Received 25 March 2007; Accepted 19 March 2008

Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
18(2): 177-182, Abr./Jun. 2008

Artigo

Trypanocidal activity of coumarins and styryl-2-pyrones from *Polygala sabulosa* A.W. Bennett (Polygalaceae)

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RESUMO: "Atividade tripanocida de cumarinas e estiril-2-pironas de *Polygala sabulosa* A.W. Bennett (Polygalaceae)". A bioatividade das frações e compostos obtidos de *Polygala sabulosa* contra as formas epimastigota, tripomastigota sanguínea e amastigota de *Trypanosoma cruzi* foram avaliadas *in vitro*. Frações diclorometano e acetato de etila mostraram potente atividade tripanocida sob as formas epimastigotas ($IC_{50} \leq 10{,}4$ μg/mL). Análises por cromatografia em camada delgada destas frações confirmaram a presença de compostos previamente descritos (dihidroestiril-2-pironas, estiril-2-pironas e 6-metoxi-7-preniloxicumarina). Após o fracionamento da fração diclorometano por cromatografia em coluna, obteve-se o composto α-espinasterol e da fração acetato de etila obtiveram-se os compostos apigenina, quercetina e uma quercetina-3-*O*-glicosídeo, todos descritos pela primeira vez para o gênero *Polygala*. 4-metoxi-6-(11,12-metilenodioxi-14-metoxidihidroestiril)-2-pirona, 4-metoxi-6-(11,12-dimetoxi-14-metoxiestiril)-2-pirona, 6-metoxi-7-preniloxicumarina e quercetina-3- *O*-glicosídeo mostraram fraca atividade contra a forma tripomastigota sanguínea ($IC_{50} \leq 1008{,}6$ μg/mL). A cumarina prenilada foi o composto mais ativo contra ambas as formas epimastigota e tripomastigota, com IC_{50} 10,5 e 88,2 μg/mL, respectivamente. A atividade hemolítica e a toxicidade celular de cada composto foram também avaliadas. Além disso, 4-metoxi-6-(11,12-metilenodioxi-14-metoxidihidroestiril)-2-pirona e 6-metoxi-7-preniloxicumarina reduziram em quatro vezes a infecção em ratos por *Vero Cells* nas concentrações de 100 e 50 μg/mL respectivamente. Esses resultados mostram, pela primeira vez, a atividade de compostos de *P. sabulosa* contra *T. cruzi*.

Unitermos: *Polygala sabulosa,* 6-metoxi-7-preniloxicumarina, estiril-2-pironas, *Trypanosoma cruzi,* atividade tripanocida.

ABSTRACT: Bioactivity of fractions and compounds obtained from *Polygala sabulosa* against *Trypanosoma cruzi* epimastigote, blood trypomastigote and amastigote forms were evaluated *in vitro*. Dichloromethane and ethyl acetate fractions showed a strong trypanocidal activity on epimastigotes ($IC_{50} \leq 10.4$ μg/mL). Chromatographic analysis by TLC of these fractions confirmed the presence of previously described compounds (dihydrostyryl-2-pyrones, styryl-2-pyrones and 6-methoxy-7-prenyloxycoumarin). The dichloromethane fraction was fractioned by silica gel column chromatography to afford the compound α-spinasterol and the ethyl acetate fraction yielded apigenin, quercetin and a quercetin-3-*O*-glucoside, being the first description for the *Polygala* genus. 4-Methoxy-6-(11,12-methylenedioxy-14-methoxydihydrostyryl)-2-pyrone, 4-methoxy-6-(11,12-dimethoxystyryl)-2-pyrone, 6-methoxy-7-prenyloxycoumarin and quercetin-3-*O*-glucoside showed a weak activity against blood trypomastigotes ($IC_{50} \leq 1008.6$ μg/mL). The prenylated coumarin was the most active compound against both epimastigote and trypomastigote forms, IC_{50} 10.5 and 88.2 μg/mL, respectively. The hemolytic activity and cell toxicity of each active compound was also assessed. Furthermore, 4-methoxy-6-(11,12-methylenedioxy-14-methoxydihydrostyryl)-2-pyrone and 6-methoxy-7-prenyloxycoumarin reduced 4 times the *T. cruzi* infection rate for Vero cells at 100 and 50 μg/mL, respectively. These results show for the first time active compounds against *T. cruzi* in *P. sabulosa*.

