

Leishmanicidal Galloylquinic Acids are Noncompetitive Inhibitors of Arginase

Lorena R. F. de Sousa,^a Suelem D. Ramalho,^a João B. Fernandes,^a
Maria Fátima das G. F. da Silva,^a Mônica R. da C. Iemma,^a Caroindes J. Corrêa,^a
Dulce H. F. de Souza,^a Maria I. S. Lima^b and Paulo C. Vieira^{*a}

^aDepartamento de Química and ^bDepartamento de Botânica, Universidade Federal de São Carlos,
Rod. Washington Luís, km 235, 13565-905 São Carlos-SP, Brazil

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 $\mu\text{mol L}^{-1}$). 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 $\mu\text{mol L}^{-1}$) 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}

*e-mail: dpcv@power.ufscar.br

N^{ω} -hydroxy-L-arginine and 2(S)-amino-6-boronoheptanoic acid are synthetic aminoacid derivatives, which inhibit in a competitive mode ARG from *L. mexicana* with K_i values of 85 and $1.3 \mu\text{mol L}^{-1}$ 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 ($\text{EC}_{50} = 2\text{-}38 \mu\text{mol L}^{-1}$), 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 ($\text{EC}_{50} = 4.4 \mu\text{g mL}^{-1}$) 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 $17.07 \mu\text{g mL}^{-1}$.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.

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 (^1H : 400 MHz; ^{13}C : 100 MHz) using D_2O and $\text{MeOH-}d_4$ 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.

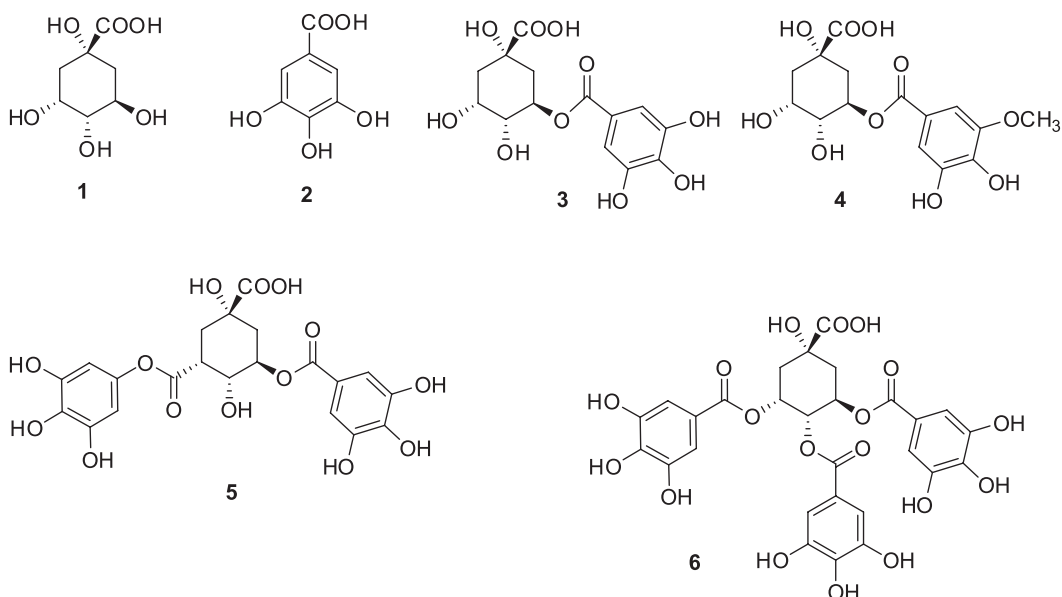


Figure 1. Compounds analysed against recombinant ARG from *L. amazonensis*.

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 ¹H and ¹³C 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 IC₅₀ 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 below.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 ($K_i = 7.2 \pm 1.4 \mu\text{mol L}^{-1}$) and epigallocatechin-3-gallate (EGCG) ($K_i = 4.0 \pm 0.5 \mu\text{mol L}^{-1}$) 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	$IC_{50} / (\mu\text{mol L}^{-1})^b$	$K_{is} / (\mu\text{mol L}^{-1})^b$	$K_{ii} / (\mu\text{mol L}^{-1})^b$	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

^aQuercitrin is a noncompetitive inhibitor used as a positive control (K_{ii} and K_{is} obtained from the reference 18); ^bdata values represent means of individual experiments \pm SD.

