Enzymatic Inhibitory Activity and Trypanocidal Effects of Extracts and Compounds from *Siphoneugena densiflora* O. Berg and *Vitex polygama* Cham.

Margareth B. C. Gallo[a,*], Anna Sylvia F. Marques[a], Paulo C. Vieira[a], Maria Fátima das G. F. da Silva[a], João B. Fernandes[a], Márcio Silva[b], Rafael V. Guido[b], Glaucius Oliva[b], Otávio H. Thiemann[b], Sérgio Albuquerque[c], and Alan H. Fairlamb[d]

[a] Departamento de Química, Universidade Federal de São Carlos, CP 676, 13565-905, São Carlos-SP, Brazil. Fax: +55-16-33518350. E-mail: margareth.gallo@gmail.com
[b] Instituto de Física de São Carlos, Universidade de São Paulo, CP 369, 13560-970, São Carlos-SP, Brazil
[c] Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café s/n, 14040-903, Ribeirão Preto-SP, Brazil
[d] Division of Molecular Parasitology & Biological Chemistry, University of Dundee, Dundee DD1 4HN, UK

* Author for correspondence and reprint requests

Z. Naturforsch. **63c**, 371–382 (2008); received June 18/December 7, 2007

Hexanic, methanolic, and hydroalcoholic extracts, and 34 isolated compounds from *Vitex polygama* Cham. (Lamiaceae, formely Verbenaceae) and *Siphoneugena densiflora* O. Berg (Myrtaceae) were screened for their trypanocidal effects on bloodstream forms of *Trypanosoma cruzi* and *T. brucei*, as well as for their enzymatic inhibitory activities on glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) and trypanothione reductase (TR) enzymes from *T. cruzi* and adeninephosphoribosyl transferase (APRT) enzyme from *Leishmania tarentolae*. In general, polar extracts displayed strong effects and some of the tested compounds have shown good results in comparison to positive controls of the bioassays.

Key words: Myrtaceae, *Trypanosoma*, *Leishmania*

Introduction

Siphoneugena densiflora O. Berg commonly known as Uvatinga belongs to the family Myrtaceae and occurs in grasslands ("campos rupestres"), deciduous forests ("cerradões") and gallery forests in Goiás, Distrito Federal, Minas Gerais and São Paulo States of Brazil. This plant takes part in a genus comprising only nine species; two of them are vulnerable to extinction in São Paulo State (Programa Biota FAPESP, 2007) and one species, *S. delicata*, has been recently described (Sobral and Proença, 2006). *S. densiflora* has been biologically and chemically researched by our group (Gallo *et al*., 2006a, b, c) since neither data about it could be found in the literature nor popular use has been registered.

Vitex polygama Cham. (Lamiaceae, formely Verbenaceae) is a widespread and well-known Brazilian species which occurs mainly in the states of Minas Gerais, Espírito Santo, Rio de Janeiro and São Paulo, and is commonly called "Tarumã". Its leaf tea is used in folk medicine to treat kidney infections (Rodrigues, 2001; Gallo *et al.*, 2008). Several of its compounds had already been characterized and bioassayed (Leitão *et al.*, 1997; Leitão and Delle Monache, 1998; Santos *et al.*, 2001; Mensor *et al.*, 2001), but none was regarded as neither potential trypanocidal nor enzyme inhibitor.

Trypanosomiasis and leishmaniasis are major diseases in developing countries which continue to infect millions of people and are responsible for a mortality rate of over one million per year. At present, only ineffective, impractical or severely toxic chemotherapeutic agents, requiring a complicated regimen, are available for the treatment of these infections (WHO, 2007); hence there is a pressing need to find novel antiprotozoal agents.

Trypanosomatids have no functional tricarboxylic acid cycle, and their bloodstream forms are highly dependent on glycolysis to the stage of pyruvate as sole energy supply. Trypanosome glycoly-

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sis occurs in a specific microbody called glycosome. Accordingly, the inhibition of enzymes of this physiologically important metabolic pathway should deprive the parasite of the energy necessary for survival (Opperdoes and Michels, 2001). The glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) enzyme of *Trypanosoma cruzi* catalyzes the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the presence of NAD^+ and inorganic phosphate. The *in silico* modeling of this pathway has indicated this catalyzed reaction as one of the preferred steps for inhibition (Bakker *et al.*, 2000). In addition, Trypanosomatidae have dithiol trypanothione [bis(glutathionyl)spermidine]-conjugated enzymes, specially trypanothione reductase (TR) (Fairlamb *et al.*, 1995), that helps to protect the parasites from oxidative stress by maintaining an intracellular reducing milieu through reduction of the N^1,N^8-(bisglutathionyl)-spermidine conjugate trypanothione ($T[S]_2$) to dihydrotrypanothione ($T[SH]_2$). TR participates in the inactivation of potentially damaging radicals and reactive oxygen species, and is re-oxidized to ($T[S]_2$) in the process. The essential role of TR in the parasite thiol metabolism and its absence from the mammalian host render the enzyme a highly attractive target for drug development against trypanosomatids (Hamilton *et al.*, 2003).

The genus *Leishmania* owns three enzymes involved in the recycling of purine nucleotides by the salvage pathway. One of them, adeninephosphoribosyl transferase (APRT), is responsible for catalyzing the conversion of adenine and α-D-5-phosphoribosyl-1-pyrophosphate (PRPP) into adenosine-5-monophosphate (AMP) and pyrophosphate (PPi) by anomeric inversion of the ribofuranose ring (Musick, 1981). APRT and gGAPDH enzymes reveal differences in the amino acid sequence when compared to corresponding mammalian enzymes, which should allow the development of selective inhibitor compounds.

