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DOI 10.1055/s-0042-112031
Planta Med 2017; 83: 239–244

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Rüdigerstraße 14
70469 Stuttgart
ISSN 0032-0943

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Trypanocidal Activity of Flavokawin B, a Component of *Polygonum ferrugineum* Wedd

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Key words

Trypanosoma cruzi, Trypanosoma brucei, flavokawin B, chalcone, trans-splicing, Polygonum ferrugineum, Polygonaceae

received March 16, 2016
revised June 26, 2016
accepted July 5, 2016

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DOI http://dx.doi.org/10.1055/s-0042-112031
Published online July 21, 2016 | Planta Med 2017; 83: 239–244

ABSTRACT

The trypanocidal potential of the natural chalcone flavokawin B, which was isolated from the hexanic extract of *Polygonum ferrugineum* Wedd., is reported here. Although flavokawin B is widespread, this is the first report about its trypanocidal properties on both *Trypanosoma cruzi* (IC_{50} = 9.5 µM, IC_{50} = 34.7 µM benznidazol, Y strain) epimastigotes and *Trypanosoma brucei* (IC_{50} = 4.8 µM, IC_{50} = 6.4 µM pentamidine, 29–13 strain) procyclic forms, which was also corroborated on *T. brucei* strain 427 (IC_{50} = 6.2 µM). In order to learn more about its properties, unspecific cytotoxicity on Hep G2 cells was investigated as well as the *trans*-splicing inhibitory potential on *T. brucei* cells. The results shown here point to flavokawin B as a candidate in the search for new agents. It is also cheaper and less toxic than the available drugs to treat trypanosomiasis with a special focus on sleeping sickness disease.

ABBREVIATIONS

HAT	human African trypanosomiasis
EHB	hexanic extract
EDB	dichloromethanic extract
EAB	ethyl acetate extract
EMB	methanolic extract
SI	safety index

Introduction

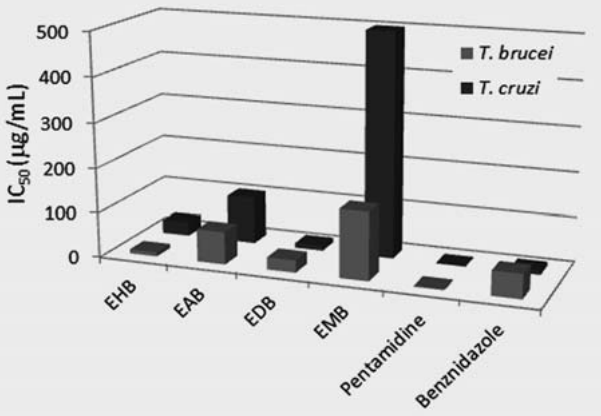
Trypanosomatid parasites are systemic protozoa causing neglected diseases related to poor sanitation and cultural factors, which affect several millions people, mostly in South America and Africa. As a consequence of globalization, American trypanosomiasis (Chagas' disease) can be now considered a worldwide illness [1]. Chagas' disease is a protozoan zoonotic disease caused by the hemoflagellate *Trypanosoma cruzi* [2]. Chagas' disease is considered the most neglected of the tropical diseases and, despite several efforts to get alternatives to benznidazole, the therapeutic scheme has remained the same for more than 40 years, and there is no effective treatment for the chronic phase of the disease yet [3].

HAT is a fatal disease caused by *Trypanosoma brucei* species, transmitted to human by flies of the *Glossina* genus, known as tsetse flies. Two subspecies of *T. brucei* are pathogenic for humans, *T. brucei* gambiense and *T. brucei* rhodesiense [4]. There are two forms of sleeping sickness, the gambiense HAT, which is endemic in West and Central Africa and causes over 95% of current cases, and the rhodesiense HAT, which is endemic in East and Southern Africa and accounts for the remainder of cases. The presence of parasites in the brain of infected patients leads to a progressive neurological breakdown. The World Health Organization's (WHO) recommended drugs to treat HAT are pentamidine and suramin for the first stage, and melarsoprol eflornithine and nifurtimox for the second stage [5]. Despite the advancements in HAT treatment, all currently available options are suboptimal and, consequently, the development of new, safer, effective, and easy to use compounds is a high priority [6].