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

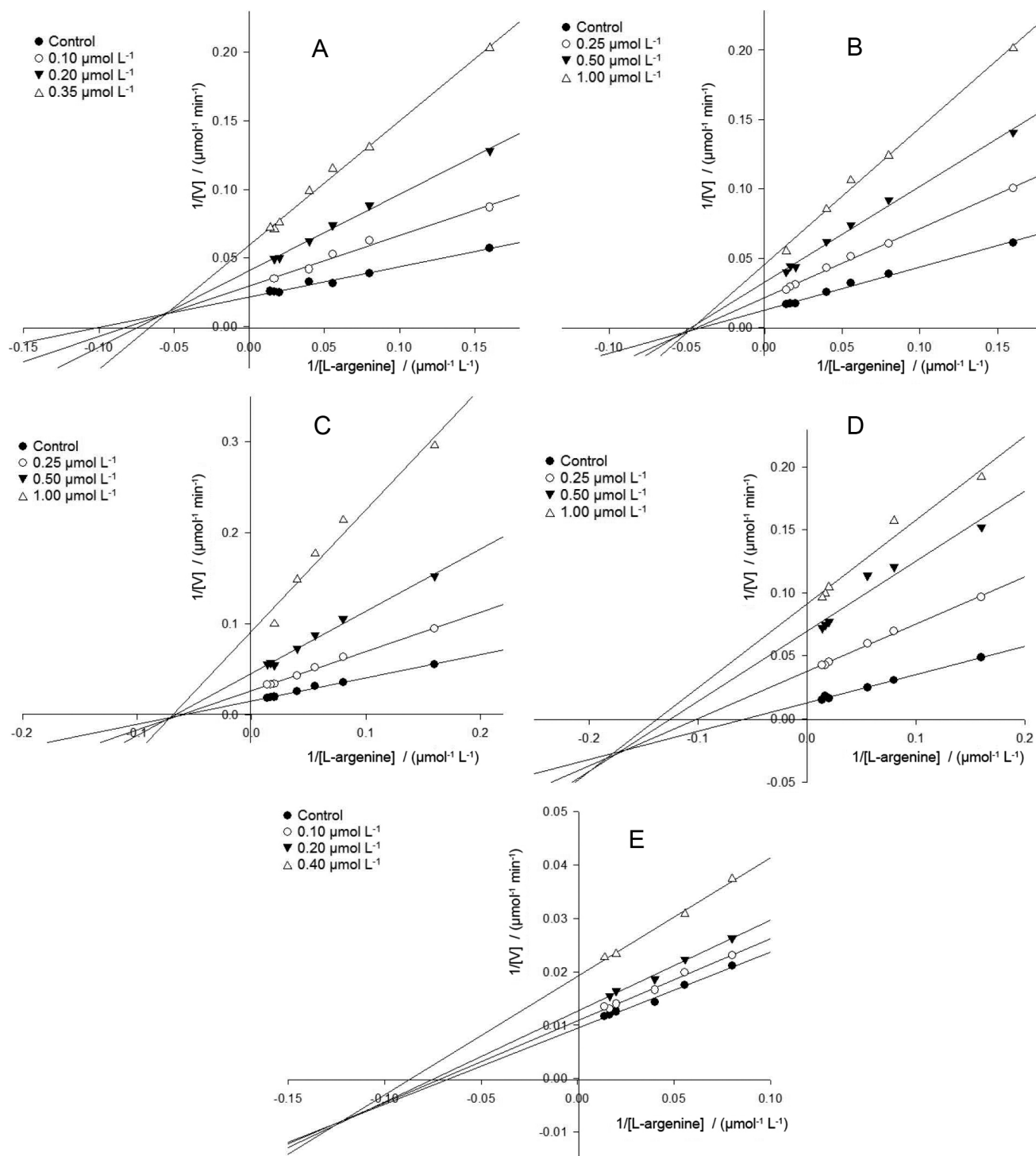


Figure 2. Lineweaver-Burk plots of gallic acid **2** (A), 5-*O*-galloylquinic acid **3** (B), 5-*O*-(3-methylgalloyl)-quinic acid **4** (C), 3,5-di-*O*-galloylquinic acid **5** (D), 3,4,5-tri-*O*-galloylquinic acid **6** (E).

$\mu\text{mol L}^{-1}$ 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.

Supplementary Information

Supplementary data (^1H , ^{13}C NMR and Dixon plots of acids) are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

Acknowledgments

This research was supported by 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.