In our investigation for enzymatic inhibitors and trypanocidal agents to be used against parasitic protozoa, we have screened hexanic, methanolic, and hydroalcoholic extracts, as well as 34 isolated compounds from the aforementioned plants for their trypanocidal effects on bloodstream forms of *Trypanosoma cruzi* and *T. brucei*. In addition, the inhibitory activities, either on gGAPDH and TR enzymes from *T. cruzi* or APRT enzyme from *Leishmania tarentolae*, were assessed.

Materials and Methods

Plant material

Vitex polygama Cham. (Lamiaceae) and *Siphoneugena densiflora* O. Berg (Myrtaceae) were collected in July 2000 in the city of Poços de Caldas, Minas Gerais, Brazil. The plants were authenticated by Dr. Fátima Regina Salimena-Pires, a taxonomic botanist from Universidade Federal de Juiz de Fora (UFJF), and Dr. Marcos Sobral from Universidade Federal de Minas Gerais (UFMG), respectively. Voucher specimens are deposited at the herbarium of Faculdade de Filosofia, Ciências e Letras of Universidade de São Paulo, campus Ribeirão Preto (SPFR), Brazil, under the acquisition number 9968, and at the Herbarium of Botany Department, Universidade de São Paulo, São Paulo, Brazil.

Extraction procedures

Extracts as well as compounds **1** to **3**, **6**, **7**, **9**, **11** to **13**, **20** to **22**, **28** to **34** were obtained in conformity with formerly described procedures (Gallo *et al.*, 2006a, b, c, 2008).

Isolation of compound **4** from the methanolic extract of stem of *Siphoneugena densiflora* (SD-MS)

A quantity of 60.7 g of SD-MS was submitted to liquid-liquid partition using solvents of increasing polarity (dichloromethane, ethyl acetate and *n*-butanol). The corresponding dichloromethane layer (SD-DMS, 1.62 g) was chromatographed over Sephadex LH-20 (60.0×2.0 cm) using a gradient elution from dichloromethane/hexane (1:1 by volume) to methanol (100%), resulting in 35 fractions of 20 mL each, which were pooled into 10 fractions (D1 to D10) according to their composition, determined by thin layer chromatography and visualized under UV light and spraying with colour reagent. Fraction D8 (600.0 mg) was rechromatographed over Sephadex LH-20 (23.0×3.0 cm) employing a step gradient from dichloromethane (100%), dichloromethane/acetone (80:20 by volume) and methanol (100%). A total of 52 fractions of 20 mL each was collected and combined in agreement with the fractions similarities, affording 16 fractions (D8a to D8p). Fraction D8i was identified as compound **4** (12.0 mg).

Isolation of compounds **5**, **14**, **15**, **16**, **17**, **19**, **24**, **25**, **26** and **27** from a dichloromethane layer of the methanolic extract of leaves of *Vitex polygama* (VP-DML)

A quantity of 38.1 g of VP-ML was submitted to liquid-liquid partition using solvents of increasing

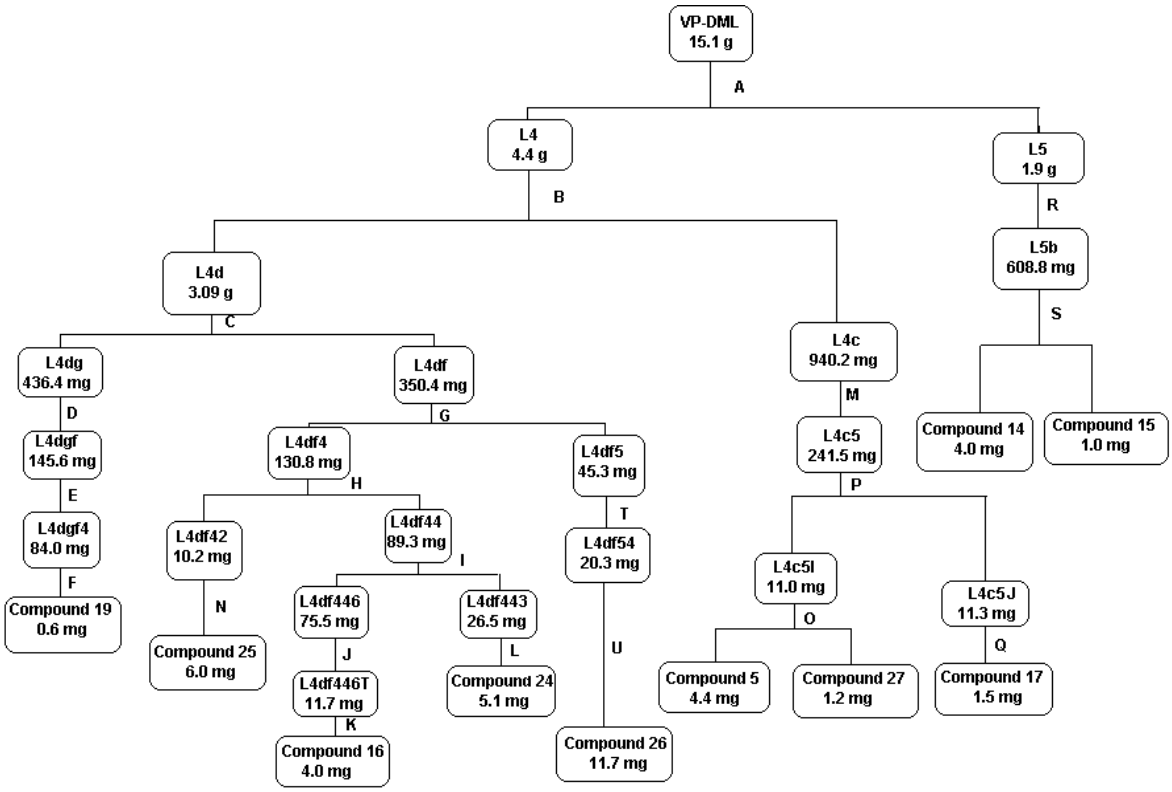


Fig. 1. Fluxogram of isolation of compounds **5**, **14** to **17**, **19**, **24** to **27** from *Vitex polygama.*