Keywords: *Polygala sabulosa*, 6-methoxy-7-prenyloxycoumarin, styryl-2-pyrones, *Trypanosoma cruzi,* trypanocidal activity.

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INTRODUCTION

Trypanosoma cruzi, the etiological agent of Chagas disease, is an obligate intracellular protozoan parasite that causes acute and chronic infection in several mammalian species, including man. Transmitted by blood-sucking triatomines (Heteroptera: Reduviidae), *T. cruzi* occurs throughout Central and South American countries including Mexico, where it is estimated that 16 to 18 million people are nowadays infected and approximately 120 million people are living in areas under risk of infection (WHO, 2002). In urban areas of several Central and South American countries, transmission of Chagas disease by means of transfusion of infected blood is still a major health problem (Schmuñis et al., 2001).

It is well established that gentian violet (crystal violet) is an effective compound for the prevention of Chagas disease by blood transfusion. However, the use of gentian violet is limited due to the toxic and other side effects of the compound such as alteration of the skin color, mucous membranes and urine (Ramirez et al., 1995). The current chemotherapy for human Chagas disease is based on only two nitro-heterocyclic derivatives named benznidazole (Rochagan®) and nifurtimox (Lampit®). Both compounds present a limited efficacy in the chronic phase of the infection and several side effects for the patients (Croft et al., 1997).

The search for new bioactive molecules on natural products has proven to be a powerful approach for discovery and development of new drugs (Cragg et al., 1997; Soejarto, 1996; Sepúlveda-Boza & Cassels, 1996; Bezerra et al., 2006; Saúde-Guimarães & Faria, 2007; Barbosa-Filho et al., 2007;), thus, outnumbered plant species used in traditional medicine have been screened for biological activity (Berger et al., 1998; Mafezoli et al., 2000; Muelas-Serrano et al., 2000; Weniger et al., 2001; Schinella et al., 2002). Among these, phytochemical and biological studies of species of the genus *Polygala* revealed the presence of a broad spectrum of secondary metabolites with promising biological activity (Pizzolatti et al., 2003). The presence of a bisanthraquinone in *P. sabulosa*, an coumarin-hemiterpene ether, as well as the styryl- and dihydrostyryl-2-pyrones, a new class of compounds for the genus *Polygala*, were recently described (Pizzolatti et al., 2000; 2004). Along with the detection of styryl-pyrones and one coumarin, already described for this species, the present work describes the isolation of a sterol (**9**) and 3 flavonoids (**10, 11, 12**) reported for the first time in *P. sabulosa*. Also, the evaluation of the *in vitro* trypanocidal activity of some compounds was carried out.

MATERIAL AND METHODS

Plant specimens

P. sabulosa was collected in November 1999 in Rancho Queimado (Santa Catarina, Brazil) and identified by Prof. Dr. Olavo A. Guimarães. A voucher specimen was deposited under number 19640 at the Herbarium of the Department of Botany at the Universidade Federal do Paraná, Brazil.