References

- Chawla, B.; Madhubala, R.; *J. Parasit. Dis.* **2010**, *34*, 1.
- <http://www.who.int/leishmaniasis/en/>, accessed in May 2014.
- Amato, V. S.; Padilha, A. R. S.; Nicodemo, A. C.; Duarte, M. I. S.; Valentini, M.; Uip, D. E.; Boulos, M.; Amato Neto, V.; *Int. J. Infect. Dis.* **2000**, *4*, 153.
- Gil, E. S.; Paula, J. R.; Nascimento, F. R. F.; Bezerra, J. C. B.; *J. Basic Appl. Pharm. Sci.* **2008**, *29*, 223.
- López, R. E. S. *Quim. Nova* **2010**, *33*, 1541.
- da Silva, M. F. L.; Zampieri, R. A.; Muxel, S. M.; Beverley, S. M.; Floeter-Winter, L. M.; *PLoS One* **2012**, *7*, e34022.
- Iniesta, V.; Gómez-Nieto, L. C.; Corraliza, I.; *J. Exp. Med.* **2001**, *193*, 777.
- Roberts, S. C.; Tancer, M. J.; Polinsky, M. R.; Gibson, K. M.; Heby, O.; Ullman, B.; *J. Biol. Chem.* **2004**, *279*, 23668.
- D'Antonio, E. L.; Ullman, B.; Roberts, S. C.; Dixit, U. G.; Wilson, M. E.; Hai, Y.; Christianson, D. W.; *Arch. Biochem. Biophys.* **2013**, *535*, 163.
- da Silva, E. R.; da Silva, M. F.; Fischer, H.; Mortara, R. A.; Mayer, M. G.; Framesqui, K.; Silber, A. M.; Floeter-Winter, L. M.; *Mol. Biochem. Parasitol.* **2008**, *159*, 104.
- Balaña-Fouce, R.; Calvo-Álvarez, E.; Álvarez-Velilla, R.; Prada, C. F.; Pérez-Pertejo, Y.; Reguera, R. M.; *Mol. Biochem. Parasitol.* **2012**, *181*, 85.
- Colotti, G.; Ilari, A.; *Amino Acids* **2011**, *40*, 269.
- Kropf, P.; Herath, S.; Weber, V.; Modolell, M.; Müller, I.; *Parasite Immunol.* **2003**, *25*, 439.
- Kropf, P.; Fuentes, J. M.; Fähnrich, E.; Arpa, L.; Herath, S.; Weber, V.; Soler, G.; Celada, A.; Modolell, M.; Müller, I.; *FASEB J. FJ Express* **2005**, *19*, 1000.
- Riley, E.; Roberts, S. C.; Ullman, B.; *J. Parasitol.* **2011**, *41*, 545.
- da Silva, E. R.; Maquiaveli, C. C.; Magalhães, P. P.; *Exp. Parasitol.* **2012**, *130*, 183.
- Cruz, E. de M.; da Silva, E. R.; Maquiaveli, C. C.; Alves, E. S.; Lucon Jr., J. F.; dos Reis, M. B.; de Toledo, C. E.; Cruz, F. G.; Vannier-Santos, M. A.; *Phytochemistry* **2013**, *89*, 71.
- Manjolin, L. C.; dos Reis, M. B. G.; Maquiaveli, C. C.; Santos-Filho, O. A.; da Silva, E. R.; *Food Chem.* **2013**, *141*, 2253.
- de Sousa, L. R. F.; Ramalho, S. D.; Burger, M. C. M.; Nebo, L.; Fernandes, J. B.; da Silva, M. F. G. F.; Iemma, M. R. C.; Corrêa, C. J.; de Souza, D. H. F.; Lima, M. I. S.; Vieira, P. C.; *J. Nat. Prod.* **2014**, *77*, 392.
- Dewick, P. M.; *Medicinal Natural Products: a Biosynthetic Approach*, 2nd ed.; John Wiley & Sons: England, 2001.
- Rawat, G.; Tripathi, P. R. K.; *Appl. Microbiol. Biotechnol.* **2013**, *97*, 4277.
- Nepka, C.; Asproдини, E.; Kouretas, D.; *Eur. J. Drug Metab. Pharmacokinet.* **1999**, *24*, 183.
- Crozier, A.; Jaganath, I. B.; Clifford, M. N.; *Nat. Prod. Rep.* **2009**, *26*, 1001.
- Fan, H.; Wu, D.; Tian, W.; Ma, X.; *Biochim. Biophys. Acta* **2013**, *1831*, 1260.
- Kolodziej, H.; Kiderlen, A. F.; *Phytochemistry* **2005**, *66*, 2056.
- Kayser, O.; Kolodziej, H.; Kiderlen, A. F.; *Phytother. Res.* **2001**, *15*, 122.
- Accioly, M. P.; Bevilaqua, C. M. L.; Rondon, F. C. M.; de Moraes, S. M.; Machado, L. K. A.; Almeida, C. A.; de Andrade Jr., H. F.; Cardoso, R. P. A.; *Vet. Parasitol.* **2012**, *187*, 79.
- Clardy, J.; Walsh, C.; *Nature* **2004**, *432*, 829.
- Guilhon-Simplicio, F.; Pereira, M. M.; *Quim. Nova* **2011**, *34*, 1032.
- Sannomiya, M.; Fonseca, V. B.; da Silva, M. A.; Rocha, L. R. M.; dos Santos, L. C.; Hiruma-Lima, C. A.; Souza Brito, A. R. M.; Vilegas, W.; *J. Ethnopharmacol.* **2005**, *97*, 1.
- de Almeida, S. C. X.; de Lemos, T. L. G.; Silveira, E. R.; Pessoa, O. D. L.; *Quim. Nova* **2005**, *28*, 57.
- Pawlowska, A. M.; De Leo, M.; Braca, A.; *J. Agric. Food Chem.* **2006**, *54*, 10234.
- Itoh, A.; Tanaka, Y.; Nagakura, N.; Nishi, T.; Tanahashi, T.; *J. Nat. Med.* **2006**, *60*, 146.
- Bouchet, N.; Levesque, J.; Blond, A.; Bodo, B.; Pousset, J. L.; *Phytochemistry* **1996**, *42*, 189.
- Moore, J. P.; Westall, K. L.; Ravenscroft, N.; Farrant, J. M.; Lindsey, G. G.; Brandt, W. F.; *Biochem. J.* **2005**, *385*, 301.
- Pauli, G. F.; Poetsch, F.; Nahrstedt, A.; *Phytochem. Anal.* **1998**, *9*, 177.
- Camacho, M. D. R.; Phillipson, J. D.; Croft, S. L.; Marley, D.; Kirby, G. C.; Warhurst, D. C.; *J. Nat. Prod.* **2002**, *65*, 1457.
- Bradford, M. M.; *Anal. Biochem.* **1976**, *72*, 248.
- da Silva, E. R.; Floeter-Winter, L. M.; *Exp. Parasitol.* **2010**, *125*, 152.
- Fawcett, J. K.; Scott, J. E.; *J. Clin. Pathol.* **1960**, *13*, 156.
- Bisswanger, H.; *Enzyme Kinetics: Principles and Methods*, 2nd ed.; WILEY-VCH Verlag GmbH & Co. KGaA: Germany, 2008.
- Dixon, M.; *Biochem. J.* **1953**, *55*, 170.
- Krupka, R. M.; *Biochem. J.* **1963**, *2*, 76.
- dos Reis, M. B. G.; Manjolin, L. C.; Maquiaveli, C. C.; Santos-Filho, O. A.; da Silva, E. R.; *Plos One* **2013**, *8*, e78387.
- Okuda, T.; Yoshida, T.; Hatano, T.; *Fortschr. Chem. Org. Naturst.* **1995**, *66*, 1.