polarity (dichloromethane and ethyl acetate). The corresponding dichloromethane layer (VP-DML, 15.1 g) was chromatographed on a silica gel 60 column (20.0 × 5.5 cm) and eluted in a step gradient from dichloromethane/methanol (95:5 by volume) to methanol (100%) (step A, Fig. 1). A total of 14 fractions of 125 mL each was collected and pooled into 8 fractions (L1 to L8). Fraction L4 (4.4 g) was submitted to column chromatography (CC) (16.5 × 5.5 cm) with silica gel 60 and gradient elution from hexane/ethyl acetate (70:30 by volume) to methanol (100%) (step B, Fig.1), resulting in 15 fractions of 100 mL each, which were combined into four fractions (L4a to L4d). Fraction L4d (3.09 g) was rechromatographed on silica gel 60 (20.0 × 5.0 cm) using a step gradient from dichloromethane (100%) to methanol (100%) (step C, Fig. 1), yielding 20 fractions of 100 mL each, which were pooled into 8 fractions (L4da to L4dh). Fraction L4df (350.4 mg) was chromatographed using step C conditions with a column of 15.0 × 4.0 cm (step G, Fig. 1) to afford 15 fractions of 150 mL each, that were combined into 8 fractions (L4df1 to L4df8). Fraction L4df4 (130.8 mg) was submitted to CC (15.0 × 4.0 cm) with silica gel 60 and gradient elution from dichloromethane/ethyl acetate (90:10 by volume) to methanol (100%) (step H, Fig. 1) to afford 11 fractions of 150 mL each, that were combined into 8 fractions (L4df41 to L4df48). Fraction L4df42 (10.2 mg) was purified on silica gel (10.0 × 3.0 cm) using isocratic elution with dichloromethane/ethyl acetate (95:5 by volume; step N, Fig. 1) to afford 14 fractions of 100 mL each, that were combined into 5 fractions (L4df421 to L4df425). Fraction L4df423 was identified as compound **25**. Fraction L4df44 (89.3 mg) was submitted to step H conditions in a column of 25.0 × 4.5 cm (step I, Fig. 1) to yield 16 fractions of 100 mL each, that were combined into 6 fractions (L4df441 to L4df446). Fraction L4df446 (75.5 mg) was chromatographed over Sephadex LH-20 (52.0 × 2.0 cm) using methanol as eluent (step J, Fig. 1) to yield 31 fractions of 15 mL each, that were combined into 4 fractions (L4df446R to

L4df446U). Fraction L4df446T (11.7 mg) was purified over a silica gel column (24.0 × 1.0 cm) using dichloromethane/ethyl acetate (90:10 by volume) as eluent (step K, Fig. 1) to yield 31 fractions of 15 mL each, that were combined into 3 fractions. Fraction 3 (L4df446Ta, 4.0 mg) was identified as compound **16**. Fraction L4df443 (26.5 mg) was submitted, consecutively, to the conditions of steps K and J (step L, Fig. 1) to afford compound **24**. Fraction L4dg (436.4 mg) was submitted repeatedly to the conditions of steps A, K and J (steps D, E and F, Fig. 1) to afford compound **19**. Fraction L4c (940.2 mg) was fractionated on silica (24.0 × 4.0 cm) using gradient elution with hexane/ethyl acetate (70:30 by volume) to yield 17 fractions of 100 mL each, that were combined into 7 fractions (L4c1 to L4c7; step M, Fig. 1). Fraction L4c5 (241.5 mg) was submitted to the conditions of step J to afford 27 fractions of 15 mL each, that were combined into 7 fractions (L4c5F to L4c5L; step P, Fig. 1). Fraction L4c5J (11.3 mg) was purified by a HPLC RP-ODS C18 system (step Q, Fig. 1) employing methanol isocratic elution (254 nm; flow rate of 2 mL/min; injection volume of 1 mL) to afford compound **17**. Fraction L4c5I (11.0 mg) was submitted to preparative TLC (20.0 × 20.0 cm, step O, Fig. 1) using dichloromethane/methanol (90:10 by volume) as eluent to yield compounds **5** and **27**. Fraction L4df5 (43.5 mg) was chromatographed on silica gel 60 (18.0 × 5.0 cm, step T, Fig. 1) under the elution conditions of step H. A total of 18 fractions of 50 mL was collected and pooled into 7 fractions (L4df51 to L4df57). Fraction L4df54 (20.3 mg) was chromatographed on silica gel 60 (15.0 × 2.5 cm, step U, Fig. 1) employing dichloromethane/ethyl acetate (70:30 by volume). A total of 10 fractions of 100 mL each was collected and pooled into 6 fractions (L4df54a to L4df54f). The constituent of fraction L4df54c was identified as compound **26** (11.7 mg). Fraction L5 (1.9 g) was subjected to CC using silica gel 60 (20.0 × 4.0 cm) and gradient elution from ethyl acetate (100%) to methanol (100%) (step R, Fig. 1), yielding 13 fractions of 50 mL each which were pooled into 6 fractions (L5a to L5f). Fraction L5b (608.8 mg) was chromatographed over Sephadex LH-20 (60.0 × 2.0 cm) using methanol as eluent for three repeated times (step S, Fig. 1) to yield fraction 4 (L5btg4, 1.0 mg) that was identified as compound **15** and fraction 5 (L5btg5, 4.0 mg) characterized as compound **14**.