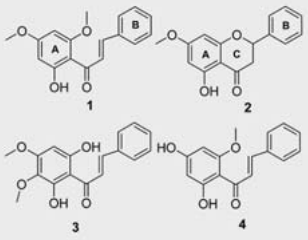
Trypanosomatid parasites utilize RNA splicing for the maturation of nuclear pre-mRNA in two distinct ways. First, as in other eukaryotes, *cis*-splicing is used for intron removal. Second, trypanosomatids process all of their nuclear pre-mRNAs by spliced leader *trans*-splicing. This process is a post-transcriptional mRNA capping mechanism and, since trypanosomatids transcribe their protein coding genes polycistronically, it resolves individual mRNAs from polycistronic precursors in conjunction with polyadenylation [7]. Spliced leader *trans*-splicing occurs in several organisms (e.g., tunicate chordates, nematodes, and trematodes), but it is not found in insects and mammalian cells, thus, it is specific to the parasites and not frequently present in the hosts of trypanosomatids [8]. Hence, factors with specific functions in the *trans*-splicing process may be potential chemotherapeutic targets [9]. The short transcript spliced leader (SL RNA) or miniexon is a primary transcript synthesized independently from pre-mRNA and *trans*-spliced in all nuclear mRNA. The majority of mRNAs is synthesized as polycistronic precursors [10]. *T. brucei* SL RNA contains an unusual cap structure consisting of 7-methylguanosine linked to four modified nucleosides. This modification of the trypanosomatid SL sequence might be required for the function of the SL RNA in *trans*-splicing and the mRNA during translation [11].

Polygonum ferrugineum Wedd. (Polygonaceae) is a bush commonly found in temperate climates of South America on the boundaries of rivers, and accumulates mostly chalcones, flavanones, and homoisoflavanones among its secondary metabolites [12]. López et al. [13] studied the antifungal activity of the species and demonstrated that chalcones pashanone and cardamonin isolated from a crude EDB of its aerial parts displayed high antifungal activity.

In this work, we present the trypanocidal activity on *T. brucei* and *T. cruzi* strains of *P. ferrugineum* extracts and the bioassay-guided fractionation of the EHB. In addition, the cytotoxicity of the isolated compounds was tested, as well as the *trans*-splicing inhibitory potential on *T. brucei* of the most active isolated compound (**1**, flavokawin B).



▶ **Fig. 1** *T. cruzi* (epimastigotes, Y strain) and *T. brucei* (procyclic forms, 29–13 strain) trypanocidal activity (IC_{50} µg/mL) of hexanic (EHB), ethyl acetate (EAB), dichloromethane (EDB), and methanol (EMB) extracts from the aerial parts of *P. ferrugineum*.



▶ **Fig. 2** Chemical structures of compounds isolated from EHB of the aerial parts of *P. ferrugineum*.

Results and Discussion

The aerial parts of *P. ferrugineum* were sequentially extracted with *n*-hexane, dichloromethane, ethyl acetate, and methanol, and the obtained extracts (EHB, EDB, EAB, and EMB, respectively) were submitted to trypanocidal evaluation on epimastigotes of *T. cruzi* Y strain and on procyclic forms of *T. brucei* 427 and 29–13 strains.

The EDB extract was the most active on the *T. cruzi* Y strain, showing an IC_{50} of 10.5 µg/mL. The best extract of all was EHB on both *T. brucei* strains evaluated (IC_{50}s of 8.6 µg/mL for strain 427 and 9.2 µg/mL for strain 29–13), which was in order with the positive controls pentamidine (IC_{50}s of 6.4 µM and 2.2 µg/mL, respectively) and benznidazole (IC_{50}s of 34.7 µM and 54.1 µg/mL, respectively) (▶ **Fig. 1**). The EHB extract was selected for further studies.

The EHB extract was subsequently fractionated by silica gel column chromatography, affording the isolation of the chalcones flavokawin B (**1**), pashanone (**3**), and cardamonin (**4**) and the flavanone pinostrobin (**2**) (▶ **Fig. 2**). The isolation and antifungal activity of compounds **3** and **4** was previously reported from the dichloromethanic extract of the aerial parts from this species [12, 13].

When the purified compounds were tested against *T. cruzi* Y strain and *T. brucei* 427 and 29–13 strains, compounds **1**, **3**, and **4** showed an inhibitory effect on *T. cruzi* with IC_{50} values of 9.5, 32.3, and 24.8 µM, respectively. Regarding *T. brucei*, only compound **1** was active, showing IC_{50} values of 6.2 µM and 4.8 µM against *T. brucei* 427 and 29–13 strains, respectively (▶ **Table 1**).

