′-Pentaacetylcatechin (**2f**) acquired at 400 MHz in MeOH-d_4.

Supporting Information

The kinetics of recombinant ARG, as represented by Michaelis−Menten and Lineweaver−Burk plots in Figure S17, and Lineweaver−Burk plots for inhibitors **2a** and **1f** in Figure S18.

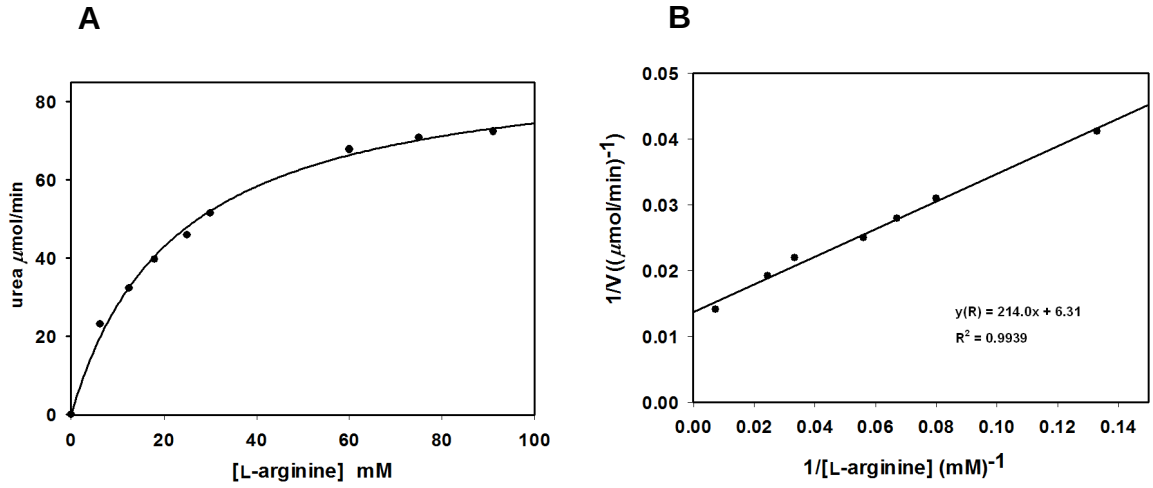

Figure S17. Michaelis–Menten (**A**) and Lineweaver–Burk (**B**) plots of kinetics of recombinant arginase of *L.* (*L.*) *amazonensis*: K_M = 22.6 mM and V_{max} = 158.5 μmol urea min^{-1} mg protein^{-1}.

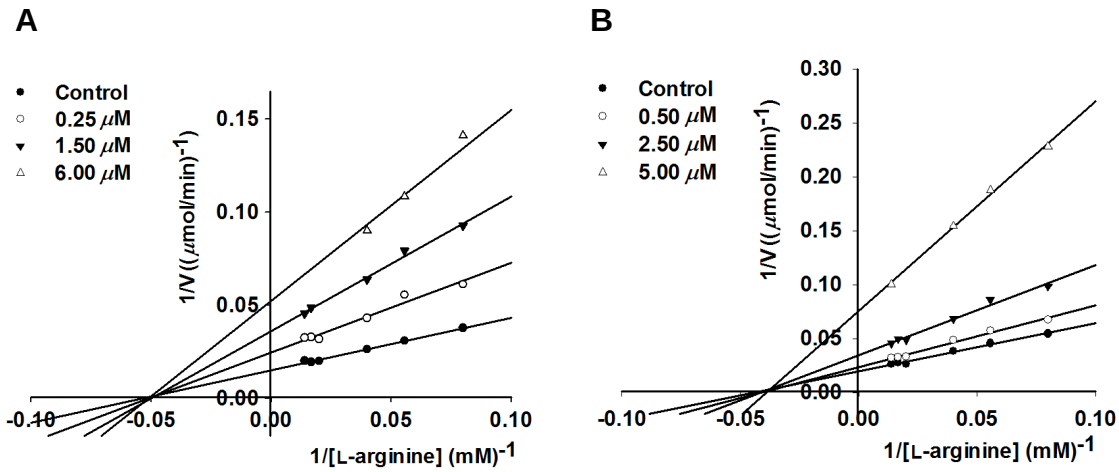

Figure S18. Lineweaver-Burk plots indicating noncompetitive ARG inhibition by (+)-catechin (**2a**) (**A**) and myricetin (**1f**) (**B**).

Supporting Information

Results of inhibition of ARG by fractions obtained from the bioactivity-guided fractionation of the EtOAc extracts of leaves and stems of *B. coccolobifolia* in Figure S19.

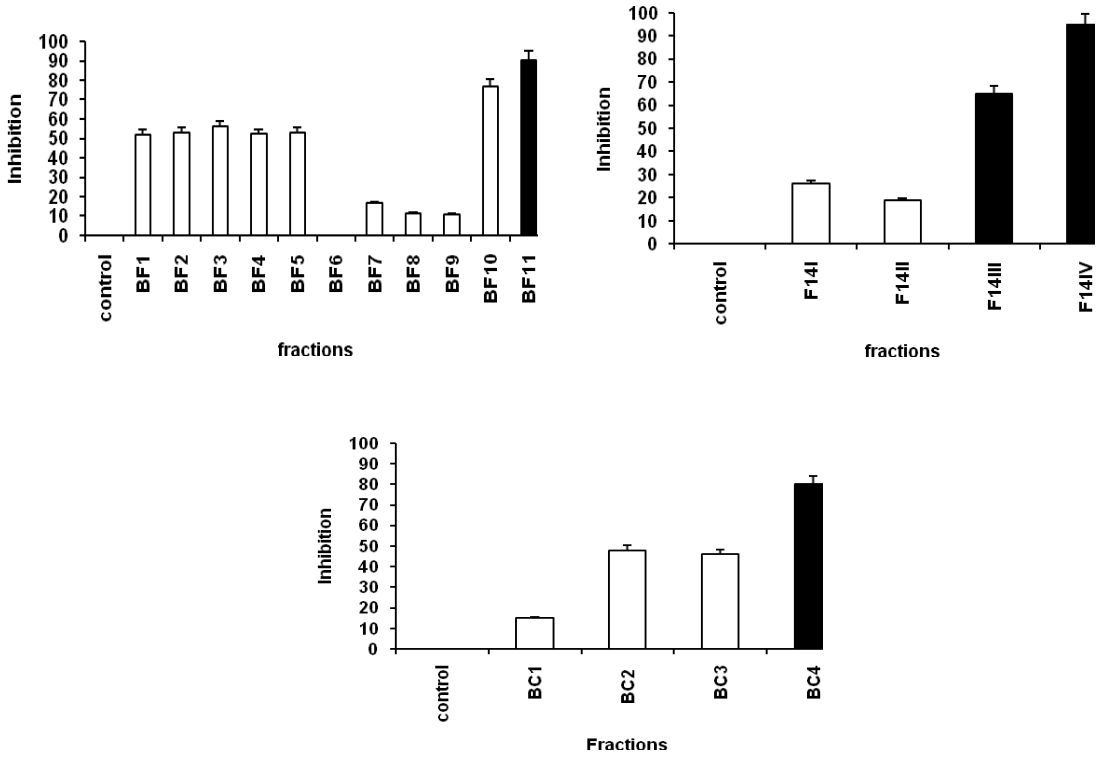

Figure S19. Inhibition of ARG by fractions tested at a concentration of 31.25 μg/mL.

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Article

Leishmanicidal Galloylquinic Acids are Noncompetitive Inhibitors of Arginase

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Ácidos galoilquínicos, substâncias que têm apresentado atividade leishmanicida, foram isolados do extrato de acetato de etila da planta *Byrsonima coccolobifolia*. Estes compostos fenólicos juntamente com o ácido gálico demonstraram ser uma nova classe de inibidores não-competitivos potentes em arginase (ARG) de *Leishmania amazonensis* (K_i variando de 0,10 a 0,68 μmol L^{-1}). O ácido quínico não apresentou atividade inibitória significativa em ARG demonstrando que a unidade galoila tem características importantes que permitem a interação enzima-inibidor. A atividade inibitória significativa do ácido gálico frente à ARG pode ser uma indicação para o entendimento da resposta imune previamente observada em *Leishmania donovani*, uma vez que a atividade enzimática da arginase está associada à diminuição dos níveis de NO no processo de infecção por *Leishmania*.

Leishmanicidal galloylquinic acids were isolated from the ethyl acetate extract of *Byrsonima coccolobifolia*. These phenols and gallic acid showed to be a new class of potent noncompetitive inhibitors of arginase ARG (K_i ranging from 0.10 to 0.68 μmol L^{-1}) from *Leishmania amazonensis*. Quinic acid did not exhibit significant inhibition of ARG, indicating that galloyl moiety has important features that allows the enzyme-inhibitor interactions. The significant inhibitory activity of gallic acid on ARG can be a clue to understand the immune response previously observed on *L. donovani*, since ARG activity is associated with the decrease of the levels of NO in *Leishmania* infection.

Keywords: arginase, *Leishmania*, galloylquinic acids, *Byrsonima coccolobifolia*

Introduction

Leishmaniasis is a deadly infectious tropical disease caused by the protozoan of the genus *Leishmania*, which affects more than 12 million people of broad geographical distribution.[1,2] The challenges for healthcare of leishmaniasis found on available drugs such as, high toxicity, undesirable side effects, high cost and parasite resistance, reveal an urgent problem and the need for new efficient drugs.[3-5]

Exploring for novel therapeutic opportunities, new biochemical targets have been investigated, in particular arginase (ARG) from *Leishmania amazonensis* have been considered an attractive target in the search for new leishmanicidal agents. The biochemical pathway that this enzyme is involved is essential for the protozoa development in their life cycle.[6-8] In addition, the crystal structure of ARG from *Leishmania mexicana* was solved,[9] making this protease more interesting to investigate for new antileishmanial compounds.

Arginase is a metalloenzyme with binuclear manganese center, which catalyzes the last step in the urea cycle in mammals, allowing hydrolyzes of L-arginine to L-ornithine and urea.[10] In infected macrophages the substrate L-arginine is used by ARG and by nitric oxide synthase (NOS) in two different metabolic pathways. The reaction catalyzed by ARG is carried out in the polyamines (PAs) metabolism, essential to preserve parasite viability, and the NOS pathway generate nitric oxide (NO) molecules, which production increases oxidative stress.[11,12] The balance between NOS and mammalian arginase is competitively regulated by TH_1 and TH_2 cytokines as protective response. *Leishmania* protozoa explore the immune response of TH_2 increasing ARG expression in host cells and consequently PAs for growth and establishment of infection.[8,13,14]

N^{ω}-hydroxy-L-arginine and 2(S)-amino-6-boronohexanoic acid are synthetic aminoacid derivatives, which inhibit in a competitive mode ARG from *L. mexicana* with K_i values of 85 and 1.3 μmol L^{-1} respectively. However, in an

in vivo study these synthetic compounds could not contain the infection completely.[7,15] Flavonoids isolated from plants have been reported as inhibitors of ARG from *L. amazonensis*. The presence of hydroxy groups in their structures showed to be important features for inhibitory activity.[16-19]

Quinic acid is a hydrated form of shikimic acid and together with galloylquinic acids (tannic acids) and gallic acid are derivatives from shikimate pathway, available and widespread in plant sources.[20,21] Tannins have been associated with many biological activities and to health beneficial effects.[22-24] Additionally, tannic acids have presented antileishmanial activity (EC_{50} = 2-38 μmol L^{-1}), and their leishmanicidal potency have been associated to the number of galloyl groups substituents in the shikimic acid moiety.[25] Gallic acid singly reduces *Leishmania donovani* amastigotes (EC_{50} = 4.4 μg mL^{-1}) in murine macrophages by activating leishmanicidal macrophage functions.[26] Also, tannins identified from fractions of cajazeira (*Spondias mombin* L.) showed leishmanicidal effect *in vitro* on amastigotes of *Leishmania chagasi*, with IC_{50} in the range of 0.61 to 17.07 μg mL^{-1}.[27] However, so far, no study has been reported on this class of secondary metabolites exploring their inhibitory activity on ARG from *L. amazonensis*.

Based on the usage of natural products as invaluable tools in the search for new drugs[5,28] and in the antiprotozoal activities previously found in *Byrsonima* species,[29,30] we performed a phytochemical study of fractions of *Byrsonima coccolobifolia* Kunth. (Malpighiaceae) extract that significantly inhibited ARG enzyme. Gallic acid (**2**) and galloylquinic acids (**3**-**6**) were isolated from ethyl acetate extract from leaves and stems of *B. coccolobifolia*, which were identified by 1D and 2D NMR spectra, and through comparison with data previously reported.[31-35] Galloylquinic acids (**3**-**6**) and gallic acid (**2**) were potent inhibitors of ARG with high affinity. Quinic acid (**1**)[36] isolated from *Myrcia lingua* (O. Berg) Mattos (Myrtaceae) was also tested on ARG to

compare inhibitory activity with those galloylquinic acid derivatives, but the inhibition of ARG found for this compound was not significant.

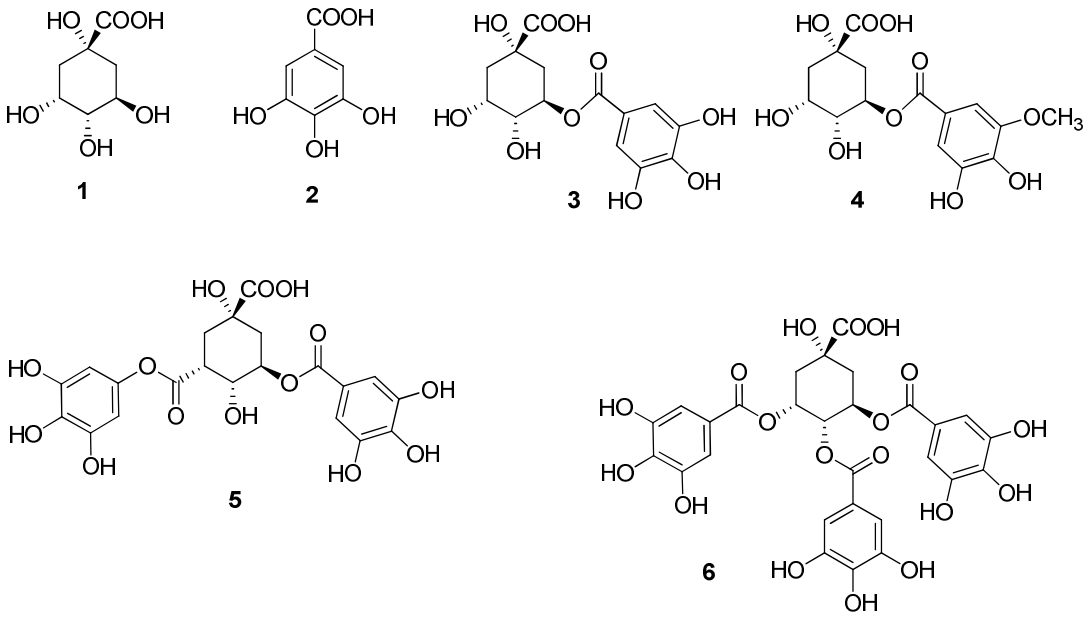

Figure 1. Compounds analysed against recombinant ARG from *L. amazonensis*

Experimental

General experimental procedures

The measurement of urea in the enzymatic assays was performed on a Beckman Coulter DU 800 spectrophotometer at 600 nm. The 1D and 2D nuclear magnetic resonance (NMR) data were acquired on a Bruker DRX-400 NMR spectrometer (^{1}H: 400 MHz; ^{13}C: 100 MHz) using D_2O and MeOH-d_4 as solvents. Silica gel 60 (Merck, 230-700 mesh) and Sephadex LH-20 (Amersham Pharmacia Biotech AB) together with thin-layer chromatography (TLC) on pre-coated aluminum silica 60 F_{254} (Merck) were used to isolate the compounds. Compounds were visualized in TLC $UV_{254/366}$ and by the usage of the stain sulfuric vanillin solution. The solvents ethanol (EtOH), methanol (MeOH), hexane, acetone, ethyl acetate (EtOAc) from Vetec were used to prepare the extracts and for chromatographic procedures.

Plant material

B. coccolobifolia leaves and stems were collected in the cerrado at Federal University of São Carlos (UFSCar), São Carlos - SP, Brazil. The plant material was identified by Dr. Maria Inês Salgueiro Lima, and deposited at the Herbarium of the Botany Laboratory (HUFSCar) in UFSCar (voucher No. 8367).

Extraction and isolation

The crude extracts from the leaves (10.0 g) and stems (30.5 g) of *B. coccolobifolia* were liquid-liquid partitioned leading to the EtOAc extracts (2.0 g of leaves and 10.0 g of stems), and both showed inhibitory activity against ARG. The procedure of extraction and some of initial chromatography steps were described previously by us.[19] The subfraction F14IV (128.0 mg) from the EtOAc extract from leaves (BcFA), after two chromatography columns using sephadex LH-20 (F14IV: 53.0 × 4.0 and 37.0 × 1.4 cm; MeOH isocratic) afforded 3,5-di-*O*-galloylquinic acid (**5**) (2.0 mg). Further purification of the resulting fraction 8 by silica flash (0.8 × 14.0 cm; 6:4 acetone/hexane isocratic) provided gallic acid (**2**) (2.0 mg).