Isolation of compounds **18** and **23** from a methanolic layer of the hexanic extract of leaves of *Vitex polygama* (VP-MHL)

The corresponding methanolic layer (VP-MHL, 171.0 mg) was chromatographed on a silica gel 60 column (18.0 × 4.5 cm) and eluted in a step gradient from dichloromethane/hexane (50:50 by volume) to methanol (100%). A total of 20 fractions of 150 mL each was collected and pooled into 7 fractions (MHL1 to MHL7). Fraction 5 (MHL5, 26.6 mg) was chromatographed over Sephadex LH-20 (52.0 × 3.0 cm) employing methanol as eluent to afford 31 fractions of 15 mL each, which were pooled into 7 fractions (MHL5a to MHL5g). Fraction 6 (MHL5f, 1.2 mg) was identified as compound **18** and fraction 5 (MHL5e, 4.2 mg) as compound **23**.

Isolation of compounds **8** and **10** from a dichloromethane layer of the methanolic extract of stem of *Siphoneugena densiflora* (SD-DMS)

A quantity of 1.62 g of SD-DMS was chromatographed over Sephadex-LH20 (28.0 × 3.0 cm) using gradient elution from dichloromethane/hexane (50:50 by volume), dichloromethane (100%), methanol/dichloromethane (50:50 by volume) to methanol (100%). A total of 34 fractions of 20 mL each was collected and pooled into 10 fractions (DMS1 to DMS10). Fraction 5 (DMS5, 75.4 mg) was rechromatographed over Sephadex-LH20 (45.0 × 2.0 cm) using methanol as eluent to afford 15 fractions of 20 mL each, which were combined into 5 fractions (DMS5m to DMS5q). Fraction 5 (DMS5q, 25.9 mg) was purified by PTLC (20.0 × 20.0 cm) using dichloromethane/acetone (90:10 by volume) as eluent twice to afford compound **8** (3.4 mg). Fraction 6 (DMS6, 115.7 mg) was submitted to CC using silica gel 230–400 mesh (23.0 × 3.0 cm) employing a gradient elution from dichloromethane (100%), dichloromethane/ethyl acetate to methanol (100%). A total of 56 fractions of 20 mL each was collected and pooled into 11 fractions (DMS61 to DMS611). Fraction 9 (DMS69, 10.4 mg) was purified by PTLC (20.0 × 20.0 cm) using dichloromethane/acetone (90:10 by volume) as eluent to afford compound **10** (5.0 mg).

T. cruzi gGAPDH inhibitory activity

The enzymatic activity of *T. cruzi* gGAPDH and the inhibitory activities of extracts and compounds against this enzyme have been determined accord-

ing to an earlier reported procedure (Vieira *et al.*, 2001). The 50% inhibitory concentration (IC_{50}), *i. e.*, the concentration necessary to inhibit 50% of enzyme activity, was estimated from graphically plotted dose-response curves by making rate measurements for at least five inhibitor concentrations, using the nonlinear curve-fitting program SigmaPlot 2001 7.0. Statistical error limits on the IC_{50} values have been calculated in amount to 10% or less. The coumarin chalepin was used as positive control (IC_{50} = 55.5 μM).

L. tarentolae APRT inhibitory activity

APRT cloning, expression, and purification have been determined according to earlier described procedures (Thiemann *et al.*, 1998). The APRT activity has been determined by spectrophotometric measurements of the formation of AMP at 259 nm after 60 s. The reaction mixture contained, in a final volume of 1.0 mL, 0.01 mM adenine, 0.5 mM PRPP, 5 mM $MgCl_2$, 100 mM Tris-HCl [2-amino-2-(hydroxymethyl)-1,3-propanediol-hydrochloride], pH 7.4, and 3.8 μL APRT solution. The reaction started by the addition of enzyme. The specific activity of the enzyme and the inhibitory activity of compounds and extracts have been calculated in agreement with the literature (Vieira *et al.*, 2001).

T. cruzi TR inhibitory activity

Enzyme activity and assay were performed according to prior descriptions (Hamilton *et al.*, 2003). Clomipramine (Novartis) was used as positive control [IC_{50} = 2.72 μM (personal communication, Alan Fairlamb)]. The IC_{50} value was determined by microplate assays in serial two-fold dilutions of the test compound. The data were processed using the GraFit version 4.5 Program (written by R. J. Laetherbarrow, Univ. London Imperial Coll. Sci. Technol. & Med., Dept. Chem., Biol. & Biophys. Chem. Sect., London SW7 2AZ, England) in order to obtain the IC_{50} values, and the results were fitted into the equation

$$Y = \{Range/[1 + (X/IC_{50})^s]\} + Background,$$

where Y is the absorbance, X the concentration of the test compound (in μM), Range the Y axis scale, Background the zero control and s the slope factor (s = 1).

T. brucei cell culture and cytotoxicity assay

Bloodstream forms of *T. brucei*, strain 427 wild type, were grown in HMI-9 medium with 10% serum following the published protocol (Hirumi and Hirumi, 1989). The effect of compounds on the viability of bloodstream forms of *T. brucei* was evaluated using the Alamar Blue® assay (Räz *et al.*, 1997). Petamidine was used as positive control (IC_{50} = 14 nM). In general, compounds were tested at a concentration of 100 μM.

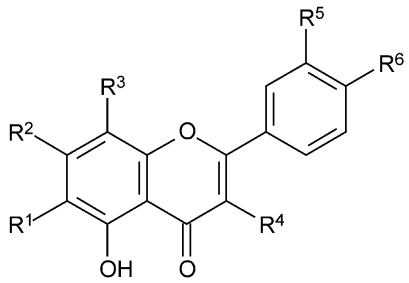
T. cruzi cytotoxicity assay

The assay was carried out using blood infected with trypomastigotes of *T. cruzi* "Y" strain collected by cardiac puncture in experimentally infected male Swiss albino mice on the parasitemic peak (seventh day) and diluted with normal murine blood to $2 \cdot 10^6$ trypomastigotes/mL. The assay was performed in triplicate on titration microplates (96 wells) containing 387.5 mL of diluted infected blood and solutions of extracts or compounds in DMSO to acquire final concentrations of 4 mg/mL, 500 μg/mL, 250 μg/mL and 100 μg/mL. The plates were incubated at 4 °C, and the number of parasites counted after 24 h. Negative and positive controls containing infected blood with either 2.5% DMSO or gentian violet (IC_{50} = 31 μg/mL or 76 μM) were run in parallel. The results were expressed as percentage of lysis of trypomastigote forms compared to the DMSO control. DMSO at 2.5% did not interfere with the parasite survival.