Extraction and isolation procedures

Whole plants were air-dried (500 g), ground to a powder and extracted with ethanol 96% at room temperature for 14 days. The alcoholic extract was then dried and the crude extract (135 g) obtained was adsorbed on silica gel (250 g) and applied at the top of a silica gel short column (5 x 5 cm). Exhaustive elution with hexane (500 mL) followed by CH_2Cl_2 (1 L), ethyl acetate (1 L) and finally ethanol (1 L) yielded the hexane (1.6 g), dichloromethane (28 g), ethyl acetate (16.8 g) and ethanol (63 g) fractions. The dichloromethane fr. (12 g) was subjected to column chromatography on silica gel (3 x 30 cm) and was eluted with a hexane/EtOAc gradient (56 fr., 75 mL each). The fractions obtained were then monitored by TLC (Thin-layer chromatography) (viewed by spraying with sulfuric vanillin reagent followed by heating at 110 °C) and similar fractions were combined. Combined fr. 10-14 was recrystallized from acetone to give α-spinasterol **9** (15 mg). Combined fr. 19-25 were dissolved in EtOAc (50 mL) and treated under heating and shaking with active coal (1 g). After filtration, drying (anhydrous Na_2SO_4) and removal of the solvent, the white residue was purified by recrystallization from hexane-EtOAc (3:1) to obtain compound **8** (400 mg). The combined fr. 30-36 gave a yellow powder containing the compounds **1**, **2**, **3** and **4** (identified by GC-MS) that have been previously described for this species (Pizzolatti et al., 2000). The major compound **2** (350 mg) was purified from combined fr. 30-36 by silica gel (230-400 mesh) flash chromatography (2 x 25 cm) using hexane-EtOAc (3:1) as the eluent. The EtOAc-soluble fr. (10 g) was further subjected to column chromatography over silica gel (3 x 30 cm) using hexane-EtOAc and EtOAc-EtOH gradient to give 72 fr. of 50 mL each. The GC-MS analysis of the combined fr. 8-13 exhibited five peaks for compounds **2**, **4**, **5**, **6** and **7** as previously described (Pizzolatti et al., 2000; 2004). Styryl-2-pyrone **7** (185 mg) was the major component among fr. 8-13 and was further purified by flash chromatography, as described above, using hexane-EtOAc 2:3. The combined fr. 19-21 was further crystallized from acetone to yield apigenin **10** (4 mg). The combined fr. 30-36 was further chromatographed on silica gel (3 x 25 cm) eluting with a hexane-acetone gradient from 90:10 to 0:100 (60 fr., 30 mL each) to obtain quercetin **11** (25 mg). The combined polar fr. (45-60) was further subjected to column chromatography on Sephadex LH-20 (1.5 x 10 cm) eluted with MeOH-H_2O (3:1, 30 mL) to give

a yellow solid that after recrystallization from acetone-MeOH, yielded quercetin-3-*O*-glucoside [3-*O*-(β-D-glycopyranosyl)-quercetin] **12** (52 mg).

Melting point, IR, NMR, GC-MS, CC, TLC and visualization were determined as previously described (Pizzolatti et al., 2000; 2004).

In vitro bioassays

The *in vitro* trypanocidal assays were performed as previously described (Pizzolatti et al., 2003). The activities against epimastigote and bloodstream trypomastigote forms were expressed as the percentage of reduction in the parasite number and the inhibitory concentration of 50% (IC_{50}) and as the percentage of parasitemia reduction, respectively. Both activities were determined by one-way analysis of variance (ANOVA) using Prism® v. 2.0 software (GraphPad Software Incorporated, San Diego). Negative tests with blood trypomastigotes, i.e., tests where no parasites were observed after incubation with the compound, were inoculated into groups of 2 healthy Swiss mice to confirm the absence of parasites. One week after inoculation, fresh blood examination was performed on each mouse every two days up to 30 days of inoculation when hemoculture in LIT medium was carried out for negative animals. Cultures were maintained at 28°C and examined for the presence of parasite epimastigotes every two weeks up to 60 days. The assays involving experimentation animals were approved by the UFSC Ethics Committee (CEUA 155/2002).

Compounds proved to be active against *T. cruzi* epimastigote and trypomastigote forms were further evaluated against intracellular forms of the parasite. For that, culture-derived *T. cruzi* trypomastigotes (Y strain) were used to infect Vero cells (ATCC-CCL81) (5:1 ratio) previously cultivated on 12 mm glass cover slips in 24-well plates. After 4 hours of cell-parasite interaction in DMEM medium supplemented with 5% of FBS at 37 °C, under a 5% CO_2 atmosphere, non-adhered parasites were removed by a single PBS washing and 0.5 mL of fresh medium was added to each well. The cells where then maintained as described above. Twenty-four hours after infection, compounds **8** (50 μg/mL)**, 7** (50 μg/mL) and **2** (100 μg/mL) were added to the cell monolayers and cultivated for 72 hours under the same conditions. Infected cells incubated with benznidazole (100 μg/mL) and in the absence of any drug or compound were used as controls. After incubation, all slides were rinsed once in cold PBS, fixed in Bouin for 15 minutes, washed in distilled water, Giemsa stained and mounted on glass slides with Entellan (Merck, Whitehouse Station). The experiments were performed in triplicate and a total of 300 randomly-chosen cells per cover slip were microscopically examined (400x) and the percentage of infected cells as well as the number of parasites/cell was determined. In addition, cell infection was followed daily for up to five days in order to evaluate the intracellular cycle of the parasite.