The EtOAc extract from stems (BcCA) was chromatographed on a silica gel column (60-200 mesh, 12.0 × 5.0 cm; 1:10 acetone/hexane isocratic) yielding four fractions. Fraction 4 (4.0 g) obtained from BcCA was then fractionated several times by sephadex LH-20 columns (50.0 × 4.5 cm; 7.0 × 5.0 cm; 1.3 × 36.0 cm; and 1.5 × 52 cm; MeOH isocratic) to give the compounds 5-*O*-galloylquinic acid (**3**) (1.5 mg), 5-*O*-(3-methylgalloyl)- quinic acid (**4**) (1.5 mg) and 3,4,5-tri-*O*-galloylquinic acid (**6**) (3.0 mg). The structures of the known compounds were determined by analysis of ^{1}H and ^{13}C NMR, DEPT-135, HSQC, and HMBC spectra, and confirmed by comparison with the literature data.[31-35]

Chemicals

Quercitrin hydrate ≥ 78% (Sigma Q3001) was purchased from Sigma-Aldrich and the natural quinic acid (**1**)[36] was previously isolated from *Myrcia lingua* (O. Berg) Mattos (Myrtaceae) in our laboratory. The Enzymatic Urea Kit was obtained from Biotécnica (Varginha, MG, Brasil).

Arginase assay

The recombinant arginase of *L. amazonensis* was expressed and purified as described previously.[19,36,37] The kinetics measurements of ARG were performed as reported before,[10,19,39] resulting a K_m value of 22.6 ± 1.7 mmol L^{-1} (R^2 = 0.996). Compounds were serially diluted, using at least 10 concentrations for IC_{50} determinations (quinic acid (**1**) was diluted from 5000 to 1.22 μmol L^{-1} and compounds (**2**-**6**), the concentrations were between 250 and 0.024 μmol L^{-1}). Mix I was prepared using 50 μL of CHES buffer solution at pH 9.6, 8 μL of arginase solution and 292 μL of water. A volume of 5 μL sample of each concentration of inhibitor was added to 35 μL of mix I, and the reaction mixture was incubated for 10 min at 37 °C. Then 10 μL of L-arginine solution was added to the reaction giving 50 mmol L^{-1} substrate and 50 mmol L^{-1} of CHES buffer at pH 9.6 in a final assay volume, which was incubated again for 10 min at 37 °C. The urea was measured spectrophotometrically at 600 nm by an enzymatic colorimetric assay,[40] using a commercially available kit (Biotécnica, Brazil). A volume of 10 μL of the enzymatic reaction was added to 500 μL of reagent 1 previously prepared (50 mmol L^{-1} phosphate buffer, pH 6.7, 60 mmol L^{-1} salicylate, 3.2 mmol L^{-1} sodium nitroprusside, and 30000 IU urease) and incubated at 37 °C for 10 min. After incubation, 500 μL of reagent 2 (140 mmol L^{-1} NaOCl and 150 mmol L^{-1} NaOH) was added and incubated at 37 °C for 10 min. The enzymatic assay was performed in duplicate and a negative control and a positive control (quercitrin)[16] were used. Type of inhibition of active compounds was evaluated using the same procedure, but increasing substrate

concentrations in the range of 6.25-72.0 mmol L^{-1}. K_{is} (affinity constant of enzyme-inhibitor) and K_{ii} (affinity constant of inhibitor with enzyme-substrate complex) for compounds (**2**-**6**) were found by double-reciprocal (Lineweaver-Burk) plots, using slopes and ordinate intercepts plotted *versus* the respective inhibitor concentrations in the abscissa. The constants are resulting from the straight lines (linear regression), which the abscissa intercepts leads to $-K_{ii}$ and $-K_{is}$. The equations used are $Sl = K_m/V + [I]\, K_m/K_{is}V$, slope (*Sl*) and $Or = 1/V + [I]/K_{ii}V$, intercept (*Or*), derived from Lineweaver-Burk equation, following bellow.[41-43] In addition, Dixon plots of 1/velocity *versus* inhibitor concentration data was also used to find K_{is} values ($1/V = [1 - (K_{is}/K_{ii})]/V_{max}$, $[I] = -K_{is}$). All data analyses were carried out with the SigmaPlot 12.0 and GraFit5 software.

$$v = \frac{V_{max}[S]}{[S]\left(1 + \frac{[I]}{K_{ii}}\right) + K_m\left(1 + \frac{[I]}{K_{is}}\right)}$$

Results and Discussion

The quinic acid esters (**3**-**6**) and gallic acid (**2**), isolated from the active EtOAc fraction of *B. coccolobifolia* strongly inhibited ARG (Table 1). However, quinic acid (**1**) did not show expressive inhibition on ARG. Furthermore, high activity was demonstrated by compounds with large number of phenolic hydroxy groups. Comparing the IC_{50} values for compounds (**2**-**6**) the potency of gallic acid (**2**) is more significant, but increasing the galloyl groups in the quinic acid moiety (**3**-**6**) did not show a considerable change in the IC_{50} values, as observed previously for leishmanicidal potency in tannic acids.[25]

Kinetics studies of compounds (**2**-**6**) showed that these inhibitors shared a common behavior acting as noncompetitive inhibitors. For compounds **2**, **5** and **6** the noncompetitive inhibition is also frequently known as mixed type, while inhibitors **3** and **4** are designated as pure noncompetitive inhibitors. Recently, gallic acid (K_i = 7.2 ± 1.4 μmol L^{-1}) and epigallocatechin-3-gallate

(EGCG) (K_i = 4.0 ± 0.5 μmol L^{-1}) showed potent ARG inhibitory activity with a noncompetitive and mixed type, respectively.[44]

Analysis of Lineweaver-Burk plot (Figure 2A) for gallic acid (**2**) showed the intersecting lines converging to the left of the y-axis and above the abscissa ($K_{ii} > K_{is}$), which means the inhibitor **2** binds with higher affinity to the free enzyme than to the enzyme-substrate complex. Additionally, when quinic acid is substituted in the position 5 by a galloyl (compound **3**) or a methoxy galloyl moieties (compound **4**), this behavior changes a little, each inhibitor (**3** and **4**) now binds with the same affinity to both, free enzyme and enzyme-substrate complex ($K_{ii} = K_{is}$). This can also be seen by the double reciprocal plots (Figures 2B and 2C), which the nest of lines converge nearly at the x-axis. On the other hand, for the substituted quinic acid with two or more galloyl moieties (compound **5** and **6**), the bind affinity is higher to the enzyme-substrate complex than to the free enzyme ($K_{ii} < K_{is}$), and the line intercept is now located below the abscissa in both plots (Figures 2D and 2E). The calculated values of affinity constants (Table 1) support the findings.

Table 1. IC_{50} and K_i values for secondary metabolites active on ARG

Compound	IC_{50} / (μmol L^{-1})[b]	K_{is} / (μmol L^{-1})[b]	K_{ii} / (μmol L^{-1})[b]	Type of inhibition
Positive control[a]	12.20 ± 1.83	7.20 ± 0.90[a]	7.20 ± 0.90[a]	Noncompetitive[a]
1	147.48 ± 15.44	–	–	–
2	0.13 ± 0.01	0.10 ± 0.03	0.18 ± 0.004	Mixed
3	0.44 ± 0.04	0.43 ± 0.014	0.41 ± 0.003	Noncompetitive
4	0.49 ± 0.04	0.16 ± 0.01	0.14 ± 0.01	Noncompetitive
5	0.46 ± 0.04	0.57 ± 0.03	0.23 ± 0.01	Mixed
6	0.31 ± 0.02	0.68 ± 0.02	0.35 ± 0.002	Mixed

[a]Quercitrin is a noncompetitive inhibitor used as a positive control (K_{ii} and K_{is} obtained from the reference 18); [b]Data values represent means of individual experiments ± SD.

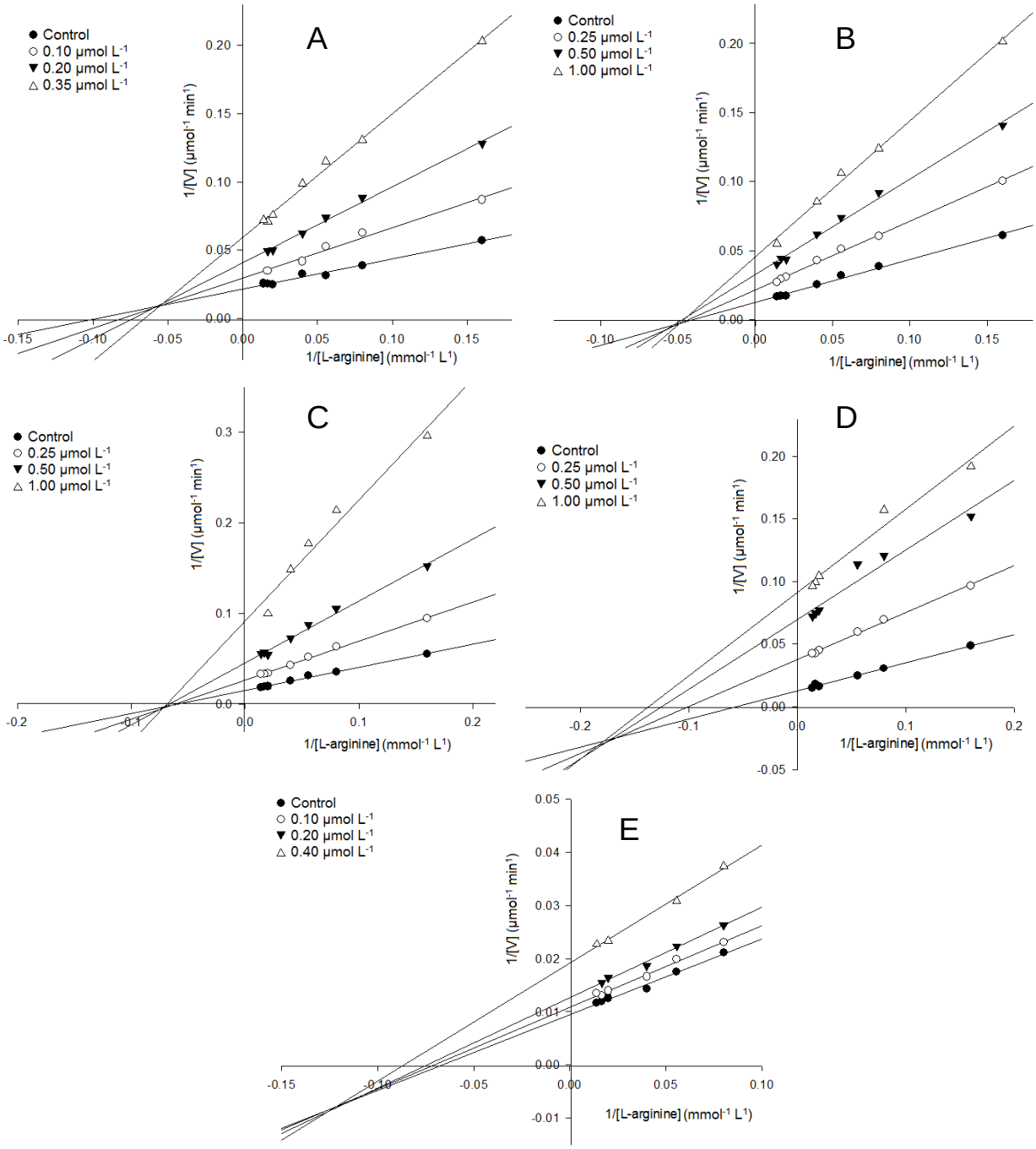

Figure 2. Lineweaver-Burk plots of gallic acid **2** (A), 5-*O*-galloylquinic acid **3** (B), 5-*O*-(3-methylgalloyl)-quinic acid **4** (C), 3,5-di-*O*-galloylquinic acid **5** (D), 3,4,5-tri-*O*-galloylquinic acid **6** (E).

Gallic acid and derivatives have been reported as responsible for benefic effects and a number of chemopreventive properties provided by green tea and wine consumption.[45,46] Also, other therapeutic effects have been described, such as anti-cancer, antimicrobial and antiviral activity.[47-50]

Additionally, some gallotannins and tannic acids have showed inhibitory potential on enzymes like α-glucosidase, fatty acid synthase (FAS) and HIV-1 protease. [24,51,52] This is contradictory with previous screening for enzyme inhibitors that showed that tannins and lauryl gallate nonspecifically inhibited enzymes through the formation of aggregates, acting as promiscuous protein binders.[53-55] The aggregate-based promiscuous inhibition could be related to the large reactive functional groups of these compounds.[55]

We have identified the inhibition mechanism of the quinic acid esters (**3**-**6**) and gallic acid (**2**) as noncompetitive and mixed inhibitors, suggesting that the inhibitory activity of ARG would not be related to the formation of aggregates. In addition, some of these phenols have already been reported as leishmanicidal compounds. Previous study showed that gallic acid increases the expression of nitric oxide synthase (iNOS) and cytokine genes in *Leishmania major* parasitised RAW 264.7 cells, as properties to combat the infection.[56] This phenol demonstrated in *in vitro* study on *L. donovani*, a nonspecific immune response recognized by release of tumour necrosis factor (TNF) and elevated nitric oxide (NO) concentration.[26]

Leishmania stimulate the PAs production for their development and decrease the level of NO prompted by NOS.[7,8,57] Thus, ARG inhibition could turn on the production of NO, instead of PAs preventing the infection.

Conclusions

Gallic acid and derivatives isolated from *B. coccolobifolia* showed to be novel noncompetitive and mixed inhibitors of ARG from *L. amazonensis*, with K_i values in the low μmol L^{-1} range. In conclusion, our results suggest that the ability of galloyl moiety in induce immunological response demonstrated earlier may be also a result of the inhibition of ARG activity. In this view, the galloylquinic acids should be considered as promising hits in the investigation of leishmanicidal compounds.

Acknowledgments

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Supplementary Information

Supplementary data (^{1}H, ^{13}C NMR and Dixon plots of acids).

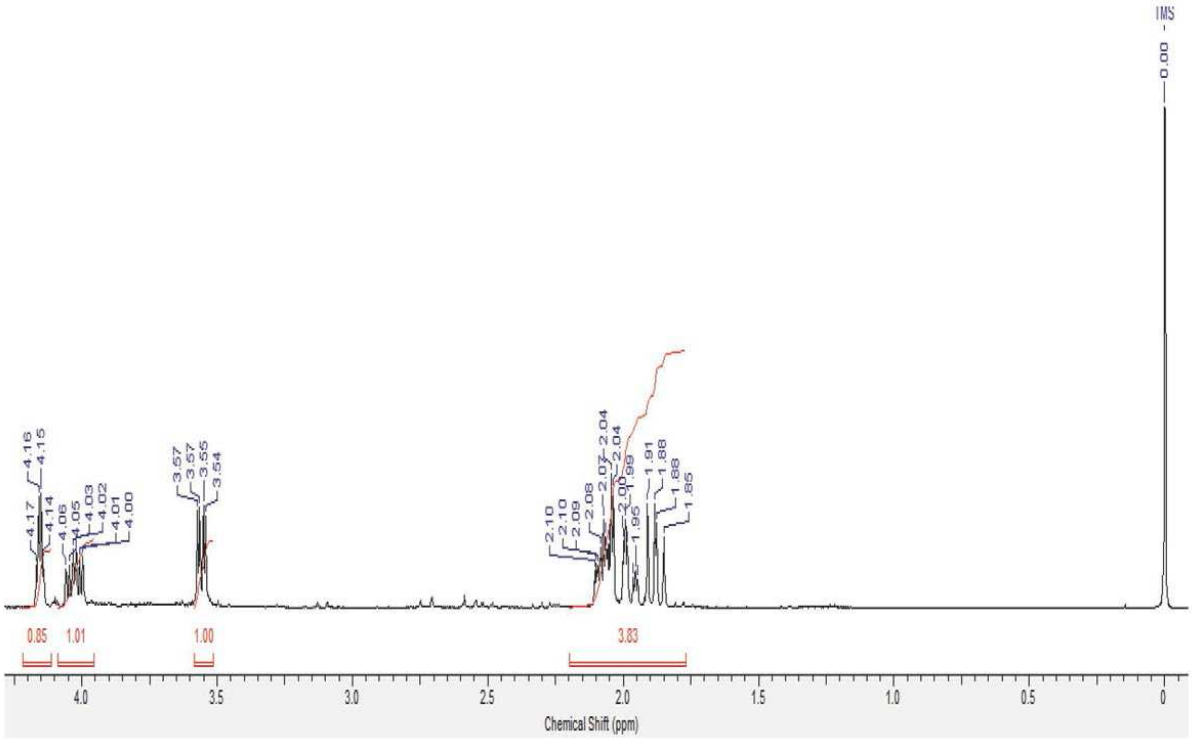

Figure S1. ^{1}H NMR spectrum of quinic acid (**1**) acquired at 400 MHz in D_2O.