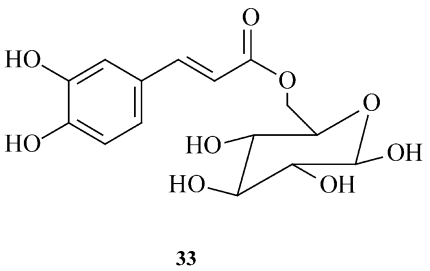
Results and Discussion

Identification of compounds

Fig. 2 shows the structures of isolated and tested compounds from *S. densiflora*: quercetin (**1**); quercitrin (**2**); gallic acid (**3**); arjunolic acid (**4**); vanillic acid (**5**); sitosterol (**6**); syringic acid (**7**); 2,4,6-trimethoxybenzoic acid (**8**); ellagic acid (**9**); 5-hydroxymethyl-2-furancarboxaldehyde (**10**); casuarinin (**11**); castalagin (**12**); ellagic acid 4-*O*-α-L-rhamnopyranoside (**13**); and from *V. polygama*: luteolin (**14**); 3-*O*-methylquercetin (**15**); chrysoeriol (**16**); 3,3′-*O*-dimethylquercetin (**17**); 3,7,4′-*O*-trimethylkaempferol (**18**); acacetin (**19**); isoorientin (**20**); schaftoside (**21**); carlinoside (**22**); penduletin 4′-*O*-methyl ether (**23**); 2α,3α-dihydroxyolean-12-en-28-oic acid (**24**); oleanolic acid (**25**); 2α,3α,19α-trihydroxyurs-12-en-28-oic acid (**26**, euscaphic acid); *p*-hydroxybenzoic acid (**27**); 20-hydroxyec-



	R1	R2	R3	R4	R5	R6
1	H	OH	H	OH	OH	OH
2	H	OH	H	α-L-rhamnopyranosyl	OH	OH
15	H	OH	H	OMe	OH	OH
17	H	OH	H	OMe	OMe	OH
18	H	OMe	H	OMe	H	OMe
23	OMe	OMe	H	OMe	H	OMe



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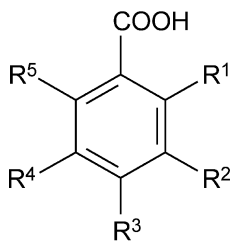

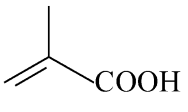

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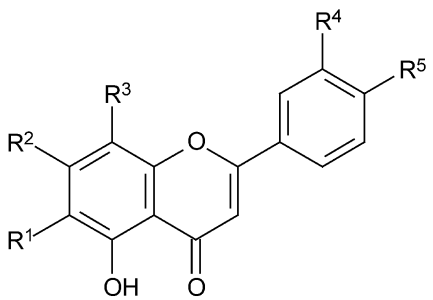
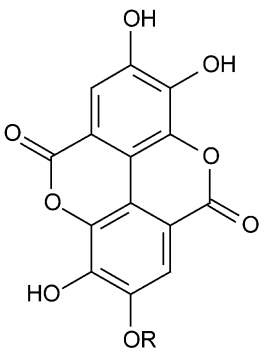
	R1	R2	R3	R4	R5
3	H	OH	OH	OH	H
5	H	OMe	OH	H	H
7	H	OMe	OH	OMe	H
8	OMe	H	OMe	H	OMe
27	H	H	OH	H	H



	R1	R2	R3	R4	R5
14	H	OH	H	OH	OH
16	H	OH	H	OMe	OH
19	H	OH	H	H	OMe
20	*C*-β-D-glucopyranosyl	OH	H	OH	OH
21	*C*-β-D-glucopyranosyl	OH	*C*- α-L-arabinopyranosyl	H	OH
22	*C*-β-D-glucopyranosyl	OH	*C*- α-L-arabinopyranosyl	OH	OH



	R
9	H
13	α-L-rhamnopyranosyl

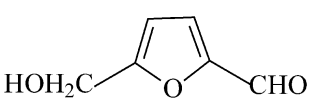


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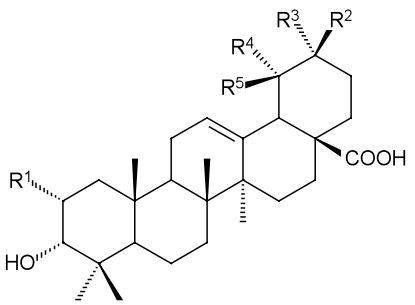
Fig. 2. Structures of compounds isolated from *V. polygama* and *S. densiflora*.

dysone (**28**, polypodine A); polypodine B (**29**); stachysterone (**30**); shidasterone (**31**); metacrylic acid (**32**); caffeoyl 6-*O*-*β*-D-glucopyranoside (**33**); sitosterol 3-*O*-*β*-D-glucopyranoside (**34**, daucosterol). The structures of the compounds were elucidated by comparison of their spectral data (^{1}H NMR, ^{13}C NMR, DEPT, HMBC, HSQC, ^{1}H-^{1}H COSY, IE-MS or ESI-MS) with earlier published data (see references in Tables I and II).