The hemolytic activity of each compound was assessed by the cyanometahemoglobin method. Briefly, 10 mL of non-infected mouse blood previously incubated with 500 μg/mL of each compound was diluted in 990 mL of a Drabkins solution in the presence of 25 mL of KCN (oxidant). The released hemoglobin is converted to cyanometahemoglobin, and the concentration was determined with a spectrophotometer (Amersham Biosciences, Piscataway) at 540 nm. The results are expressed as a percentage of hemolysis (Koga, 2003).

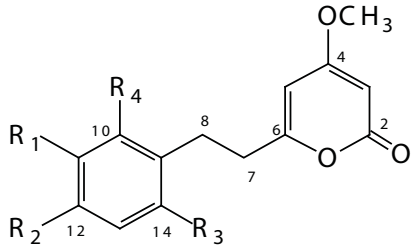
The cytotoxicity of the herein described compounds was determined through the MTT assay as formerly described (Sieuwerts et al., 1995). Vero cells were cultivated in 96-wells micro plates in DMEM medium supplemented with 5% of FBS, 100 UI of penicillin and 100 μg/mL of streptomycin/mL and incubated in a 5% CO_2 atmosphere, 95% humidity at 37 °C. After 24 hours, a new culture medium was added and the cells were incubated for 72 hours under the same conditions in the presence of 100, 50 and 25 μg/mL of compounds **2**, **7** and **8**. As controls, cells were cultivated in the absence of any drug or compound, with 1% DMSO and with benznidazole (100 μg/mL). Optical density was determined in a Bio-Tek micro reader (Molecular Devices, Sunnyvale) at 540 nm and the results expressed as the percentage of cytotoxicity.

RESULTS AND DISCUSSION

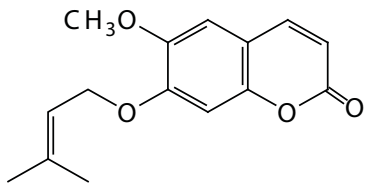
During the screening test carried out with the 4 fractions obtained from *P. sabulosa* against *T. cruzi* epimastigotes *in vitro*, no activity was observed for the ethanol fr. and a similar activity to benznidazole was observed for the hexane, EtOAc and CH_2Cl_2 frs. Despite these promising results against epimastigote forms, only the CH_2Cl_2 fr. revealed a similar activity to gentian violet, being in accordance with our previous results (Pizzolatti et al., 2003) (Table 1).

Fractionation of the CH_2Cl_2 fr. allowed the isolation of a α-spinasterol **9** $[\alpha]^{20}_D$ -2.5º, which was characterized by physical properties (TLC, $[\alpha]^{20}_D$ and m.p.) and comparison of their ^{1}H-NMR, ^{13}C- NMR and MS with known standards (Itoh et al., 1981). Two other compounds, 6-methoxy-7-prenyloxycoumarin **(8)** and 4-methoxy-6-(11,12-methylenedioxy-14-methoxydihydrostyryl)-2-pyrone **(2)**, were also isolated and identified by spectroscopy and comparison to authentic samples (Pizzolatti et al., 2000).

Fractionation of the EtOAc fr. on silica gel and Sephadex LH-20 revealed two major compounds, styryl-2-pyrone **7** as previously reported (Pizzolatti et al., 2000) and quercetin-3-*O*-glucoside **12**, and also two minor flavones (apigenin **10** and quercetin **11**), identified by spectral analyses and by comparison of the spectrum with literature data (Agrawal, 1989). The flavonoids



1. R_1-R_2= OCH_2O; R_3=R_4- H,
2. R_1-R_2= OCH_2O; R_3=OCH_3; R_4- H,
3. R_1-R_2= OCH_2O; R_3=R_4= OCH_3;
4. R_1-R_2= OCH_2O; R_3=R_4- H, Δ^{7-8}= double bond
5. R_1-R_2= OCH_2O; R_3=OCH_3; R_4- H, Δ^{7-8}= double bond
6. R_1-R_2= OCH_2O; R_3=R_4= OCH_3, Δ^{7-8}= double bond
7. R_1=R_2= OCH_3; R_3=R_4- H, Δ^{7-8}= double bond