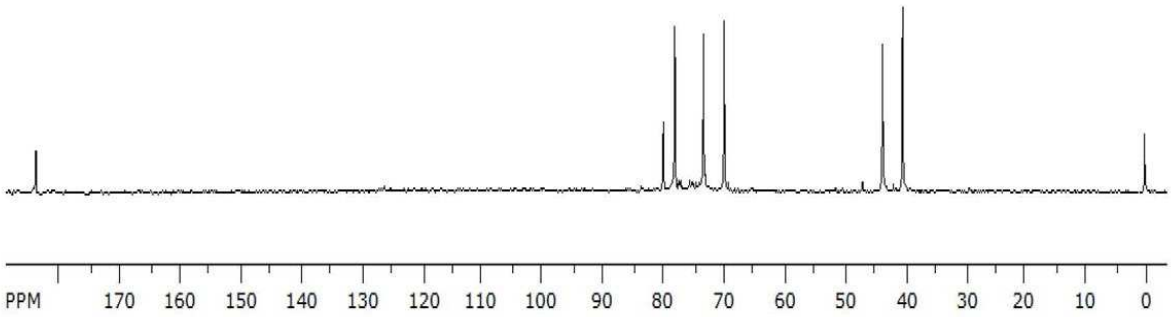

Figure S2. ^{13}C NMR spectrum of quinic acid (**1**) acquired at 100 MHz in D_2O.

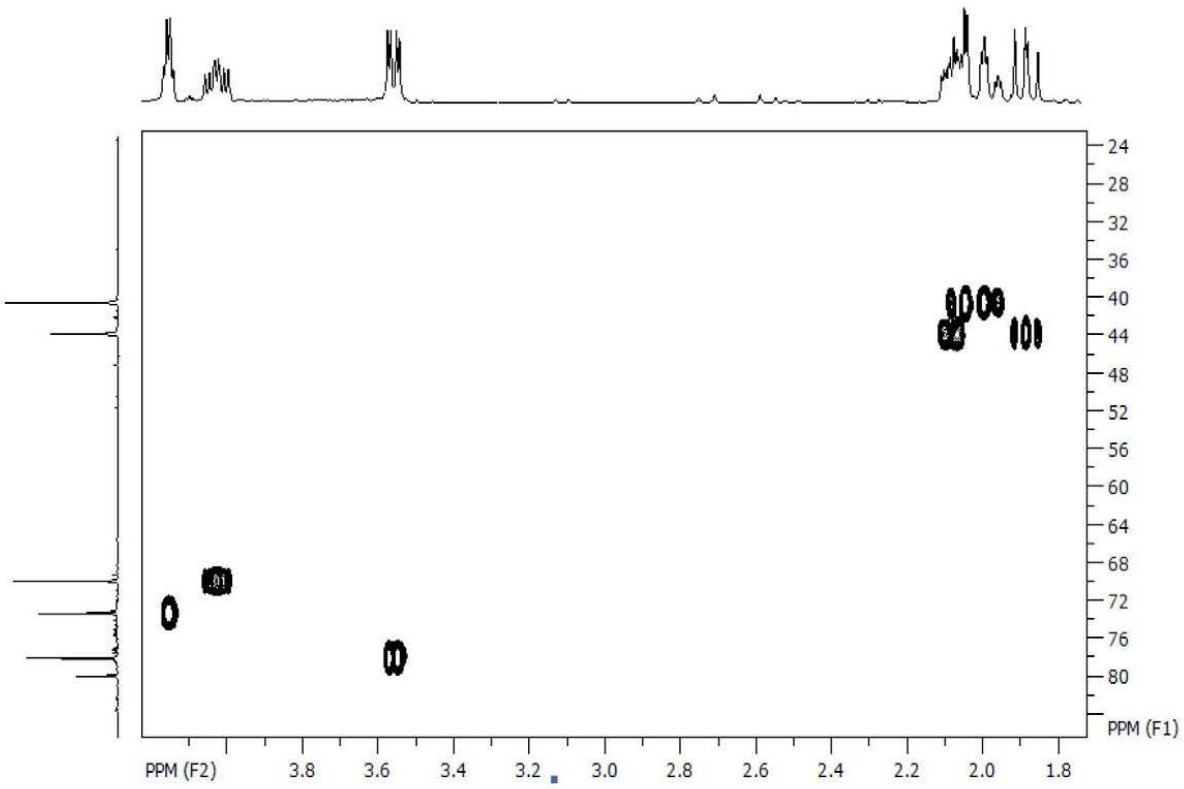

Figure S3. HSQC of quinic acid (**1**) acquired at 400 MHz in D_2O.

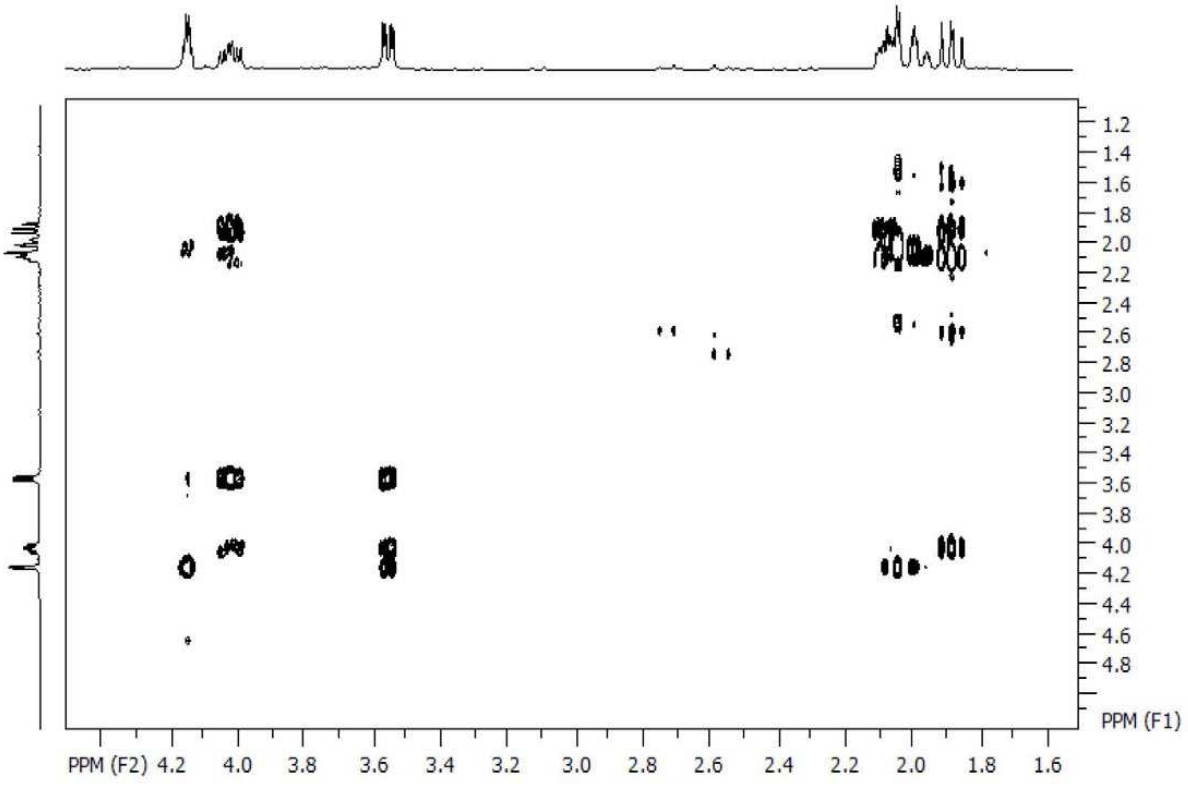

Figure S4. COSY of quinic acid (**1**) acquired at 400 MHz in D_2O.

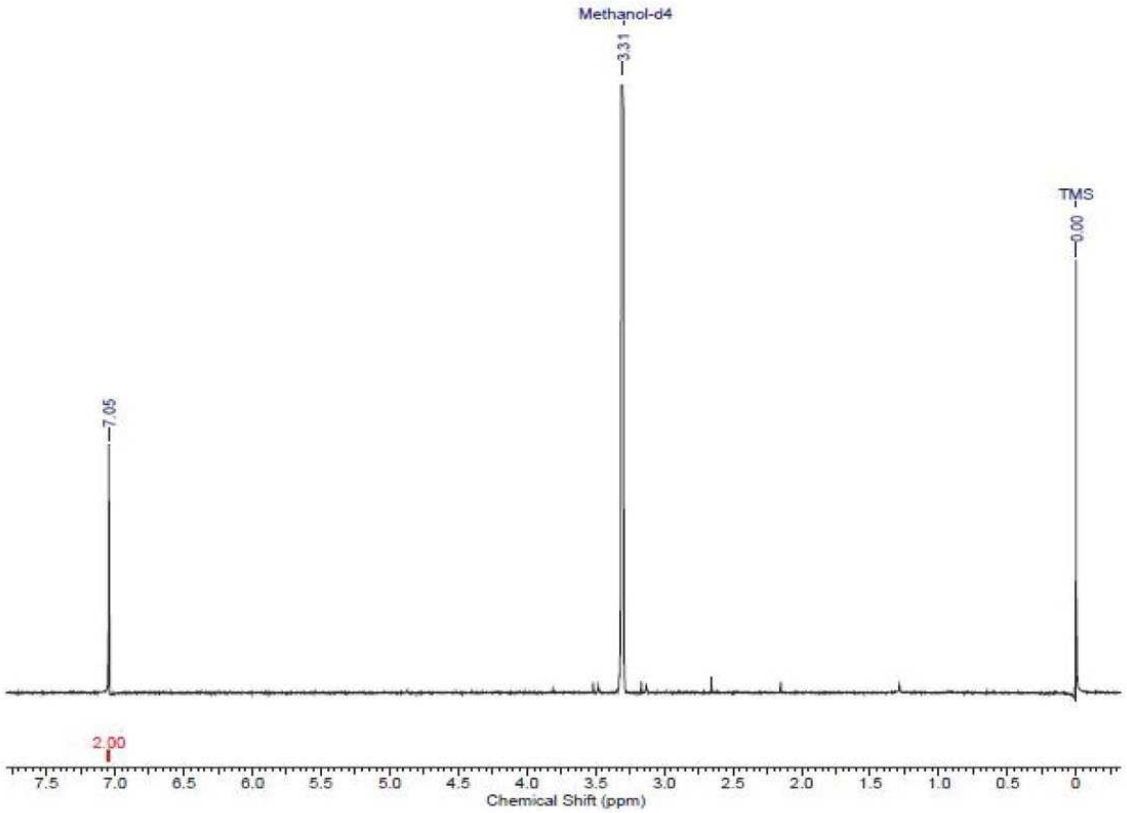

Figure S5. ^{1}H NMR spectrum of gallic acid (**2**) acquired at 400 MHz in MeOH-d_4.

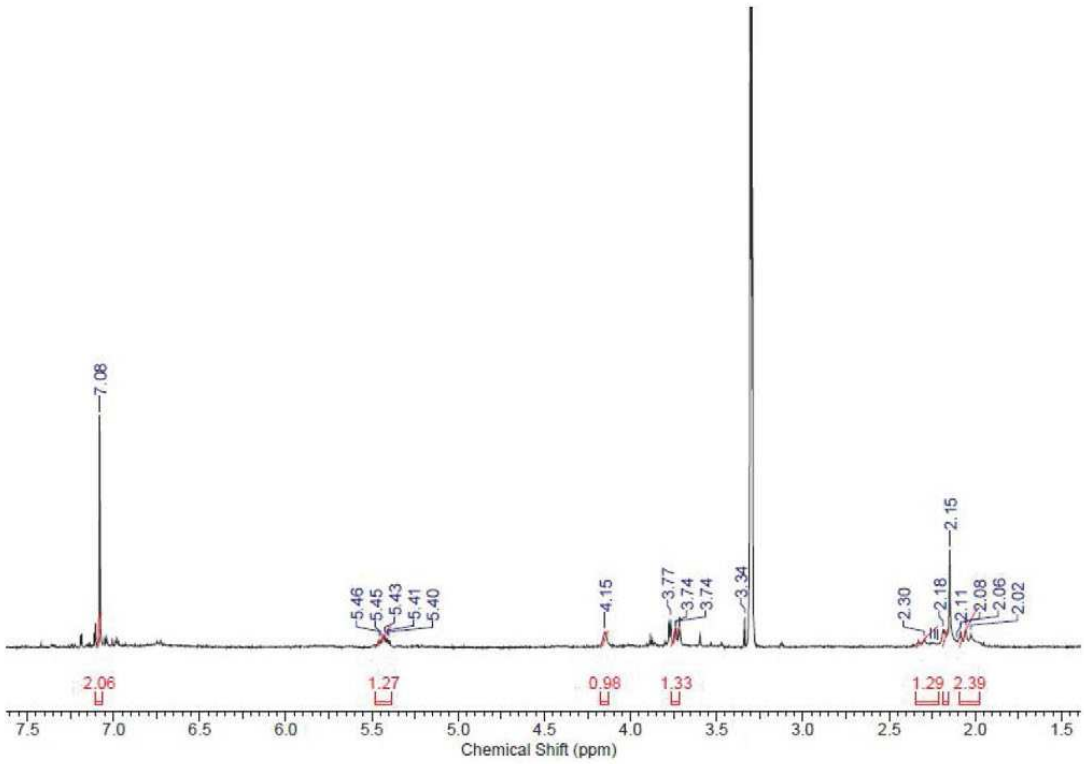

Figure S6. ^{1}H NMR spectrum of 5-*O*-galloylquinic acid (**3**) acquired at 400 MHz in MeOH-d_4.

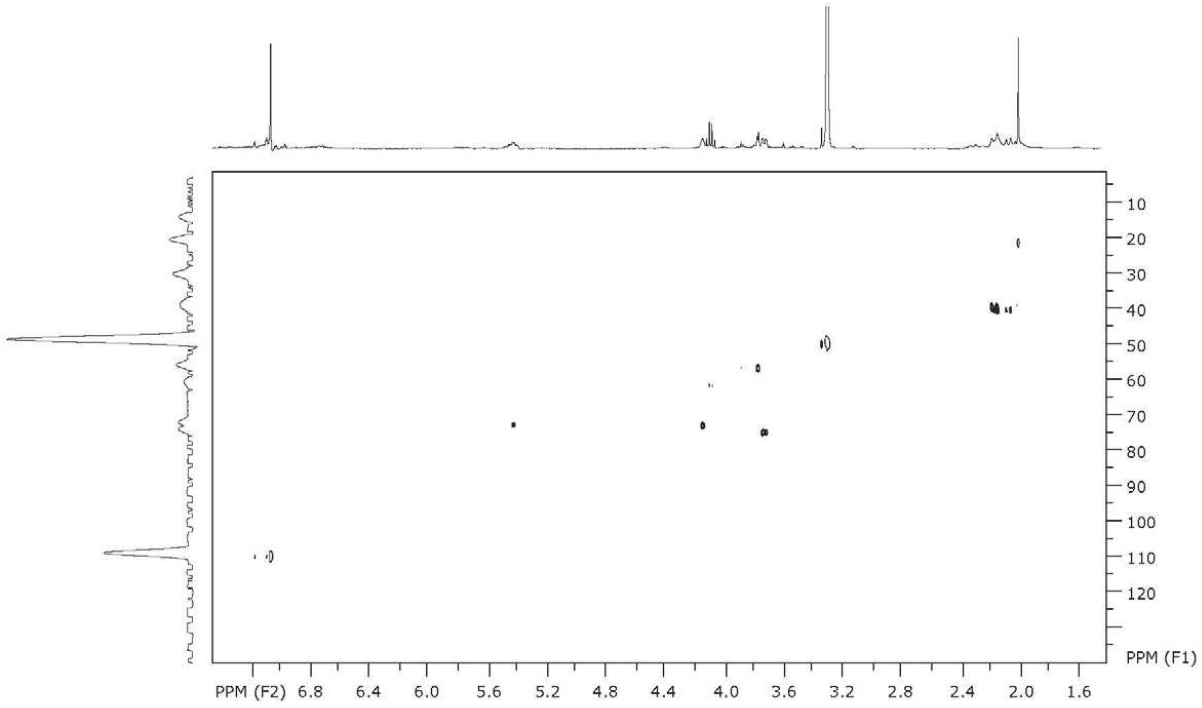

Figure S7. HSQC of 5-*O*-galloylquinic acid (**3**) acquired at 400 MHz in MeOH-d_4.