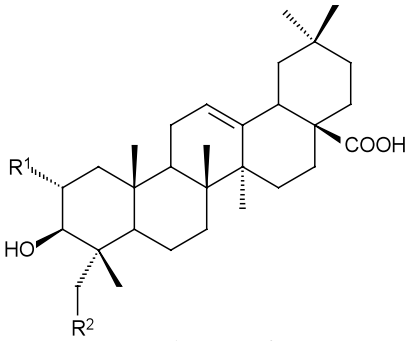
Trypanocidal activity

Among the 21 extracts and 13 partition fractions tested against trypomastigote forms of *T. cruzi*, 15 revealed significant activities (> 60%, Table III) and were considered as promising sources of active substances.

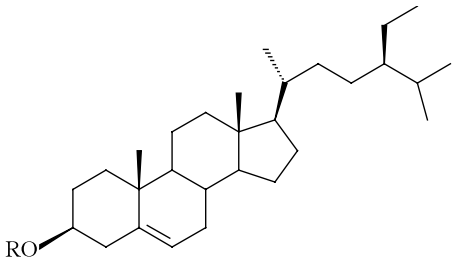
Partition of VP-HL produced fractions with poorer activities (Table III). ^{1}H NMR analysis of



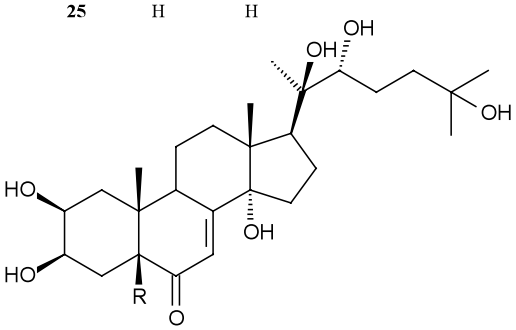
	R^1	R^2	R^3	R^4	R^5
24	OH	Me	Me	H	H
26	OH	H	Me	OH	Me



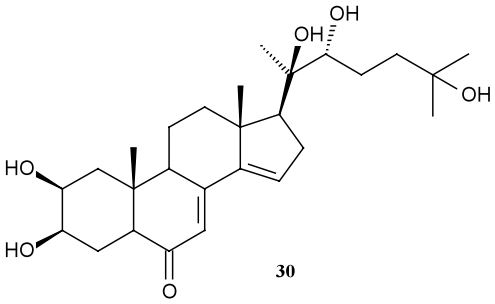
	R^1	R^2
4	OH	OH
25	H	H



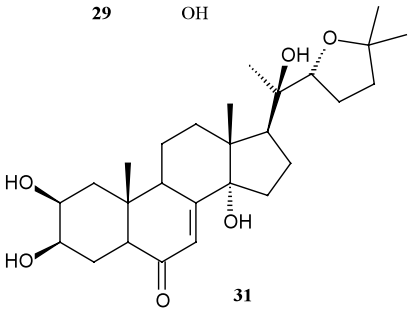
	R
6	H
34	β-D-glucopyranosyl



	R
28	H
29	OH



30



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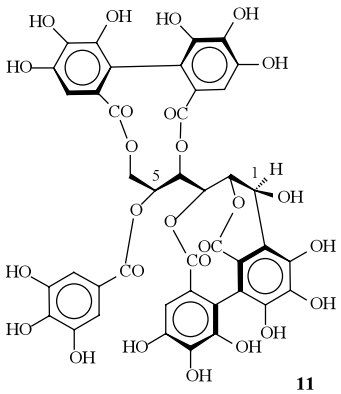

11

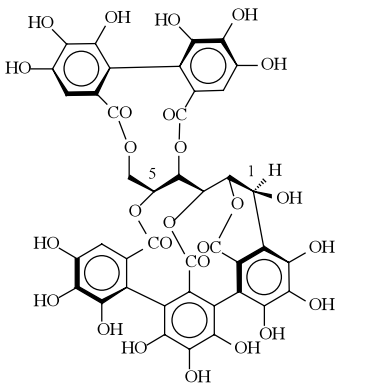

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Fig. 2 (continued).

Table I. *In vitro* antiprotozoal and enzymatic inhibitory activities of compounds from *Siphoneugena densiflora.*

Compound	Extract origin	% lysis Tc [mM]	% inhibition gGAPDH (200 μM)	% inhibition APRT (100 μM)	% inhibition TR (100 μM)	% growth inhibition Tb (100 μM)	Reference
1	SD-EML	34 (1.7)	91[a]	97	NT	NT	Gallo *et al.*, 2006a
2	SD-EML	30 (1.1)	19	89	4	I	Gallo *et al.*, 2006a
3	SD-EML SD-MR SD-MS	19 (2.9)	I	I	5	I	Gallo *et al.*, 2006b
4	SD-MS	90 (1.0) 61 (0.5) 9 (0.2)	I	13	NT	NT	Mahato and Kundu, 1994
5	SD-MS	NT	I	I	NT	NT	Sakushima *et al.*, 1995
6	SD-MR	NT	I	I	NT	NT	Gallo *et al.*, 2006b
7	SD-EML SD-MR	16 (2.5)	I	I	14	I	Gallo *et al.*, 2006a
8	SD-MS	NT	I	24	NT	NT	Russel *et al.*, 1990
9	SD-MR	41 (1.7)	90[b]	80	NT	NT	Gallo *et al.*, 2006a
10	SD-MS	NT	I	I	46	I	Kulkarni *et al.*, 1989
11	SD-MS SD-MR SD-EML	28 (0.5)	c	d	87[g]	I	Gallo *et al.*, 2006a
12	SD-MS SD-MR	11 (0.5)	e	f	87[g]	I	Gallo *et al.*, 2006a
13	SD-MS SD-MR SD-HAS	12 (1.1)	86 (239 μM) 33 (119 μM)	NT*	NT	NT	Gallo *et al.*, 2006a

NT, not tested; NT*, compound absorbed at the same wavelength of enzyme assay; I, inactive; Tc, trypomastigotes forms of *Trypanosoma cruzi*; Tb, bloodstream forms of *T. brucei*; TR, trypanothione reductase.
[a] $IC_{50} = (24 \pm 3.9)$ μM.
[b] $IC_{50} = (25 \pm 2.2)$ μM.
[c,d] $IC_{50} = (1.8 \pm 1.5)$ μM.
[e] $IC_{50} = (7.5 \pm 0.7)$ μM.
[f] $IC_{50} = (3.3 \pm 2.3)$ μM.
[g] $IC_{50} = (179.3 \pm 1.5)$ μM.