46. Paixão, N.; Pereira, V.; Marques, J. C.; Camara, J. S.; *J. Sep. Sci.* **2008**, *31*, 2189.
47. Liu, X.; Kim, J.; Li, Y.; Li, J.; Liu, F.; Chen, X.; *J. Nutr.* **2005**, *135*, 165.
48. Ximenes, V. F.; Lopes, M. G.; Petronio, M. S.; Regasini, L. O.; Silva, D. H.; da Fonseca, L. M.; *J. Agric. Food Chem.* **2010**, *58*, 5355.
49. Giftson, J. S.; Jayanthi, S.; Nalini, N.; *Invest. New Drugs* **2010**, *28*, 251.
50. Morais, M. C.; Luqman, S.; Kondratyuk, T. P.; Petronio, M. S.; Regasini, L. O.; Silva, D. H.; Bolzani, V. S.; Soares, C. P.; Pezzuto, J. M.; *Nat. Prod. Res.* **2010**, *24*, 1758.
51. Flausino Jr., O. A.; Dufaua, L.; Regasini, L. O.; Petrônio, M. S.; Silva, D. H. S.; Rosec, T.; Bolzani, V. S.; Reboud-Ravauxa, M.; *Curr. Med. Chem.* **2012**, *19*, 4534.
52. Wana, C.; Yuan, T.; Li, L.; Kandhi, V.; Cech, N. B.; Xie, M.; Seeram, N. P.; *Bioorg. Med. Chem. Lett.* **2012**, *22*, 597.
53. Pohjala, L.; Tammela, P.; *Molecules* **2012**, *17*, 10774.
54. Coan, K. E.; Shoichet, B. K.; *Mol. Biosyst.* **2007**, *3*, 208.
55. Coan, K. E.; Maltby, D. A.; Burlingame, A. L.; Shoichet, B. K.; *J. Med. Chem.* **2009**, *52*, 2067.
56. Radtke, O. A.; Kiderlen, A. F.; Kayser, O.; Kolodziej, H.; *Planta Med.* **2004**, *70*, 924.
57. Gaur, U.; Roberts, S. C.; Dalvi, R. P.; Corraliza, I.; Ullman, B.; Wilson, M. E.; *J. Immunol.* **2007**, *179*, 8446.

Submitted on: March 10, 2014

Published online: May 23, 2014

FAPESP has sponsored the publication of this article.