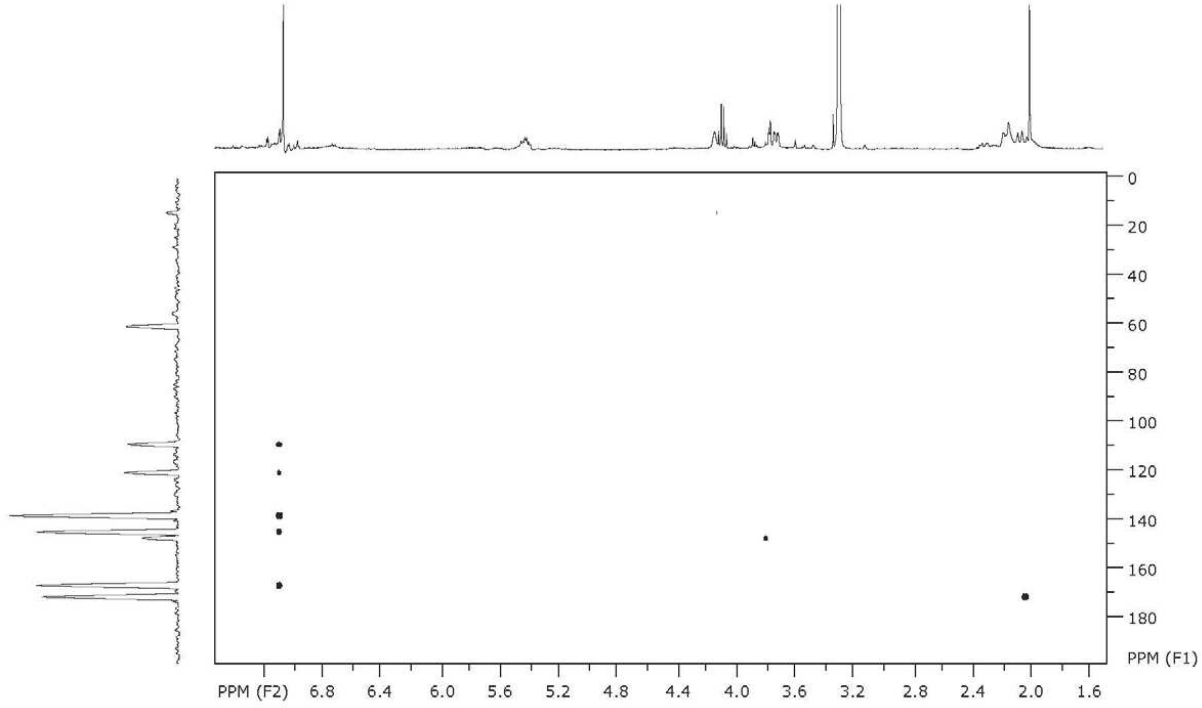

Figure S8. HMBC of 5-*O*-galloylquinic acid (**3**) acquired at 400 MHz in MeOH-d_4.

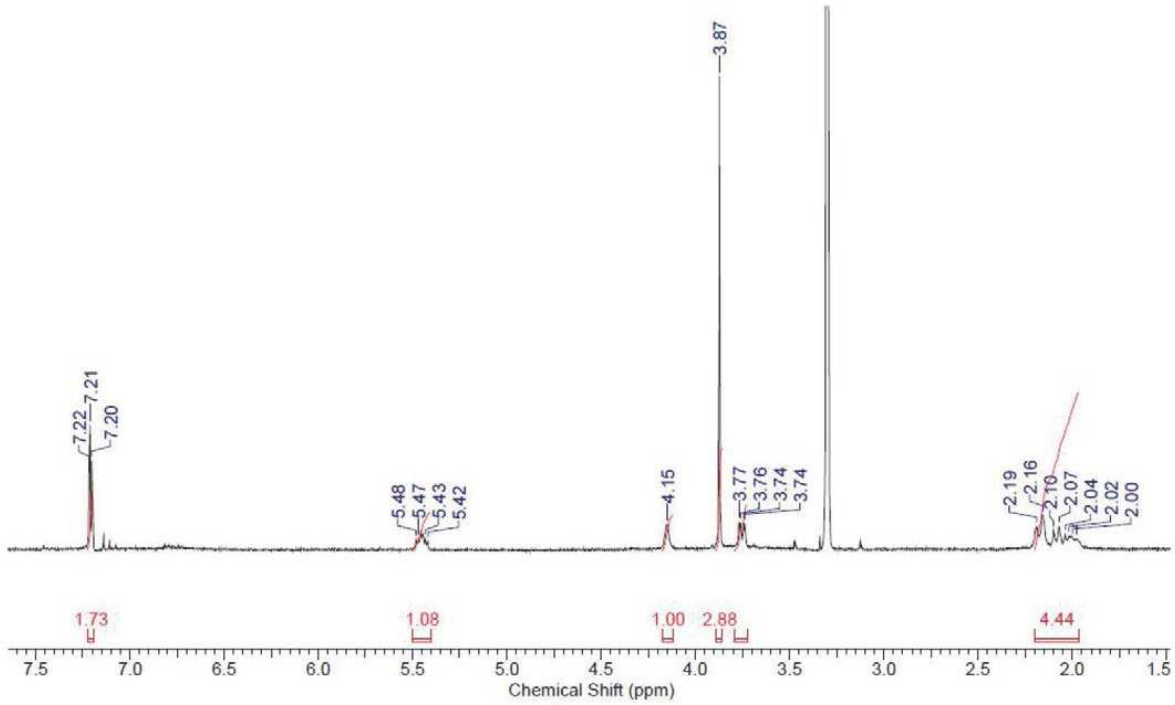

Figure S9. ^{1}H NMR spectrum of 5-*O*-(3-methylgalloyl)-quinic acid (**4**) acquired at 400 MHz in MeOH-d_4.

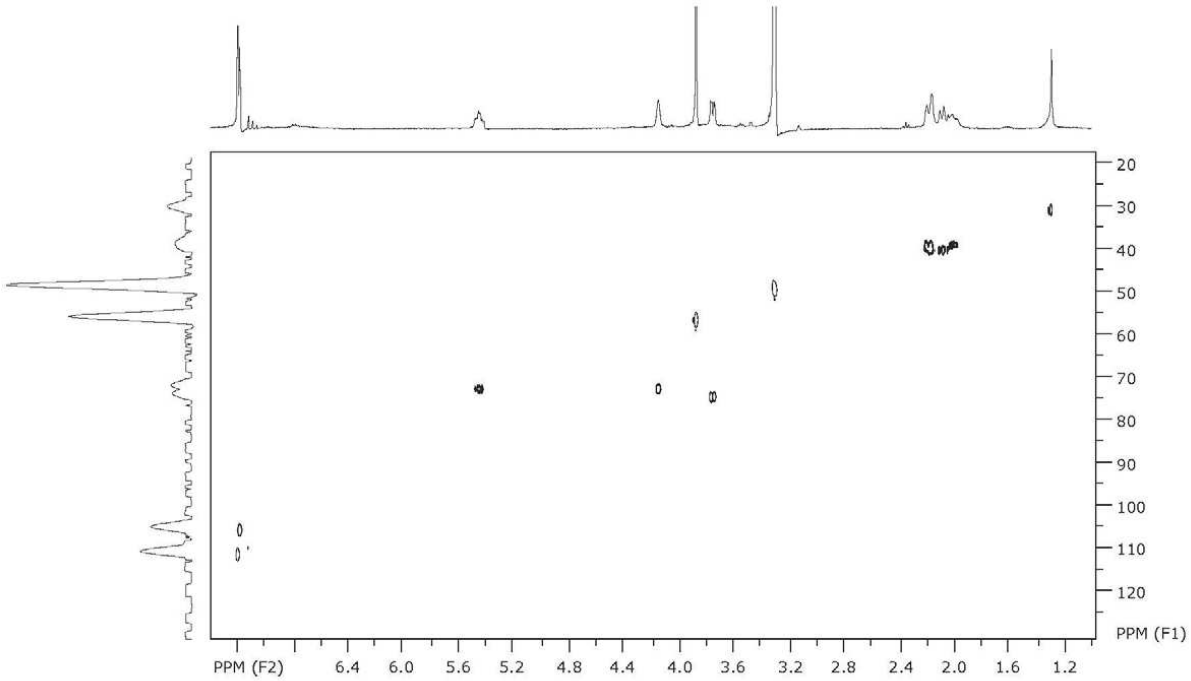

Figure S10. HSQC of 5-*O*-(3-methylgalloyl)-quinic acid (**4**) acquired at 400 MHz in MeOH-d_4.

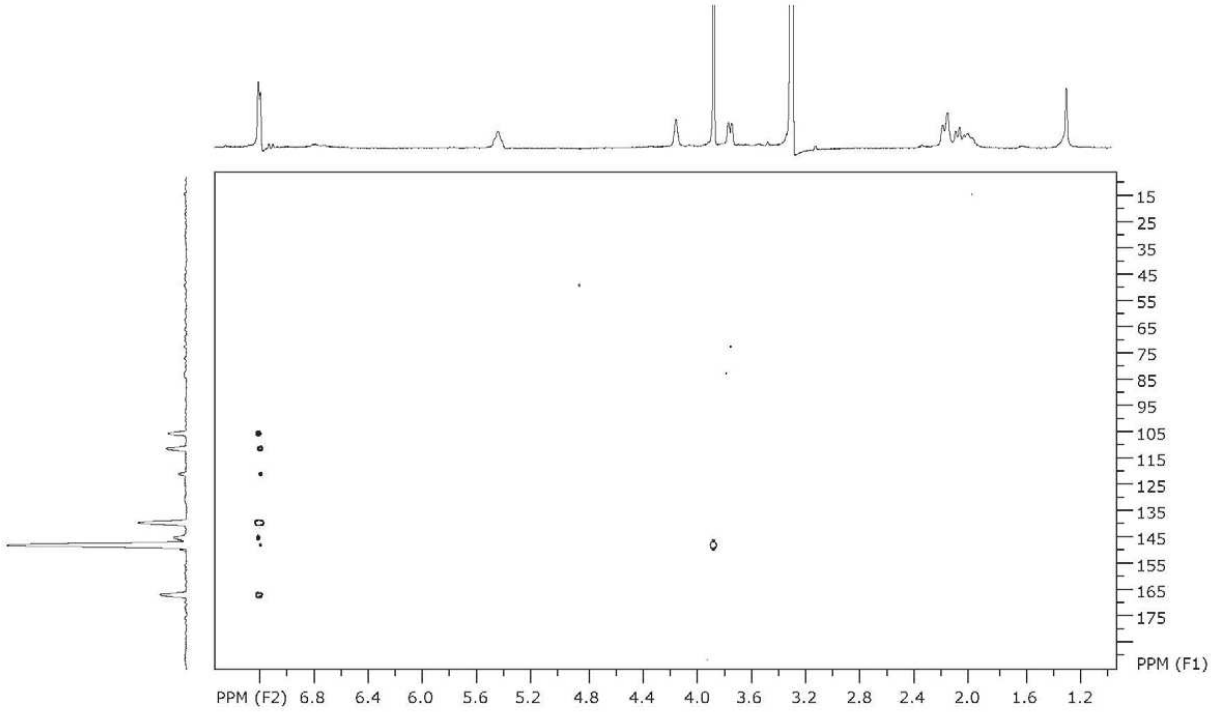

Figure S11. HMBC of 5-*O*-(3-methylgalloyl)-quinic acid (**4**) acquired at 400 MHz in MeOH-d_4.

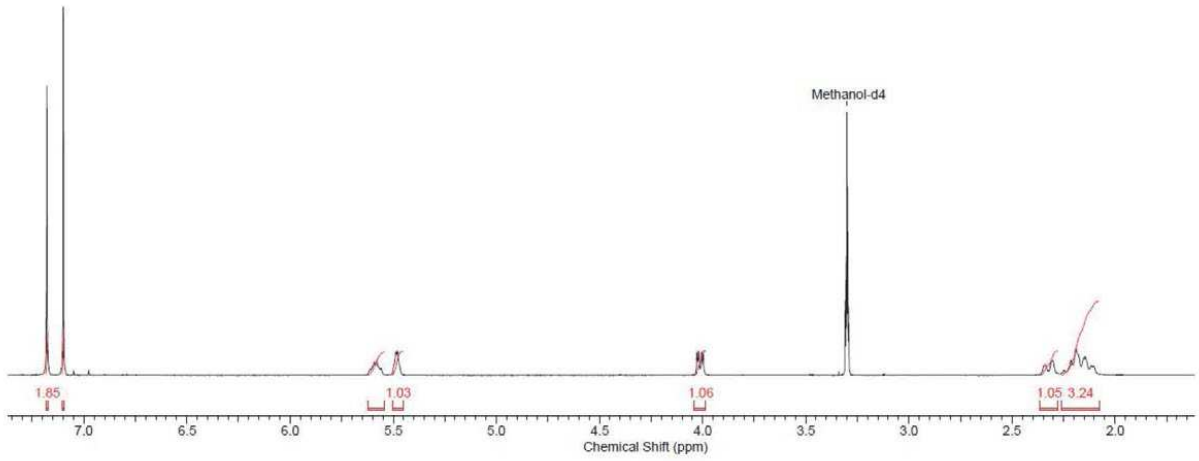

Figure S12. ^{1}H NMR spectrum of 3,5-di-*O*-galloylquinic acid (**5**) acquired at 400 MHz in MeOH-d_4.

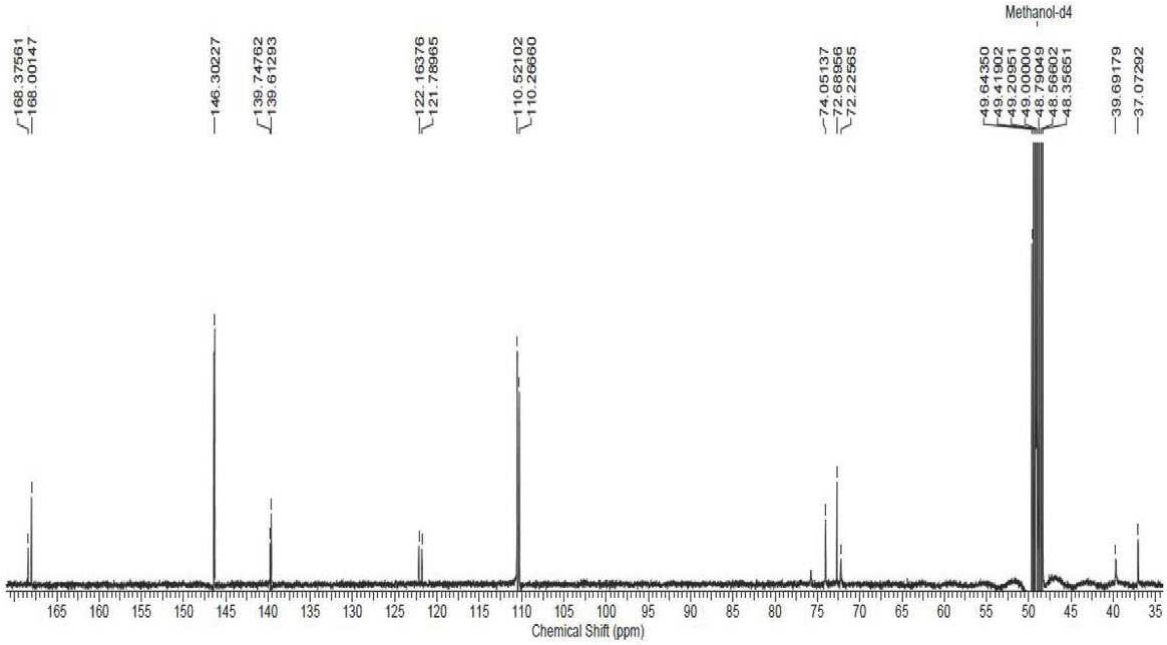

Figure S13. ^{13}C NMR spectrum of 3,5-di-*O*-galloylquinic acid (**5**) acquired at 100 MHz in MeOH-d_4.

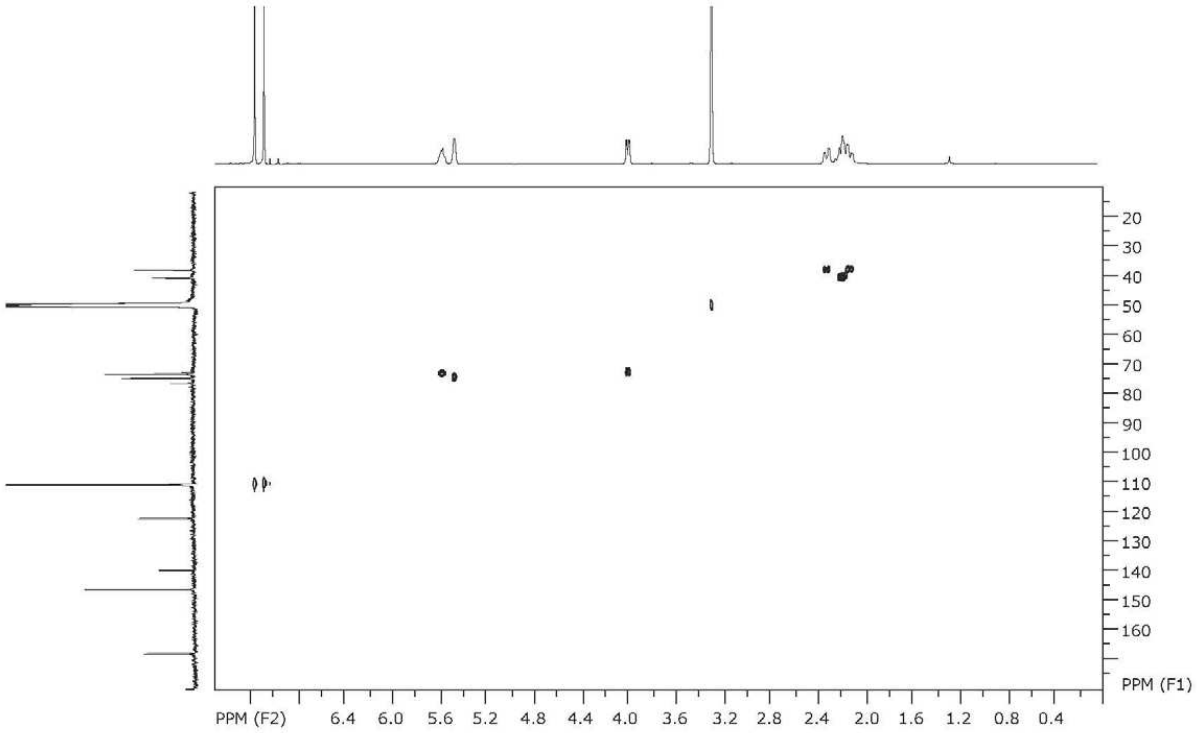

Figure S14. HSQC of 3,5-di-*O*-galloylquinic acid (**5**) acquired at 400 MHz in MeOH-d_4.