VP-HHL (53%) showed characteristic signals of hydrocarbons and fatty acids. VP-DHL (38%), when submitted to column chromatography, yielded a fraction containing a mixture of steroids which was responsible for 93% of Tc lysis at 500 μg/mL (this information is not displayed in the table). VP-EHL (36%) yielded compound **18** (31%), and VP-MHL (52%) afforded compounds **18** and **23** (16%).

VP-DML, after fractionation, afforded compounds **16**, **17**, and a mixture of flavonoids containing quercetin 3-*O*-β-D-glucopyranoside and quercetin 4′-*O*-β-D-glucopyranoside, which caused 97% Tc lysis at 500 μg/L. The same mixture inhibited the gGAPDH activity by 92% at 100 μg/mL (this information is not displayed in the table), which may explain the mechanism of action of the mixture on the trypomastigote forms of the parasite. **16** performed a better activity against *T. cruzi* (33%, Table II) in comparison with compound **14** (27%), indicating that *O*-methylation of the 3′-hydroxy group was important to improve the activity.

VP-BHAL showed a good trypanocidal activity against *T. cruzi* (60%, Table III), however, the compounds isolated from this fraction did not. Compound **33** (11%, 1.5 mM, Table II) was much less active than its aglycone tested previously (IC_{50}

Table II. *In vitro* antiprotozoal and enzymatic inhibitory activities of compounds from *V. polygama*.

Compound	Extract origin	% lysis Tc [mM]	% inhibition gGAPDH (200 μM)	% inhibition APRT (100 μM)	% inhibition TR (100 μM)	% growth inhibition Tb (100 μM)	Reference
14	VP-DML	27 (1.7)[a]	56	40	46	I	Gallo *et al.*, 2008
15	VP-DML	NT[b]	73[c]	84	NT	NT	Roitman and James, 1985
16	VP-DML	33 (1.7)	75	80	18	I	Brieskorn and Riedel, 1977
17	VP-DML	NT	37	25	NT	NT	Chiappini *et al.*, 1982
18	VP-MHL VP-EHL	31 (1.5)	41 (305 μM)	NT	NT	NT	Suttanut *et al.*, 2007
19	VP-DML	NT	22	16	NT	NT	Pettit *et al.*, 1987
20	VP-BHAL	13 (1.1)	63	63	17	I	Gallo *et al.*, 2008
21	VP-BHAL	NT	10	47	5	I	Besson *et al.*, 1985
22	VP-BHAL	NT	7	14	NT	NT	Besson *et al.*, 1985
23	VP-MHL	16 (1.4)	20	12	NT	NT	Southwick *et al.*, 1972
24	VP-DML	76 (1.1) 5 (0.5)	44	71	NT	NT	Mahato and Kundu, 1994
25	VP-DML	38 (1.1)	40	68	NT	NT	Mahato and Kundu, 1994
26	VP-DML VP-DMT	90 (1.0) 87 (0.5) 16 (0.2)	I	10	29	I	Mahato and Kundu, 1994
27	VP-DML	NT	I	I	NT	NT	Dayrit *et al.*, 1987
28	VP-EMT	71 (1.0) 70 (0.5) 4 (0.2)	6	41	NT	NT	Suksamrarn and Sommechai, 1993
29	VP-EMT	NT	27	84	NT	NT	Gallo *et al.* 2006a
30	VP-EMT	NT	10	12	NT	NT	Gallo *et al.* 2006c
31	VP-EMT	NT	14	58	NT	NT	Gallo *et al.* 2006c
32	VP-AHAL	8 (5.8)	I	I	NT	NT	Davidson *et al.*, 1989
33	VP-BHAL	11 (1.5)	I	I	11	I	Gallo *et al.* 2006a
34	VP-DML	14 (0.9)	I	40	NT	NT	Gallo *et al.* 2006b

NT, not tested; I, inactive; Tc, trypomastigotes forms of *Trypanosoma cruzi*; Tb, bloodstream forms of *T. brucei*; TR, trypanothione reductase.
[a] IC_{50} = 132.6 mM according to Grael *et al.* (2005).
[b] 63% lysis (1.7 mM) according to Takeara *et al.* (2003).
[c] IC_{50} = (36 ± 9.63) μM.

2.1 mM, Grael *et al.*, 2005), showing that the glycose moiety, making the molecule more polar, had a bad influence on the biological action.

The polar extracts from *S. densiflora* (SD-HAS, SD-MT, SD-HAT, SD-MR, and SD-HAR, Table III) exhibited a strong effect on *T. cruzi*; on the other hand, compounds isolated from them did not (**3**, **6**, **7**, **9**, **11**, **12** and **13**; Table I). Maybe the compound(s) responsible for the original extract activity had not been identified yet.

Table III. *In vitro* antiprotozoal and enzymatic inhibitory activities of *Siphoneugena densiflora* (SD) and *Vitex polygama* (VP) extracts and fractions.