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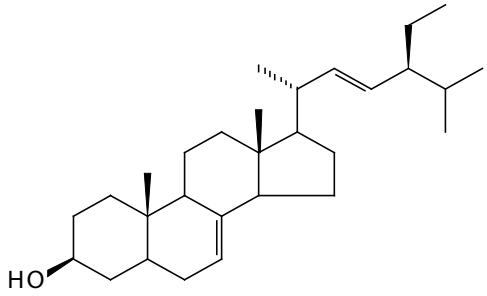
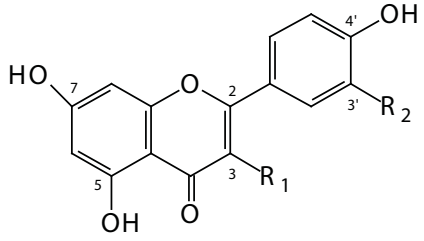


9



10. R_1-R_2= H,
11. R_1-R_2= OH,
12. R_1= β-D-glycopyranosyl; R_2= OH

10-12 and the α-spinasterol **9** herein described are the first report of these compounds for *P. sabulosa*. The trypanocidal activity of compound **10** was not determined because only a little amount was isolated and compound **11** is an already known substance and its activity has already been determined (Tasdemir et al., 2006; Silveira et al., 2005).

Four isolated compounds (**2**, **7**, **8** and **9**) showed interesting trypanocidal activity against epimastigote forms (IC_{50} 10.5 up to 33.5 μg/mL) but only compound **8** revealed to be active against trypomastigotes (IC_{50} 88.2 μg/mL) (Table 2).

In order to assess the activity of prenyloxycoumarin **8** against *T. cruzi* blood trypomastigotes, infected mouse blood was incubated *in vitro* at 4 ºC for 48 h with 250 μg/mL and 500 μg/mL of the compound. After incubation, the presence of live parasites was microscopically assessed and the treated blood was inoculated into healthy mice in order to further assess the presence of parasites. As observed for the control group treated with 250 μg/mL of gentian violet, no parasites were observed by microscopy in blood treated with both concentrations of prenylcoumarin **8**. However, mice inoculated with blood treated with 250 μg/mL of compound **8** presented detectable parasitemia in the second post-inoculation week and showed positive hemoculture. In contrast, all mice inoculated with infected blood treated with 500 μg/mL of compound **8** presented negative hemoculture. The hemolysis rate to mouse erythrocytes was tested using 500 μg/mL of each fraction or compound and varied from 3% - 9% and from 2% - 6%, respectively. This result allows concluding that parasite clearance is not due to unspecific lysis activity.

In the search for new chemoprophylatic agents for Chagas disease, Chiari et al. (1996) studying ethanol extracts from 47 Asteraceae species showed that 11 extracts reduced by 100% blood trypomastigotes *in vitro* at a concentration of 12 mg/mL. Zani et al. (1995) evaluating trypanocidal activity of ethanol extracts of 52 Asteraceae species showed that 10% of them reduced by 70% the number blood trypomastigotes *in vitro* at a concentration of 250 μg/mL. Mafezoli et al. (2000), studying 25 fractions from 9 species of the Rutaceae family, showed that 6 fractions produced an 80 - 100% parasitemia reduction of *T. cruzi* trypomastigotes *in vitro* at concentrations of 2 mg/mL. Scio et al. (2003a) reported that a diterpene compound from *Alomia myriadenia* at 250 μg/mL produced a parasitemia reduction of 100% for *T. cruzi* blood trypomastigotes *in vitro*.

Trypanocidal activity of a coumarin from *Kielmeyera albopunctata*, a plant species from the Brazilian Cerrado, produced an 80% mortality rate of *T. cruzi* blood trypomastigotes at 125 μg/mL in a 24 hour period (Scio et al., 2003b). In the present study, coumarin **8** obtained from *P. sabulosa* showed *in vitro* activity against both cultured epimastigotes and blood trypomastigotes (IC_{50} 10.5 and 88.2 μg/mL, respectively). However, only concentrations ≤ 500 μg/mL of the compound was able to prevent mouse infection *in vivo*. The other compounds **2**, **7** and **12** presented a

Table 1. *In vitro* trypanocidal activity of fractions obtained from *Polygala sabulosa* against *Trypanosoma cruzi* (Y strain) culture epimastigote and blood trypomastigote forms and percentage of hemolysis of mouse erythrocytes.