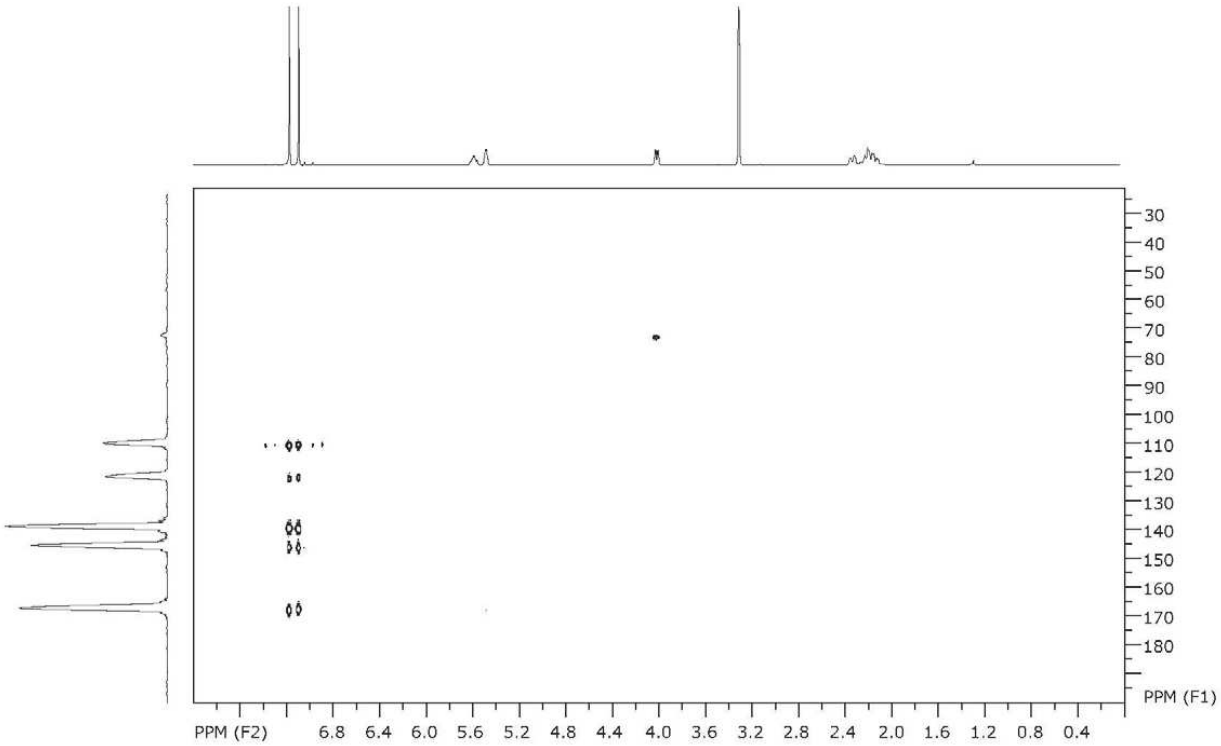

Figure S15. HMBC of 3,5-di-*O*-galloylquinic acid (**5**) acquired at 400 MHz in MeOH-d_4.

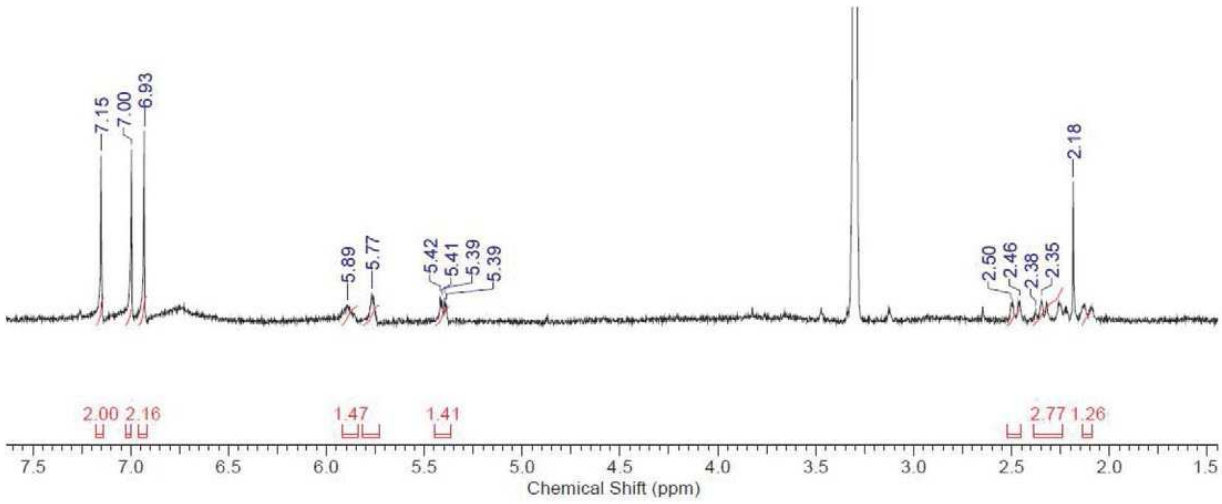

Figure S16. ^{1}H NMR spectrum of 3,4,5-tri-*O*-galloylquinic acid (**6**) acquired at 400 MHz in MeOH-d_4.

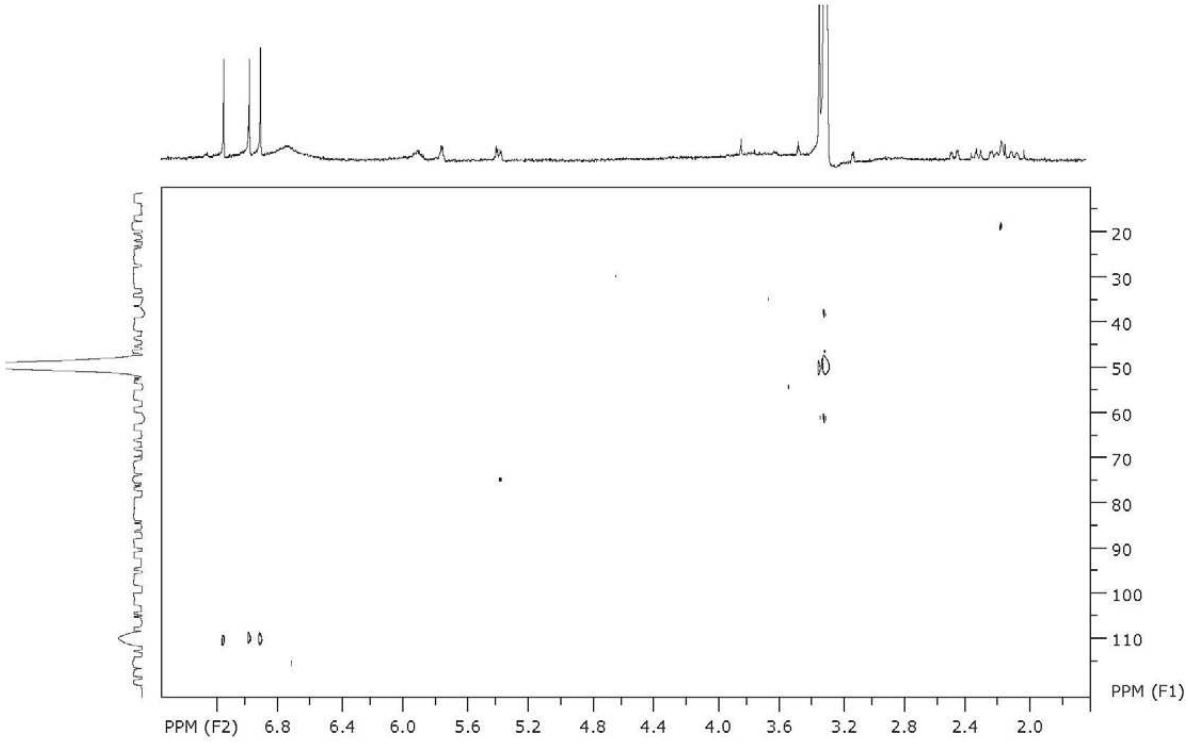

Figure S17. HSQC of 3,4,5-tri-*O*-galloylquinic acid (**6**) acquired at 400 MHz in MeOH-d_4.

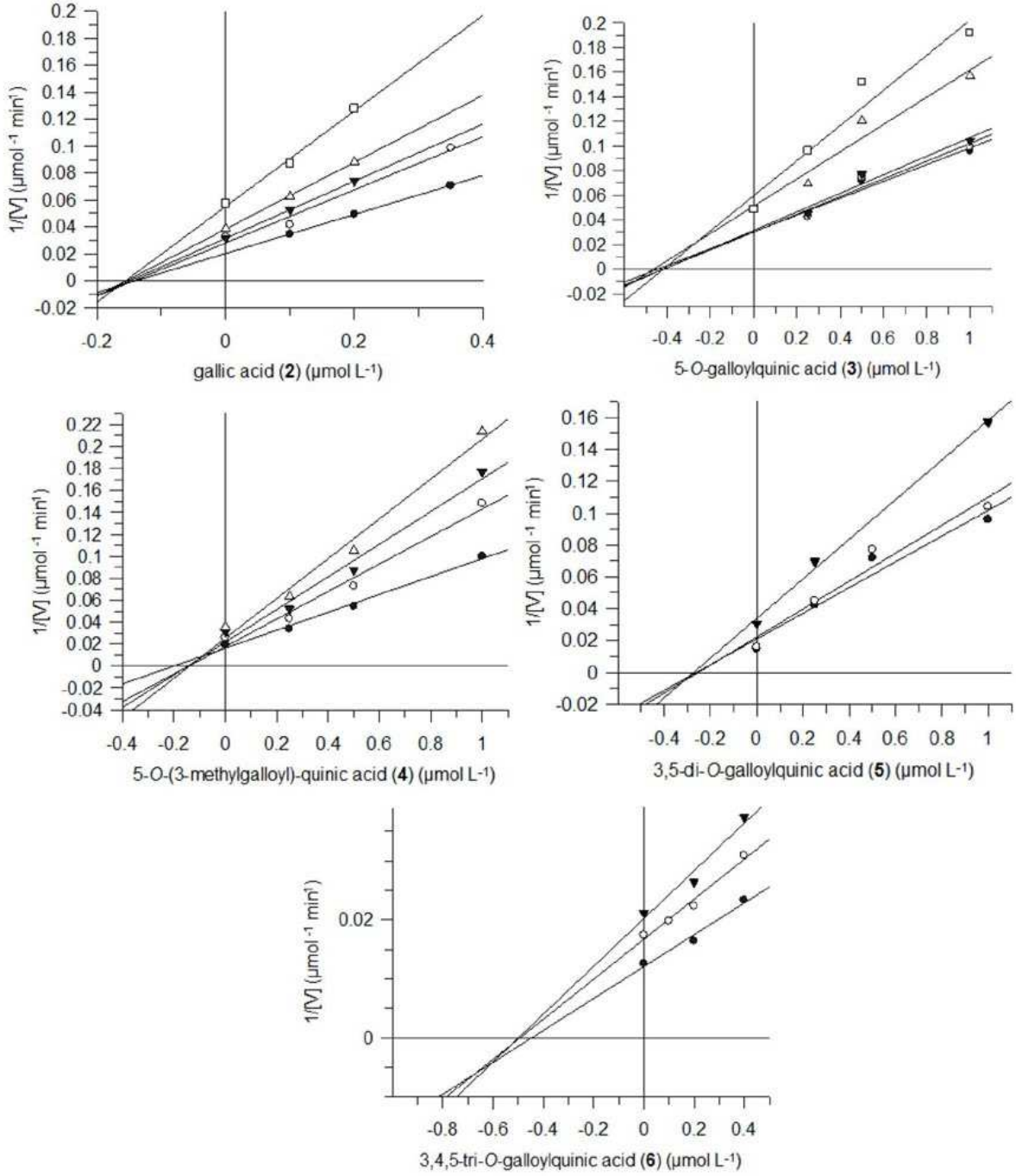

Figure S18. Dixon plots of gallic acid **2** (A), 5-*O*-galloylquinic acid **3** (B), 5-*O*-(3-methylgalloyl)-quinic acid **4** (C), 3,5-di-*O*-galloylquinic acid **5** (D), 3,4,5-tri-*O*-galloylquinic acid **6** (E).

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Flavonoids as noncompetitive inhibitors of Dengue virus NS2B-NS3 protease: Inhibition kinetics and docking studies

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ABSTRACT

NS2B-NS3 is a serine protease of the Dengue virus considered a key target in the search for new antiviral drugs. In this study flavonoids were found to be inhibitors of NS2B-NS3 proteases of the Dengue virus serotypes 2 and 3 with IC_{50} values ranging from 15 to 44 μM. Agathisflavone (**1**) and myricetin (**4**) turned out to be noncompetitive inhibitors of dengue virus serotype 2 NS2B-NS3 protease with K_i values of 11 and 4.7 μM, respectively. Docking studies propose a binding mode of the flavonoids in a specific allosteric binding site of the enzyme. Analysis of biomolecular interactions of quercetin (**5**) with NT647-NHS-labeled Dengue virus serotype 3 NS2B-NS3 protease by microscale thermophoresis experiments, yielded a dissociation constant K_D of 20 μM. Our results help to understand the mechanism of inhibition of the Dengue virus serine protease by flavonoids, which is essential for the development of improved inhibitors.

1. Introduction

Dengue fever is a disease caused by a virus, which is transmitted to humans by *Aedes aegypiti* and *Aedes albopictus* mosquitos.[1–3] Some of the obstacles in the discovery of specific drugs for the treatment of Dengue virus infections are the different serotypes of virus (DENV 1, 2, 3, 4 and the recently discovered 5),[4] and the difficulty in understanding the molecular pathways that

can lead to severe infections such as Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS), which have a high mortality.[5–9]

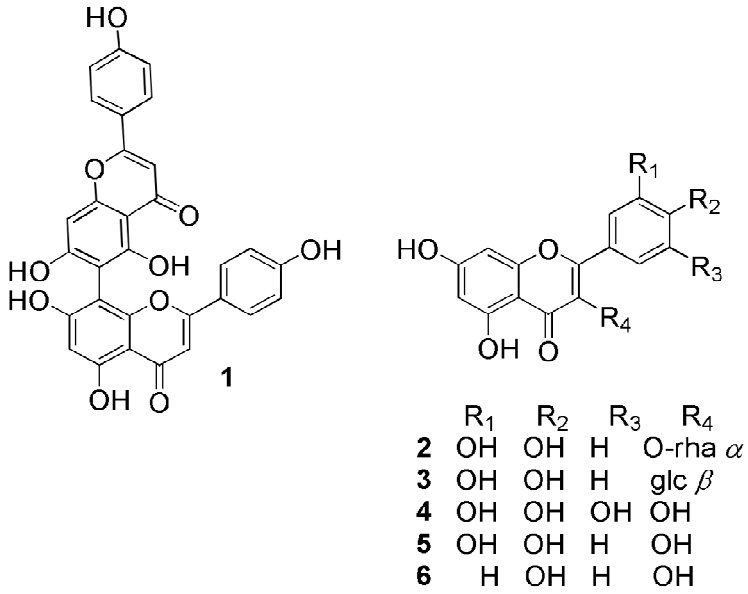
In the search for new drugs against this infection, the NS2B-NS3 protease of the dengue virus has been investigated as molecular target.[10–12] NS2B is a cofactor of the NS3 serine protease, which is crucial in the virus replication process.[12–16]

Natural products have been shown to be effective inhibitors of viral replication in vitro.[17–22] Among the phytochemicals related to antiviral activity some flavonoids have demonstrated significant anti-DENV2 inhibitory activity. Quercetin (**5**), baicalein and fisetin have IC_{50} values of 35.7 µg mL^{-1}, 6.46 µg mL^{-1}, and 55.0 µg mL^{-1} (118 µM, 23.9 µM and 192 µM calculated values), respectively, against DENV2, affecting the replication of virus inside cells.[20,23,24] A series of dialkylated flavanones and chartaceones isolated from *Crypotocarya chartacea* Kosterm., a plant from *Lauraceae* family, demonstrated inhibitory activity against DENV NS5 RNA-dependent RNA polymerase (IC_{50} values ranging from 1.8 to 4.2 µM).[25]

Additionally some secondary metabolites isolated from plants such as flavonoids, chalcone derivatives, and biflavonoids show inhibitory activity against the DENV2 NS2B-NS3 serine protease.[26,27] The 4-hydroxypanduratin A and panduratin A isolated from *Boesenbergia rotunda* (L.) Mansf. Kulturpfl., a species belonging to the *Zingiberaceae* family, were characterized as competitive inhibitors of DENV2 NS2B-NS3 (K_i = 21 and 25 µM, respectively), and pinostrobin and cardamonin showed to be noncompetitive inhibitors.[26] The allosteric pocket of DENV2 NS2B-NS3 protease, which is close to its catalytic triad, have been showed to be a promising drug target, albeit few noncompetitive inhibitors of dengue virus serine protease were discovered so far.[26,28,29]

In this communication we present results from screening assays of flavonols and a biflavone against NS2B-NS3 protease of DENV2 and DENV3.