Extract (obtained amount, g)[a]	% lysis Tc (4 mg/mL)	% inhibition gGAPDH (100 μg/mL)	% inhibition APRT (50 μg/mL)
SD-HL (8.2)	82	28	NT
SD-ML (164.8)	34	74	97
SD-DML (9.5)	NT	NT	30
SD-EML (14.6)	NT	90	73
SD-BML (12.0)	NT	NT	68
SD-AML (12.5)	NT	NT	75
SD-HAL (22.1)	49	82	NT*
SD-HS (0.9)	65	I	14
SD-MS (177.6)	47	91	NT*
SD-HAS (5.5)	100	93	NT*
SD-HT (1.0)	33	I	30
SD-MT (131.1)	83	94	28
SD-HAT (5.7)	100	92	73
SD-HR (0.3)	64	26	4
SD-MR (73.2)	88	92	NT*
SD-HAR (2.4)	61	94	NT*
VP-HL (5.1)	94	NT	NT
VP-HHL (1.08)	53	I	NT
VP-DHL (1.84)	38	I	NT
VP-EHL (1.94)	36	22	NT
VP-MHL (0.19)	52	I	NT
VP-ML (40.4)	58	3	NT
VP-DML (15.1)	NT	NT	74
VP-EML (8.0)	NT	NT	62
VP-HAL (31.0)	41	81	NT
VP-EHAL (2.2)	62	I	56
VP-BHAL (12.8)	60	I	67
VP-AHAL (22.6)	40	76	NT
VP-HT (0.85)	75	I	NT
VP-MT (19.3)	28	I	NT
VP-EMT (3.9)	NT	NT	51
VP-DMT (2.6)	NT	NT	52
VP-HAT (2.3)	81	39	NT
VP-HF (1.1)	80	I	NT
VP-MF (2.0)	80	NT	26
VP-HAF (0.9)	39	NT	NT

[a] H, hexane extract; HH, hexane partition of H; DH, dichloromethane partition of H; EH, ethyl acetate partition of H; MH, methanol partition of H; M, methanol extract; BM, *n*-BuOH partition of M; DM, dichloromethane partition of M; EM, ethyl acetate partition of M; AM: aqueous partition of M; HA, hydroalcoholic extract; EHA, ethyl acetate partition of HA; BHA, *n*-BuOH partition of HA; AHA, aqueous partition of HA; L, leaves; S, stem; T, twigs; R, root bark; F, fruit.

Tc, trypomastigotes forms of *Trypanosoma cruzi*; NT, not tested; NT*, the extract absorbed at the same wavelength of the enzyme assay and was not tested; I, inactive.

The outcomes from triterpenes **4** (Table I), **24**, **25** and **26** (Table II) point out a clear correlation between increasing number of hydroxy groups and higher activity, which justifies a meticulous investigation on structure-activity relationships in this case.

All the substances tested against *T. brucei* were inactive, perhaps because of the low concentration (100 μM) tested in contrast to the concentration tested on *T. cruzi* (500 μg/mL, see Tables I and II).

gGAPDH inhibition

On the whole, 26 extracts and subfractions were tested. Eleven of them, the most polar (SD-methanolic and hydroalcoholic extracts), showed outcomes above 70% (Table III), and the majority belonged to *S. densiflora* (SD). The ascription of such activities to the hydrolysable tannins **11**, **12**, **13**, and ellagic acid (**9**) may be considered due to the low IC_{50} values accomplished by these compounds (Table I).

Among the flavonoids, the simple and highly oxidized flavonol quercetin (**1**) was the most active. The flavone luteolin (**14**), without a hydroxy group at C-3, had a reduced performance. Though, its activity seemed to be improved by the introduction of the methoxy group at position C-3 (**15**) or C-3′ (**16**), the presence of two or more methoxy groups at the flavone skeleton did significantly lower the inhibitory activity of the three tested enzymes (for comparison, see activities of compounds **17**, **18** and **23** in Table II). The *O*-glycosylated flavonol quercitrin (**2**) had a minor outcome in contrast to its aglycone **1**. Lio *et al.* (1985) observed the same behaviour with these compounds on xanthine oxidase, concluding that glycosylated derivatives are more hydrophilic and bulky, what reduces their contact with the active site of the enzyme. This observation fits with the small activities also performed by compounds **21** and **22** (Table II). The triterpenes were no efficient inhibitors of the gGAPDH enzyme neither a mixture of them (see Tables I and II). An unseparated mixture of lupeol, α-amyrin and β-amyrin, isolated from the dichloromethane layer of the methanolic extract of leaves from *S. densiflora* (SD-DML), tested at 200 μM, was inactive (this information is not shown in the table). At a similar concentration, a mixture of compound **24** and its isomer 2α,3β-dihydroxyurs-12-en-28-oic acid, isolated from VP-DML, was also inactive, while compound **24** inhibited 44% of the enzyme activity (Table II).

APRT inhibition

From 17 extracts and subfractions tested, eight presented results greater than 60%. Several could not be screened because they absorbed at the same wavelength used in the bioassay. The activity of the methanolic extract from leaves of *S. densiflora* was increased after fractionation (from 74 to 90%, Table I). Furthermore, quercetin (**1**) as well as quercitrin (**2**), from the ethyl acetate layer (SD-EML), showed inhibitory activities similar to their original extracts (97 and 89%, respectively, Table I). Altogether, flavonoids and triterpenes had activities similar to those performed on gGAPDH (Tables I and II). When analyzing the results displayed by ecdysteroids, we can notice that their activities were strictly linked to the number of hydroxy groups. Accordingly, compound **29**, bearing an extra hydroxy group in relation to **28** and two in relation to **30**, was twice more active than **28** and seven times more than **30** (Table II).

TR inhibition

All tested substances were practically inactive toward the TR enzyme except for casuarinin (**11**) and castalagin (**12**) which performed an IC_{50} value of 179.3 μM on enzyme inhibition, although this result is not promising in comparison with clomipramine, the positive control.

Acknowledgements

The authors are grateful to Dr. Marcos Sobral (UFMG) and Fátima Regina Salimena-Pires (UFJF) for botanical identifications, to ALCOA Alumínio S/A for facilitating the plant collections, to Ahilan Saravanamuthu for supplying TR enzyme, and to Dr. John Lee Richardson for valuable discussions. This research has been supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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