Fractions and Controls	IC_{50} μg/mL*		Hemolysis rate at 500 μg/mL (%)
	Epimastigote	Trypomastigote	
Hexane	1.0 (0.6-2.1)	>1000	ND
Aqueous	NA	748.2 (708.6-790.1)	9
Ethyl acetate	10.3 (5.8-18.4)	993.6 (845.2-1168.1)	3
Dichloromethane	10.4 (5.1-21.1)	147.6 (96.9-224.9)	3
Benznidazole	13.6 (8.5-21.6)	ND	-
Gentian violet	ND	115.1 (100.6-126.8)	ND

NA = not active; ND = not determined.
*Results represent the mean of 3 independent experiments with a 95% confidence interval in parentheses.

Table 2. *In vitro* trypanocidal activity of compounds isolated from *Polygala sabulosa* against *Trypanosoma cruzi* (Y strain) culture epimastigote and blood trypomastigote forms and percentage of hemolysis of mouse erythrocytes.

Compound and Controls	IC_{50} μg/mL*		Hemolysis rate at 500 μg/mL (%)
	Epimastigote	Trypomastigote	
8	10.5 (4.9-22.0)	88.2 (57.3-35.7)	3
2	13.7 (6.3-29.9)	636.3 (601.9-672.9)	2
7	25.9 (17.8-37.5)	1008.6 (919.7-1106.0)	6
9	33.5 (28.0-40.3)	ND	-
12	326.3 (244.5-435.5)	945.6 (845.6-1057.2)	5
Gentian violet	ND	115.1 (100.6-126.8)	ND
Benznidazole	13.6 (8.5-21.6)	ND	ND

ND = not determined.
* Results represent the mean of 3 independent experiments with a 95% confidence interval in parentheses

Table 3. Cell toxicity, infection rate and number of intracellular amastigotes/cells of Vero cells infected with *Trypanosoma cruzi* (Y strain) and treated with different concentrations of compounds isolated from *Polygala sabulosa*.

Compounds and Controls	Concentration (μg/mL)	Cell toxicity (%)	Vero cell	
			Cell infection rate (%)	Number of amastigotes/cell
Benznidazole	100	25	0	0
8	50	29	4	4 ± 1.5
7	50	26	14	8 ± 1.9
2	100	22	5	4.2 ± 1.8
Negative Control	-	-	16	8.0 ± 3.1

Results represent the mean of 2 independent experiments.

weak activity against *T. cruzi* blood trypomastigotes and none of them produced a 100% parasite clearance in infected blood. All active compounds described herein showed a low hemolysis rate (2 - 6%) even at high concentrations (2 mg/mL), suggesting a selective trypanocidal activity.

Based on the obtained IC_{50} values, we can conclude that cytotoxicity of the studied compounds toward Vero cells proved to be concentration-dependent (data not shown). Prenylcoumarin **8** and styryl-2-pyrone **7** were the most toxic compounds, whereas dihydrostyryl-2-pyrone **2** presented a similar toxicity to that of benznidazole. Infected Vero cells treated with prenylcoumarin **8** (50 μg/mL) and dihydrostyryl-2-pirone **2** (100 μg/mL) for 72 hours revealed a 3-4-fold decrease in the percentage of infected cells and a 2-fold reduction in the number of intracellular parasites/cell in comparison to the control. Compound **7** did not show activity against amastigotes at any tested concentration it was observed in cells treated with benznidazole (100 μg/mL). *In vitro* assays to determine the intracellular trypanocidal activity showed that compounds **8** and **2** produced a 3-4-fold reduction in cell infection by *T. cruzi* as well as in the number of intracellular amastigotes. However, the most active compound **8** showed a high cytotoxicity (50%) toward Vero cells even at low concentrations (~100 μg/mL) (Table 3).

Four out of five active compounds from *P. sabulosa* presented a significant growth inhibition of *T. cruzi* epimastigotes, and two of them also revealed activity against blood trypomastigotes and intracellular amastigotes *in vitro*, possibly constituting styryl-2-pirone and coumarin new groups of anti-*T. cruzi* compounds.

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