Additionally, we determined the inhibition types of the most promising compounds (Fig. 1). Moreover, docking studies were performed, proposing a binding mode consistent with the inhibition mechanism, and the inhibition was investigated by a newly established substrate-free microscale thermophoresis (MST) assay.



	R_1	R_2	R_3	R_4
2	OH	OH	H	O-rha α
3	OH	OH	H	glc β
4	OH	OH	OH	OH
5	OH	OH	H	OH
6	H	OH	H	OH

Figure 1. Flavonoids that showed inhibitory activity against DENV2 and DENV3 NS2B-NS3 proteases.

2. Materials and methods

2.1. Materials

The proteases NS2B-NS3 of DENV2 and DENV3 were recombinantly expressed as described previously.[30] The fluorogenic peptide substrate Boc-Gly-Arg-7-amino-4-methylcoumarin was acquired from Bachem (Bubendorf, Switzerland). The Monolith NT^{TM} Protein Labeling Kit and the instrument NanoTemper Monolith NT.115 used for thermophoresis analysis were purchased from NanoTemper Technologies GmbH (Munich, Germany). In vitro DENV2 and DENV3 protease assay were performed using an Infinite

F200PRO fluorescent plate reader (TECAN, Männedorf, Switzerland). Agathisflavone (**1**),[31, 32] quercitrin (**2**),[33] and isoquercitrin (**3**),[34] were previously isolated and characterized by NMR (^{1}H and ^{13}C, DEPT-135, HSQC, and HMBC) and mass spectroscopy as described.[31–36] Myricetin (**4**) ≥ 96% (Sigma 70050), quercetin hydrate (**5**) ≥ 95% (Aldrich 337951), kaempferol ≥ 97% (**6**) (Sigma 60010) were acquired from Sigma Aldrich.

2.2. Computational docking

For molecular docking of compounds **1** and **4**–**6**, the recently solved crystal structure of DENV3 NS2B-NS3 in complex with the aldehyde inhibitor Bz-nKRR-H (pdb: 3U1I) was used.[37] Possible docking modes between ligands and the protease were studied using the FlexX docking approach of the LeadIT 2.1.6 suite (BioSolveIT, Germany).[38] Energies of the compound structures were minimized by using the MOE software (Molecular Operating Environment, 2012.10). All water and ligand molecules were deleted from the protease structure. The binding site was defined on a specific allosteric pocket, which is proximal to its catalytic triad.[39]

2.3. In vitro DENV2 and DENV3 protease assays

Screening of the compounds was performed using a final concentration of 50 µM (dissolved in DMSO). For compounds with inhibitory activities greater than 50% the IC_{50} values were determined. The enzymatic reaction solution (200 µL) contained a standard buffer solution with pH = 9.5 (200 mM Tris HCl, 6 mM NaCl, 30% glycerol), 47.63 nM DENV2 serine protease, and 100 µM of the substrate Boc-Gly-Arg-Arg-7-amino-4-methylcumarin. The protease assay against DENV3 NS2B-NS3 was carried out similarly but using another buffer solution containing 50 mM Tris HCl (pH = 9.0), 1 mM CHAPS, and 50 nM of DENV3 protease. Inhibition measurements were carried out in three independent determinations which were performed in

duplicate as described previously.[40–42] Hydrolysis of the substrate Boc-Gly-Arg-Arg-AMC was monitored at excitation and emission wavelengths of 380 and 460 nm, respectively. For IC_{50} determination the same procedure was used as described above with serial dilutions of the inhibitors in the range of 0.5−200 μM. Kinetic studies were performed for flavonoids **1**and **4**, where the incubation mixtures were prepared similarly by using different concentrations of substrate (25−200 μM) and fixed flavonoids concentrations (10−32 μM). The enzyme activity for increasing substrate concentrations were analysed by Lineweaver−Burk and Dixon plots. K_i values for compounds **1** and **4** were determined by using the secondary plots of slopes values from a double-reciprocal plot versus compound concentrations, and additionally by using Dixon plots of 1/velocity versus inhibitor concentration data. The K_i values for DENV2 and 3 proteases were also calculated by using the Dixon equation $v_o/v_i = 1 + ([I]/K_i^{app})$ and correction to zero substrate concentration with $K_i = K_i^{app} / (1 + [S]/K_m])$ with [S] = 100 μM and K_m = 143 μM for DENV2 NS2B-NS3 and K_m = 707 μM for DENV3 NS2B-NS3. The experimental data were analysed with the program GraFit® .[43]

2.4. Microscale thermophoresis

Protein NS2B-NS3 of Dengue virus serotype 3 was labeled with the NT-647-NHS fluorescent dye according to the supplied labeling protocol (Monolith NT™ Protein Labeling Kit). A series of dilutions of quercetin (**5**) were prepared using a buffer solution containing 50 mM Tris HCl (pH = 9.0), 1 mM CHAPS, 10% of DMSO, and 0.05% Tween 20. The enzyme dilution was prepared with the same buffer used for quercetin dilutions but without Tween 20 and DMSO. The solution of labeled NS2B-NS3 was mixed 1:1 with different concentrations of compound **5** yielding a final concentration of 25 nM of protein, and the final inhibitor concentrations in the range of 0.06 to 1000 μM. After 10 min the capillaries NT.115 (NanoTemper Technologies) were filled

with the enzyme reaction solution and the thermophoresis was measured at a LED power of 20% and an MST power of 40%. The K_D was determined by nonlinear fitting of the thermophoresis responses using the NTAnalysis software.

3. Results and discussion

The biflavonoid agathisflavone (**1**)[31,32,36] was isolated and identified previously in our laboratory. Quercitrin (**2**) and isoquercitrin (**3**) were isolated from the ethyl acetate extract from the leaves of *Byrsonima coccolobifolia* Kunth (Malpighiaceae) by chromatography techniques and characterized by spectrometric methods (1D and 2D NMR spectra and mass spectroscopy).[34] The obtained data were in agreement to those reported in literature.[31–36] The detailed methods of isolation and identification of these flavonoids were described previously.[34] Compounds **4-6** were purchased from Sigma.

The natural products (**1**–**6**) were evaluated for their ability to affect the DENV2 and DENV3 NS2B-NS3 proteases activity by fluorometric enzyme assays. The IC_{50} values of these flavonoids were determined and among all substances the biflavonoid (**1**) was the most active one with an IC_{50} of 15.1 ± 2.2 and 17.5 ± 1.4 µM against DENV2 and DENV3 protease, respectively. The flavonols (**2**–**6**) showed a moderate inhibitory activity on proteases of both serotypes. No considerable differences were found between the glycoflavonols compared to the aglycones (Table 1). The IC_{50} values for inhibition of DENV2 and DENV3 NS2B-NS3 (Fig. 2) by the compounds (**3**–**5**), which only differ in the number of hydroxy groups in the B ring moiety, were not affected either (Table 1). Kinetics studies were performed for compounds **1** and **4**, which were both found to be reversible inhibitors with a noncompetitive behavior for inhibition of DENV2 NS2B-NS3 protease as revealed by Lineweaver-Burk and Dixon plots (Fig. 3).

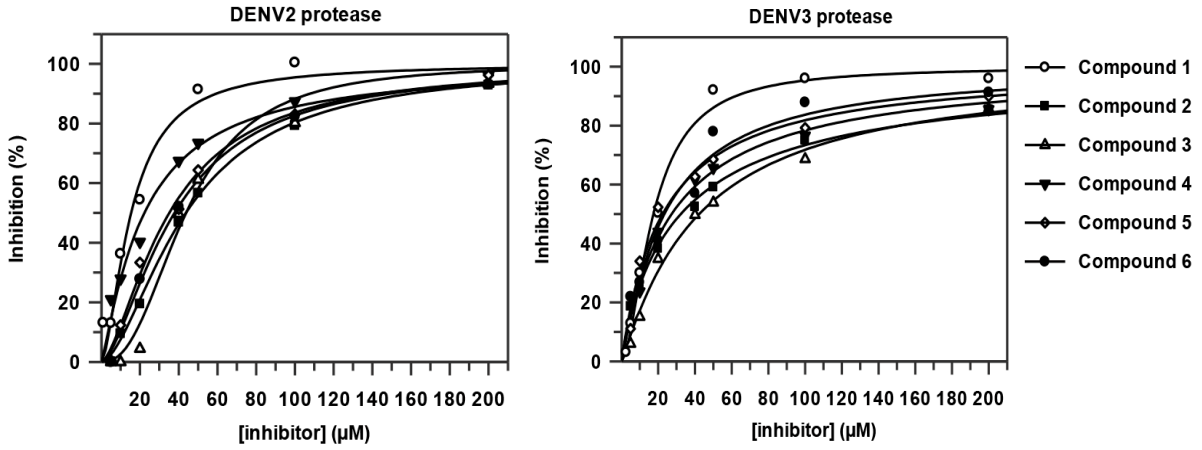


Figure 2. Inhibition of NS2B-NS3 proteases of DENV2 and DENV3 by compounds **1** to **6**. The $IC_{50's}$ were determined by fitting the inhibition data to a 2 parameter equation where the lower data limit is 0, the data are background corrected and the upper data limit is 100. The equation $y = 100\%/ 1 + (x/IC_{50})^s$ assumes that y falls with increasing x. Data are expressed as the mean of three independent assays. In addition the calculation with a 4-parameter equation did yield the same results.

Table 1

Inhibition of DENV2 and DENV3 proteases by compounds **1**–**6** (IC_{50} and affinity constants).[a]

	DENV2 NS2B-NS3		DENV3 NS2B-NS3	
Compd	IC_{50} (µM)	K_i[b] (µM)	IC_{50} (µM)	K_i[b] (µM)
1	15.1 ± 2.2	11.1 ± 0.7[c]	17.5 ± 1.4	15.3 ± 0.9
2	43.6 ± 1.7	25.7 ± 0.9	32.0 ± 1.4	28.0 ± 0.4
3	44.0 ± 2.8	25.9 ± 2.6	42.4 ± 3.4	37.1 ± 0.1
4	22.3 ± 1.8	4.7 ± 0.5[c]	29.3 ± 3.3	25.7 ± 1.1
5	35.2 ± 2.3	20.7 ± 2.4	22.7 ± 2.3	19.9 ± 0.5
6	37.8 ± 1.4	22.3 ± 0.7	27.7 ± 3.2	24.2 ± 0.8

[a]Data are expressed as the mean of three independent assays ± SD.

[b] K_i values were calculated using the Dixon equations $v_o/v_i=1 + ([I]/K_i^{app})$, $K_i = K_i^{app} / (1 + [S]/K_m)$, with the K_i^{app} values calculated using the GraFit 5.0 software. [41]

[c] K_i values are results from Dixon plots (1/velocity versus inhibitor concentration) (Fig. 3).

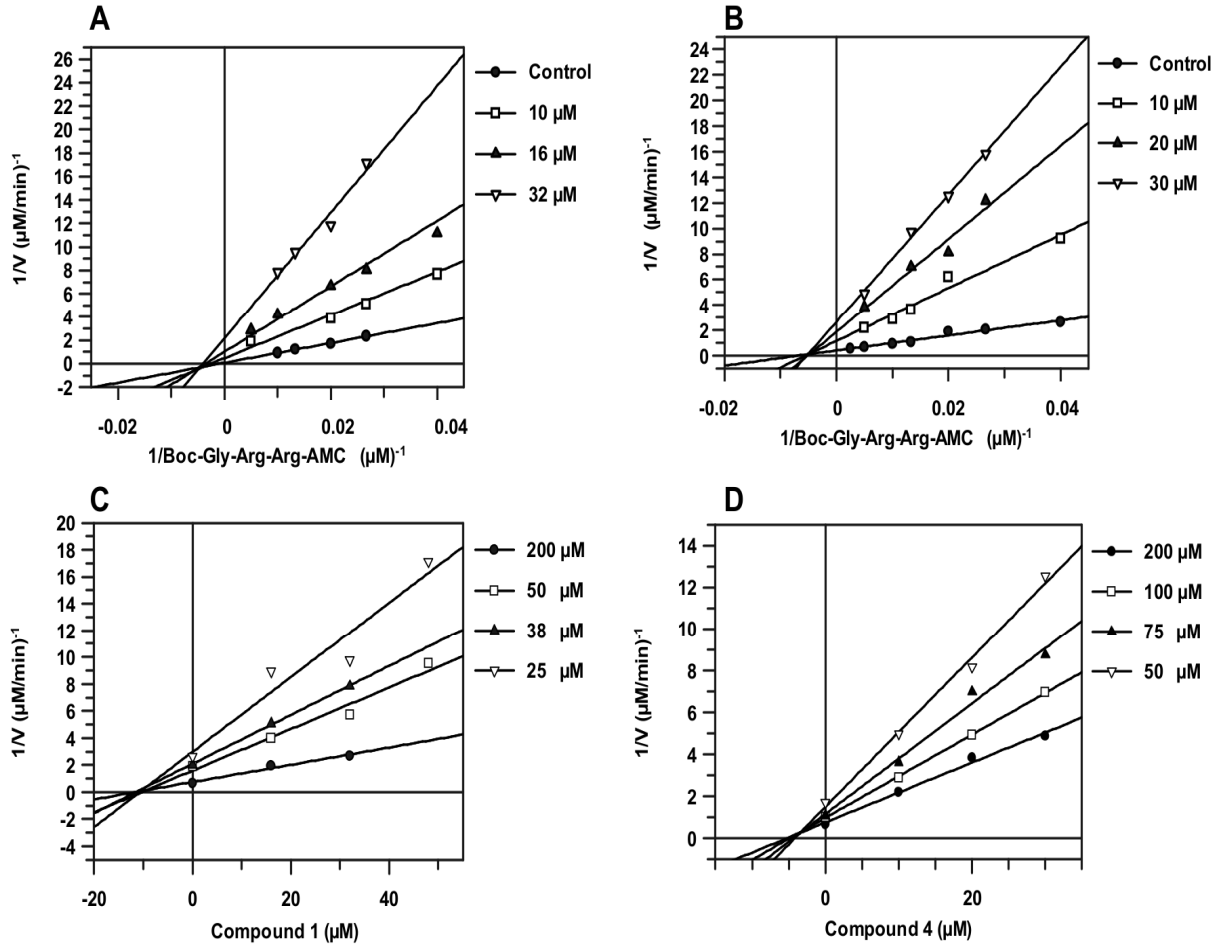


Figure 3. Noncompetitive inhibition of DENV2 NS2B-NS3 by compounds **1** and **4**. K_i values were determined using the secondary replot of Lineweaver–Burk plots (A–B) (slope values versus various compounds concentrations) and using Dixon plots (C–D); (A) and (C), compound **1** (K_i = 11.1 ± 0.7 µM); (B) and (D), compound **4** (K_i = 4.7 ± 0.5 µM). The conversion of fluorescence to concentration of AMC $(\mu M/min)^{-1}$ was made by a standard curve determined by the substrate total hydrolysis. Data are expressed as the mean of three independent assays.

To understand the binding interactions between the inhibitors and the NS2B-NS3 protease, molecular docking studies of compounds **1** and **4–6** were performed using LeadIT-FlexX (version 2.1.6)[38] with the recently solved crystal structure of DENV3 protease in complex with the aldehyde inhibitor Bz-nKRR-H (pdb: 3U1I),[37] which is the only available structure of a DENV protease in complex with a low-molecular weight inhibitor. The binding site was defined on a specific allosteric pocket, which is proximal to its catalytic triad

and was shown to be an allosteric binding site for other noncompetitive inhibitors.[39] All the tested inhibitors are predicted to bind similarly into the docking pocket.For compound **4**–**6**, three hydroxy groups of the “left” hand side of the molecule are proposed to form hydrogen bonds with Gln88, Gln167, and Gly124, respectively (Figure 4B). On the “right” hand side of the molecule, the *para*-hydroxy group of the phenyl ring forms hydrogen-bonding interactions with the side chain of Asn152 and with the backbone of Lys73 (Figure 4B). No interactions are observed between both *meta*-hydroxy groups and the binding pocket, which might explain the similar inhibitory activity observed with derivatives **4**–**6**. Additionally, hydrophobic interactions of compounds **4**–**6** with Lys74, Ile123, and Gln167 are proposed.

Microscale thermophoresis (MST), a new technology based on the directed movement of particles in a temperature gradient,[44–46] was used to verify the affinity in a substrate-free assay. The MST experiment was carried out using the NT647-NHS -labeled DENV3 NS2B-NS3 protease in constant concentration together with a series of dilutions of quercetin (**5**) (Fig. 5). After the temperature perturbation in the capillaries a different intensity of the fluorescence signals for each inhibitor dilution was recorded. Data fitting showed that compound **5** binds to DENV3 NS2B-NS3 with a dissociation constant K_D of 20.0 ± 1.6 µM.

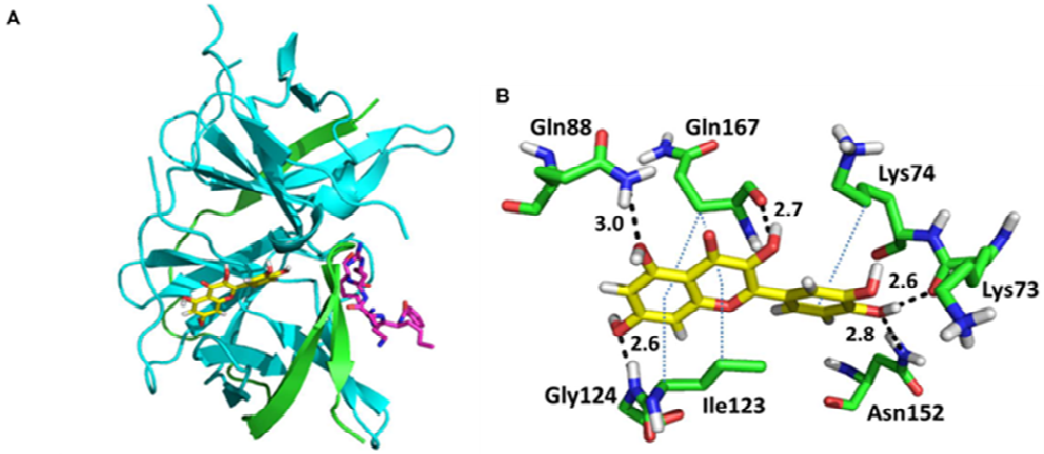


Figure 4. (A)View of quercertin **5** in stick representation (yellow) at the allosteric binding site of DENV3 NS2B-NS3 (cartoon, NS3 in cyan and NS2B in green), whereas the inhibitor Bz-nKRR-H (stick, purple) bound with DENV3 at the active site; (B) binding mode of compound **5** showing the H-bonds (black) between hydroxy groups and the amino acids Gln88, Gln167, Gly124, Lys73, Asn152, and hydrophobic interactions (blue) with Lys74, Ile123, and Gln167. The pictures were generated by PyMol [http://www.pymol.org/]

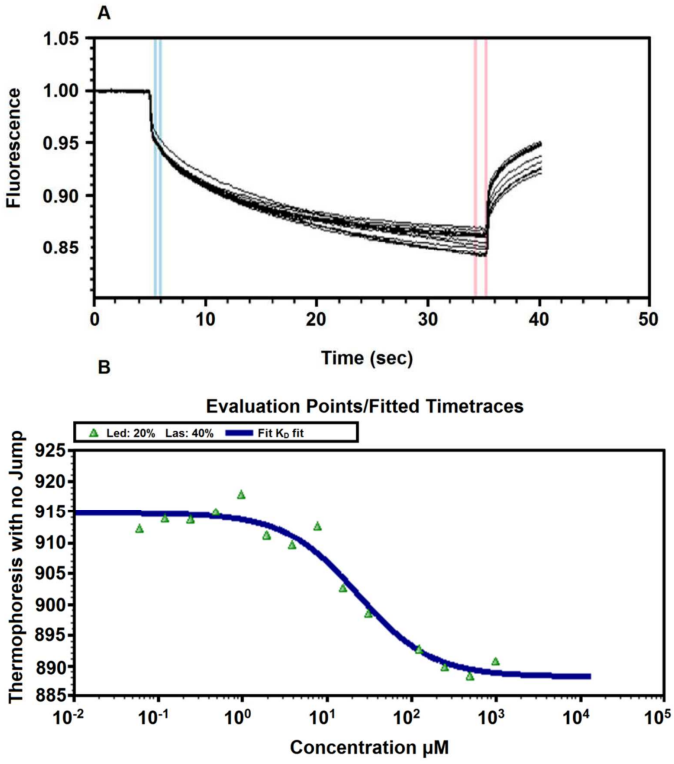


Figure 5. Microscale thermophoresis (MST) of quercetin (**5**). (A) MST signals for each capillary containing compound **5** in concentrations in the range of 0.06 to 1000 µM; (B) thermophoresis assay to determine the K_D of **5** with DENV3 NS2B-NS3. Data are expressed as the mean of three independent assays.

4. Conclusions

The screening led to bioactive flavonoids against DENV3 and DENV2 NS2B-NS3 proteases, which showed noncompetitive mode of inhibition. Although flavonoids have been deemed as compounds with activity against the Dengue virus, the mechanism is still unknown. Therefore, the inhibitory activity against DENV3 and DENV2 serine proteases by these compounds can be regarded as important features for the development of compounds with anti-Dengue activity. Additionally the allosteric interactions found for flavonoids experimentally and using in silico study can be further explored to develop highly selective inhibitors, making this class of compounds interesting for the design of DENV protein inhibitors.

Acknowledgments

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3.3 - PAPER 4 – not submitted

Natural products as inhibitors of recombinant cysteine protease L of *Leishmania mexicana*

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ABSTRACT

Cysteine proteases from *Leishmania* spp. are promising molecular targets against Leishmaniasis. *Leishmania mexicana* cathepsin L (CPB) is essential in the parasite life cycle and a pivotal in virulence factor in mammals. Natural products that have been shown to display antileishmanial activity were screened as part of our ongoing efforts to design inhibitors against the *L. mexicana* cathepsin L-like rCPB2.8. Among them, agathisflavone (**1**), tetrahydrorobustaflavone (**2**), 3-oxo-urs-12-en-28-oic acid (**3**), and quercetin (**4**) showed significant inhibitory activity on rCPB2.8 with IC_{50} values ranging from 0.4 to 18.0 µM. The mechanism of inhibition for compounds **1**−**3**, which showed K_i values in the low micromolar range (K_i = 0.14−1.3 µM) were determined. The biflavone **1** and the triterpene **3** are partially noncompetitive inhibitors, whereas biflavanone **2** is an uncompetitive inhibitor. The mechanism of action established for these leishmanicidal natural products provide a new outlook in the search for drugs against *Leishmania*.

Keywords

Cathepsin L; *Leishmania mexicana*; rCPB2.8; biflavonoid; triterpene.

1. Introduction

Leishmaniasis is a tropical disease caused by parasites of *Leishmania* spp. which is transmitted to mammalian host by the bite of sand flies from *Lutzomyia* genus (WHO, 2014). The different species of parasite can lead to cutaneous leishmaniasis (*Leishmania major*, *Leishmania tropica*, *Leishmania mexicana*, *Leishmania braziliensis*), mucocutaneous leishmaniaisis (*Leishmania braziliensis*) or visceral leishmaniasis (*Leishmania donovani*, *Leishmania infantum*/ *chagasi*) (Berman, 1997). This disease has been a global problematic that ask for new safe treatment, since the available chemotherapeutics have many issues related to toxicity, efficacy and administration (Santos et al., 2008; Gómez et al., 2014).

Leishmania infection is established when the parasite inside macrophage cells proliferate evading and manipulating the human immune defense mechanism (Wilson et al., 2005; Sharma and Singh, 2009). This suppression of the antileishmanial immune response in mammalian host can be directly related to the activity of cysteine proteases L (CPA and CPB) and B (CPC) from *Leishmania* spp. parasites (Selzer et al., 1999; Buxbaum et al., 2003; Onishi et al., 2004). Previously, studies demonstrated by genetic approach with deletion of cathepsins (CPs) genes in *L. mexicana*, that these enzymes represent a key determinant for virulence in *Leishmania* infection (Alexander et al., 1998; Denise et al., 2003; Montram et al., 2004).

The recombinant rCPB2.8 (cathepsin L-like) from *L. mexicana* is an protease without the C-terminal extension that have been used as target in the search for new leishmanicidal compounds (Alves et al., 2001; Desai et al., 2006; Steert et al. 2010; Gontijo et al., 2012; Judice et al., 2013; Schröder et al., 2013). The structure of this enzyme has the amino acid residues Asn^{60}, Asp^{61} and Asp^{64} in the α-helices (wall of the active site), and previous NMR experiments revealed that rCPB2.8 adopts a type of immunoglobulin-like fold (Juliano et al., 2004; Smith et al., 2006).

Natural products are an important source for antiparasitic drug discovery (Kayser et al., 2003; Newman and Gragg, 2007; Ndjonka et al., 2013). Particularly triterpenes and flavonoids isolated from plants have been revealed antiprotozoal potential (Suárez et al., 2003; Weniger et al., 2004; Mbwambo et al., 2006; Tasdemir et. al., 2006; Gontijo et al., 2012; Al Musayeib et al., 2013). Tingenin B (22β-hydroxytingenone), a triterpene isolated from *Elaeodendron schlechteranum* (Loes.) Loes. (Celastraceae), presented activity against *Trypanosoma cruzi* (IC_{50}<0.25 µg/mL), *Trypanosoma brucei* (<0.25 µg/mL), *L. infantum* (0.51µg/mL), and *Plasmodium falciparum* (0.36 µg/mL) (Maregesi et al., 2010). The biflavonoids 2,3-dihydrohinokiflavone and isoginkgetin showed strong leishmanicidal activity, with IC_{50} values of 1.6 and 1.9 µM, respectively (Kunert et al., 2008; Weniger et al., 2006).

In an effort toward the discovery of new inhibitors of cathespin L-Like rCPB2.8, this work report the results of a screening of natural products isolated from plants as well the inhibition type presented by the promising compounds. A triterpene with ursolic skeleton and two biflavonoids (**Fig. 1**) have showed significant inhibitory activity against rCPB2.8. The inhibition mechanism of these compounds showed that they were not competing directly for the active site.

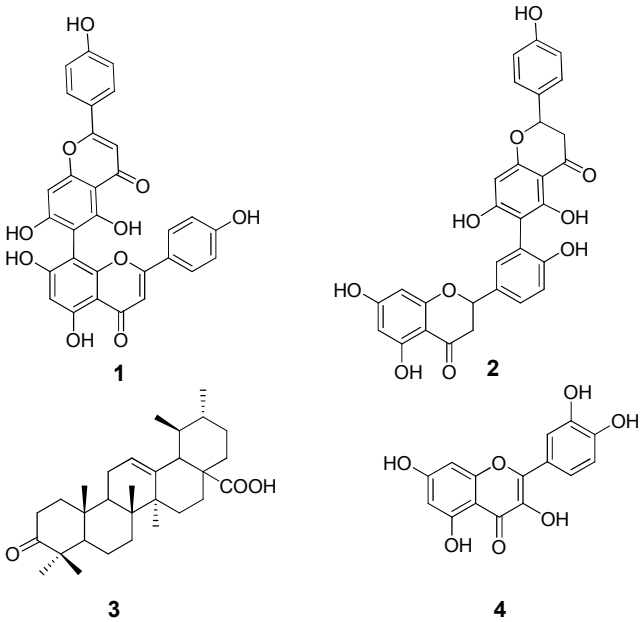

Fig. 1. Structures of the natural compound inhibitors of rCPB2.8 activity.

2. Materials and methods

2.1 Materials

The recombinant protease rCPB2.8 of *L. mexicana* was expressed and purified as described previously (Sanderson et al., 2000). The mammalian cathepsin L (human liver; EC 3.4.22.15) and B (human liver; EC 3.4.22.1), and the E-64 protease inhibitor were acquired from Calbiochem. The substrate, Z-Phe-Arg-7-amino-4-methylcoumarin hydrochloride was acquired from Bachem (Bubendorf, Switzerland). The fluorescence protease activity assay was performed using an Infinite F200PRO fluorescent plate reader spectrophotometer (TECAN, Männedorf, Switzerland). The natural compounds agathisflavone (**1**) (Alves et al., 2012; Mbing et al., 2006; Correia et al., 2006), tetrahydrorobustaflavone (**2**) (Kassem et al., 2004; Correia et al., 2006) and 3-oxo-urs-12-en-28-oic acid (**3**) (de Sousa et al., 2014a), were isolated in our laboratory and characterized by NMR (^{1}H and ^{13}C, DEPT-135, HSQC, and HMBC) and mass spectroscopy comparing to literature data. Quercetin hydrate (**4**) ≥ 97% was purchased from Sigma Aldrich (337951).

2.2 In vitro protease assay

The protease assays were performed by spectrofluorometric measurements in 96 well microtiter plates, all assays were performed in duplicate. The natural products samples dissolved in dimethyl sulfoxide (DMSO) were first screened with a final concentration of 20 µM. Hydrolysis of the fluorogenic peptide substrate Z-Phe-Arg-7-amino-4-methylcoumarin (Z-Phe-Arg-AMC) of human cathepsins L and B (cat L and B) were carried out in 50 mM Tris-HCl buffer (pH 6.5), containing 5 mM EDTA, 2 mM DTT, 200 mM NaCl, 0.005% Brij 35, 6.25 µM Z-Phe-Arg-AMC (cat L) and 100 µM Z-Phe-Arg-AMC (cat B) at 25 °C, as previously described (Viciket al., 2006; Ehmke et al., 2011).

The enzymatic reaction of *L. mexicana* rCPB2.8 was carried out in 50 mM phosphate- buffer (disodium phosphate and monopotassium phosphate), 5 mM DTT, 5 mM of EDTA, 10 µM Z-Phe-Arg-AMC, pH 6.5 at 25 °C (Alves et al., 2001). Thereafter, the proteolytic activity was monitored by the release of 7-amino-4-methylcoumarin (AMC), the fluorescence of which was measured at λ_{ex} = 380 nm and emission at λ_{em} = 460 nm over the period of 10 min. The inhibitory activity was calculated using the enzyme activity of the negative control (without inhibitor). The assay was also performed using a positive control, the irreversible inhibitor E-64 (Matsumoto et al., 1999). The serial dilutions were used for compounds **1** (0.025−10 µM), **2** and **3** (0.1−40 µM), and **4** (5−200 µM) to determine the IC_{50} values.

2.3 Kinetic studies

The type of inhibition was determined for biflavonoids **1**and **2**, and for the triterpene **3**. The experiment was performed similarly as described in *2.2* but keeping the inhibitor concentration constant with serial dilutions for the Z-Phe-Arg-AMC (0.375−3.75 µM). The inhibitor concentrations used were 0−1.0 µM for compound **1**; 0−5.0 µM for compound **2**; and 0−12.0 µM for compound **3**, to analyse the behavior when increasing the substrate concentration. The rates of the reaction were measured for each inhibitor concentration with serial dilutions of substrate.

2.4 Data analysis

The experimental data were processed in the*GraFit® 5.0.13* (Erithacus Software Ltd.: Horley, Surrey, UK, 2006). The analysis of kinetic experiments data was done using Lineweaver−Burk and the double reciprocal plot of 1/V versus 1/[substrate]. The Lineweaver−Burk plots represent a valuable tool for characterization of the inhibition type. The replots of slopes and intercepts auxiliary can distinguish among a simple linear inhibition and a

hyperbolic or partial inhibition. Jointly the graphical features can estimate the reversible types of inhibition. The equation of Lineweaver-Burk used for linear inhibition of the compound **2**, an uncompetitive inhibitor can be written as (1):

$$v = \frac{V_{max}[S]}{[S]\left(1 + \frac{[I]}{K_i}\right) + K_m} \qquad (1)$$

In the simple linear inhibition, $1/V_{max}^{app}$ (intercept) and K_m^{app}/V_{max}^{app} (slope) functions display a simple linear dependence on the inhibitor concentration. The uncompetitive inhibitor constant K_i is obtained from the secondary plot of the reciprocal ordinate intercept (*Or*) against the inhibitor concentrations ($Or = 1/V + [I]/K_iV$) derived from the equation above, which yields the K_i value (Dixon, 1953; Copeland, 2005; Bisswanger, 2008). However, in the hyperbolic inhibition the replots of slopes and intercepts will display a nonlinear dependence on the inhibitor concentrations, therefore, this type of inhibition is usually called a hyperbolic or partial inhibition. Another kinetic representation used to distinguish between complete or partial inhibition, it is by using graphical plot of the fractional velocity $[v/(V_0 - v)]$ versus reciprocal of inhibitor concentration 1/[Inhibitor], where a straight line converging at the origin characterize a complete noncompetitive inhibition, and when converging on the abscissa but away out of the origin a partial noncompetitive inhibition type (Whiteley, 1999).

In the case of compounds **1** and **3**, where the shape of the plots showed to be of hyperbolic noncompetitive inhibition type, the equation can be written as shown in (2). The α factor value shows how the inhibitor binding affects the affinity of the enzyme for the substrate. The magnitude of the β factor is related to the activity of the ESI and ES complex. When $\alpha = 1$ and $\beta = 0$, it is pure noncompetitive inhibition and when $\alpha = 1$ and $0 < \beta < 1$, it is partial noncompetitive inhibition (Leskovac, 2003; Bisswanger, 2008).

$$v = \frac{V_{max}[S]}{[S]\left(\frac{1 + \frac{[I]}{\alpha K_i}}{1 + \frac{\beta[I]}{\alpha K_i}}\right) + K_m\left(\frac{1 + \frac{[I]}{K_i}}{1 + \frac{\beta[I]}{\alpha K_i}}\right)} \qquad (2)$$

The K_i values for compounds **1**−**4** were determined using the Dixon equation $v_o/v_i = 1 + ([I]/K_i^{app})$ and correction to zero substrate concentration with $K_i = K_i^{app} / (1 + [S]/K_m])$ (Cer et al., 2009), with [S] = 10 µM and K_m = 5.0 µM for *L. mexicana* rCPB2.8 (Alves et al., 2001).

3. Results

The recombinant protease rCPB2.8 of *L. mexicana* and the mammalian cathepsin L and B were characterized previously and exhibited K_m values of 5.0 µM, 6.5 µM and 150 µM, respectively (Sanderson et al., 2000; Alves et al., 2001; Vicik et al., 2006; Ehmke et al., 2011).

The compounds were subjected to protease inhibition assays against mammalian proteases cathepsin L and B, and *L. mexicana* cathepsin-L like rCPB2.8 protease. The analysis of compounds in a concentration of 20 µM revealed ≥ 50% of inhibition on rCPB2.8 for the compounds **1**−**4** as shown in **Fig. 2**. The compounds inhibited the tested cathepsin L-like enzymes (rCPB2.8 and human cathepsin L) with slight selectively, except for compound **4** (**Fig. 2**). Comparing human cathepsin L and B inhibition by these compounds, there was no selectivity presented, only compound **3** showed some preference by inhibition of rCPB2.8 face up to human cathepsin L. The IC_{50} and K_i values for compounds **1**−**4** are summarized in **Table 1**. Compounds **1**−**3** were investigated further and the kinetics study showed that agathisflavone (**1**) and 3-oxo-urs-12-en-28-oic acid (**3**) are partially noncompetitive inhibitors, whereas tetrahydrorobustaflavone (**2**) is an uncompetitive inhibitor.

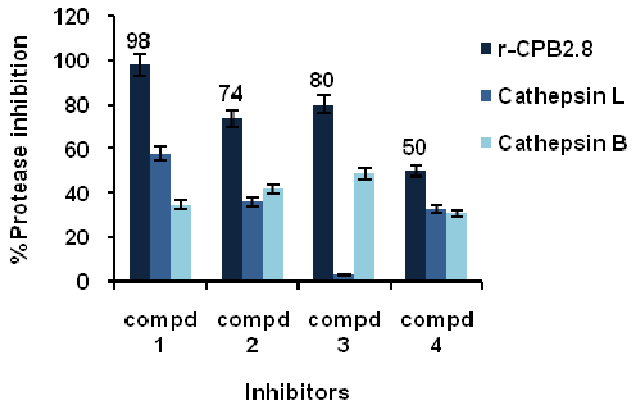

Fig. 2. Inhibitory activity by compounds **1**−**4** (concentration of 20 μM) against cathepsin L-like enzymes and cathepsin B.

Table 1

Inhibition of cathepsin L-like rCPB2.8 protease from *L. mexicana* by compounds **1**–**4**

Compd	IC_{50} (μM)	K_i[a] (μM)	Type of inhibition
1	0.43 ± 0.04	0.14 ± 0.04	partially noncompetitive
2	2.2 ± 0.2	0.7 ± 0.1	uncompetitive
3	3.8 ± 0.4	1.3 ± 0.1	partially noncompetitive
4	18.0 ± 1.9	6.0 ± 0.7	nd

[a] K_i values were calculated using the Dixon equations $v_o/v_i=1 + ([I]/K_i^{app})$, $K_i = K_i^{app} / (1 + [S]/K_m)$, with the K_i^{app} values calculated using the GraFit 5.0 Software (*GraFit® 5.0.*13, 2006); nd not determined. Data presented are average values from at least two independent assays.

4. Discussion

Cysteine proteases L and B from *Leishmania* spp. are crucial in the parasite life cycle, becoming important drug targets. Inhibitors of cysteine proteases from *Leishmania* with selectivity to the homologous host enzymes would be ideal drug candidates (Montram et al., 2004; Onishi et al., 2004). However, cathepsins have overlapping substrate specificity, which makes the discovery of specific inhibitors using conventional strategies a tough task (Drag and Salvesen, 2010).

The usage of plants as natural sources of novel antiprotozoal compounds, have contributed to develop new antiparasitic drugs (Kayser et al., 2003; Ndjonka et al., 2013). Natural products that have been displayed antiprotozoal activity were screened in this work, regarding hits into discovery of new specific inhibitors of CPB from *Leishmania*.

Among the compounds tested quercetin presented moderate inhibition on rCPB2.8 (IC_{50} = 18.0 ± 1.9 μM), and it previously has shown antileishmanial activity *in vitro* and *in vivo* (Muzitano et al., 2006; Muzitano et al., 2009), with mechanism related to the production of reactive oxygen species (Fonseca-Silva et al., 2013). Interestingly, this flavononol has demonstrated inhibitory activity on other enzymes from *Leishmania* spp., such as arginase (da Silva et al., 2012; de Sousa et al., 2014b), ribonucleotide reductase (Sen et al., 2008) and topoisomerase II (Mittra et al., 2000), suggesting that the leishmanicidal action by quercetin is an outcome of the interference in diverse metabolic pathways.

The triterpene 3-oxo-urs-12-en-28-oic acid (**3**) showed an IC_{50} of 3.8 ± 0.4 μM against rCPB2.8. Previously, the ursolic acid, similar to compound **3** except for a hydroxy group in position 3, was found to display antileishmanial activity with an IC_{50} value of 3.7 μg/mL (IC_{50} = 8.1 μM) (da Silva Filho et al., 2009).

The primary double reciprocal plot of inhibitor **3** (1/V versus 1/S) ($0 < \alpha < 1$, $\beta > \alpha$, with $0 < \beta < 1$) together with the secondary replots of Lineweaver–Burk, and the graphical plot of the fractional velocity showed a hyperbolic behavior (partial inhibition) (**Fig. 3**−C, **Fig. 4** and **Fig. 5**). In this case, at a fixed inhibitor concentration, compound **3** acts as an inhibitor if is above a certain fixed concentration of substrate. Unlike linear inhibition, which represents a complete enzyme inhibition, the partial inhibition is characterized by still active ESI complex.

The rCPB2.8 assays of biflavone agathisflavone (**1**) and the tetrahydrorobustaflavone (**2**) showed significant inhibitory activity with IC_{50} of 0.43 ± 0.04 and 2.2 ± 0.2 µM, respectively. Previously, some leishmanicidal biflavones such as morelloflavone, morelloflavone-7,4′,7″,3‴,4‴-penta-*O*-acetyl, morelloflavone-7,4′,7″,3‴,4‴-penta-*O*-methyl and morelloflavone-7,4′,7″,3‴,4‴-penta-*O*-butanoyl, presented also strong inhibitory activity of rCPB2.8 from *L. mexicana* (IC_{50} values of 0.42, 0.67, 1.26 and 1.01 µM, respectively) (Gontijo et al., 2012).

The kinetic data of agathisflavone (**1**) are characteristic of partial inhibition, likewise the Dixon plot and the secondary plots (slopes and intercept versus inhibitor as variable) are curved (**Fig. 3**−A and **Fig. 4**). In addition the graphical plot of the fractional velocity generated a straight line that intercepts the abscissa at a point away from the origin (**Fig. 5**). Despite the inhibition type of compound **1** to be similar to **3** (hyperbolic noncompetitive inhibitors), in the case of **1** the intercepts and slopes increase with increasing inhibitor concentration ($0 < \alpha < 1$, $\alpha > \beta$, which $0 < \beta < 1$).

Tetrahydrorobustaflavone is a linear inhibitor (**Fig. 3**−B) leading to an inactive enzyme-substrate complex. The Lineweaver–Burk diagram for compound **2** revealed graphically parallel lines, a form of uncompetitive inhibitor, which binds exclusively to the ES complex with little or no affinity for the free enzyme.

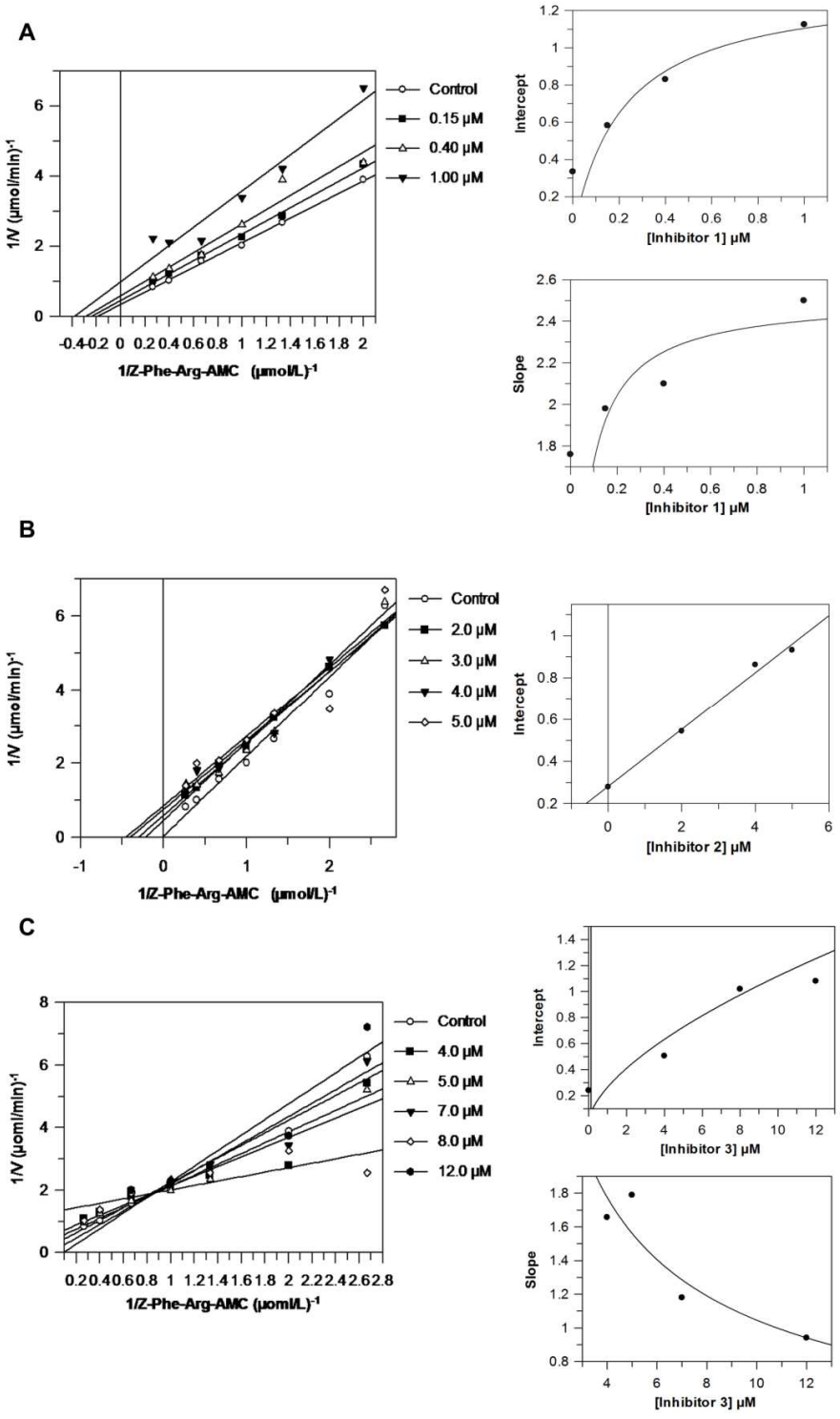

Fig. 3. (A) Lineweaver–Burk plot for inhibitor **1** and the secondary replots of the primary reciprocal plots of (A) (slopes and ordinate intercepts set against inhibition concentrations), showing hyperbolic noncompetitive inhibition; (B) Lineweaver–Burk plot for **2** and the secondary replot of the primary reciprocal plots of (B), the intercept displays a simple linear dependence on the inhibitor concentration, which is a characteristic of linear inhibition with uncompetitive behavior; (C) Lineweaver–Burk plot for inhibitor **3** and the secondary replots of the primary reciprocal plots of (C), showing a hyperbolic noncompetitive inhibition type.

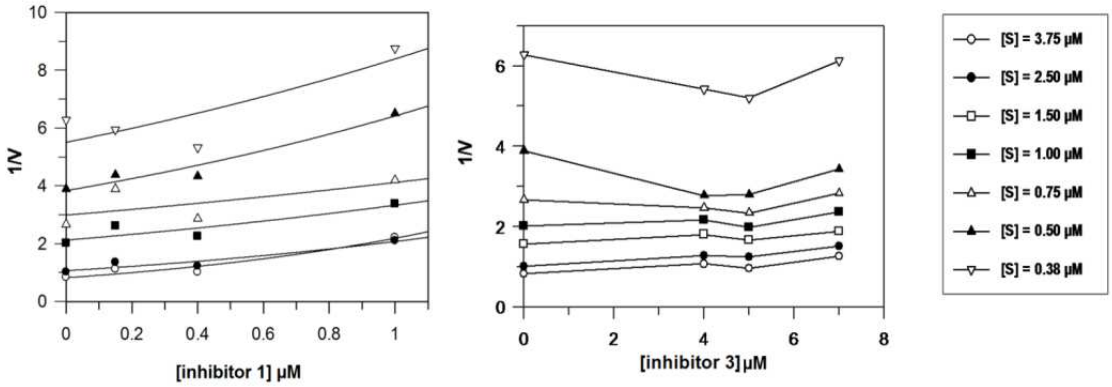

Figure 4. Dixon plots of agathisflavone (**1**) and 3-oxo-urs-12-en-28-oic acid (**3**) do not yield straight lines (partial inhibitors).

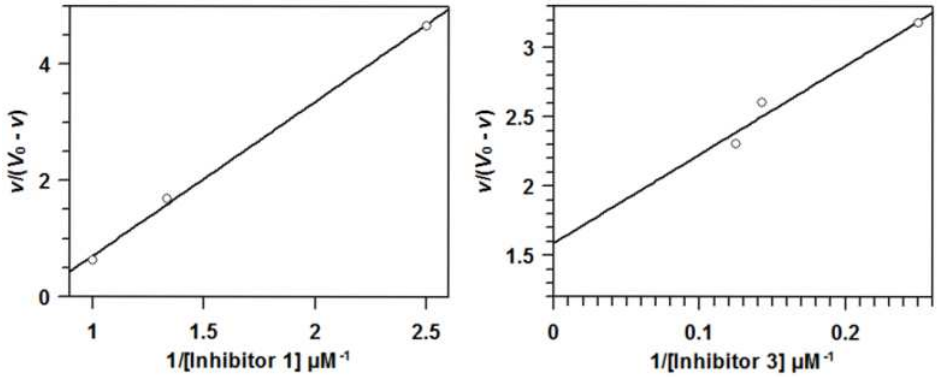

Figure 5. Graphical plots of the fractional velocity versus 1/[inhibitor] of agathisflavone (**1**) and 3-oxo-urs-12-en-28-oic acid (**3**) yield straight lines that intercept the abscissa at a point away from the origin showing partial inhibitors type.

5. Conclusions

The mechanism of inhibition presented by compounds **1**−**3** can be further explored as a newsworthy approach to develop highly selective inhibitiors. Thereafter, the interest in allosteric and exosite interactions have emerged in this area recently, since some inhibitors that operate by allosteric mechanism were successful (Drag and Salvesen, 2010). Furthermore, flavonoid and triterpene secondary metabolites can be used to design specific and tight inhibitors of *L. mexicana* cathepsin L-like rCPB2.8, in order to discover an inhibitor to deliver a *Leishmania* therapeutic outcome.

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4 – GENERAL CONCLUSIONS

Several compounds were isolated in the bioactivity-guided study from *B. coccolobifolia* extracts using arginase, and most of the secondary metabolites derived from the active fractions induced an evidential decrease of ARG activity. Accordingly, flavonoids and galloylquinic acids were potent noncompetitive inhibitors of ARG representing the observant compounds from the active extract. The flavonoids are known compounds, but the galloylquinic acids were new in the phytochemistry of *B. coccolobifolia*, and the syringaresinol (**3**) and trigonostemone (**4**) were reported for the first time in *Byrsonima* genus, showing the relevance of the results in chemistry terms.

The screening of natural products against several proteases associated to Neglected Tropical Diseases (dengue fever, African trypanosomiasis, chagas, leishmaniasis and malaria), led to the discovery of new noncompetitive inhibitors of dengue NS2B-NS3 serine proteases and partially noncompetitive and uncompetitive inhibitors of the *L. mexicana* cathepsin L-like r-CPB2.8.

The results described in this thesis were summarized in papers published in the journals *Journal of Natural Products*, *Journal of Brazilian Chemical Society*, *Bioorganic & Medicinal Chemistry*, and the fourth manuscript (Paper 4) still must be submitted for publication.

Our results contributed with the research on this area, once the search brought new inhibitors of proteases associated to NTDs. Furthermore, the new prototypes inhibitors of cathepsin L like-rCPB2.8, ARG and NS2B-NS3 proteases can be further optimized to improve the inhibitors features in a future prospect for leishmanicidal and antiviral drugs design.

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