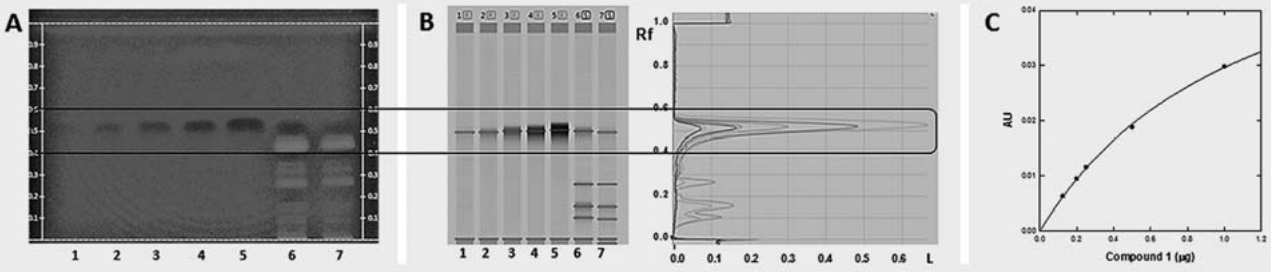
Cytotoxicity of the isolated compounds was assessed in Hep G2 cells and SIs were calculated (▶ **Table 1**). None of the compounds tested showed cytotoxicity up to 500 µg/mL. The pentamidine SI was 25.1, while the compound **1** SIs were 86.3 and 112.3 for *T. brucei* strains 427 and 29–13, respectively. Regarding *T. cruzi*, the SIs were greater than 50, less toxic than the positive control drug benznidazole (SI of 62.5).

Compounds **1**, **3**, and **4** possess a chalcone scaffold (▶ **Fig. 2**), with differences in the substitution pattern of ring A [14]. Chalcones, or 1,3-diaryl-2-propen-1-ones, are an abundant group of natural compounds belonging to the flavonoid class that possess

▶ **Table 1** *T. brucei* and *T. cruzi* trypanocidal activity (IC_{50} µM), Hep G2 cytotoxicity (IC_{50} µM), and safety index (SI) of the isolated compounds from the EHB of the aerial parts from *P. ferrugineum*.

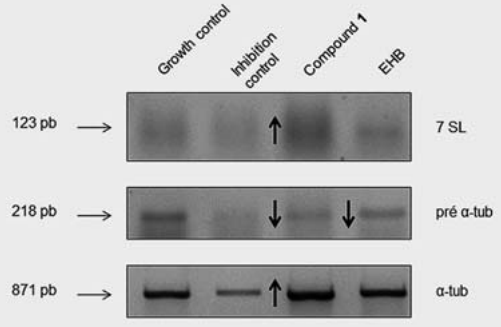
Sample	Hep G2 cells	*T. brucei* strain 427	S.I. *T. brucei* strain 427	*T. brucei* strain 29–13	S.I. *T. brucei* strain 29–13	*T. cruzi* Y strain	S.I. *T. cruzi* Y strain
1	537.8	6.2	86.3	4.8	112.3	9.5	56.6
2	1851.2	I*	[a]	I*	[a]	I*	[a]
3	> 1666.7	I*	[a]	I*	[a]	33.0	50.5
4	> 1851.8	I*	[a]	I*	[a]	24.8	74.6
Pentamidine	159.7	6.4	24.8	6.4	24.8	4.6	34.9
Benznidazole	2126.2	207.6	10.2	149.6	14.2	34.7	62.5

IC_{50} values were calculated applying probit analysis. I* = IC_{50} > 100 µg/mL; [a] SI not determined



▶ **Fig. 3** HPTLC densitometric quantification of compound **1** in the EHB extract. **A** Image of the HPTLC Si 60 F_{254} plate under UV 366 nm (mobile phase: hexane : ethyl acetate 8 : 2). Tracks 1 to 5: 0.125, 0.25, 0.5, and 1 µg of purified compound **1**; tracks 6 and 7: 2 and 1 µg of EHB extract. **B** Pick assignment (gel view) and scan plots at UV 366 nm for tracks 1 to 7 (absorbance mode). **C** Titration curve for compound **1**. Symbols represent the area under the curve for the peaks at Rf = 0.53 for different amounts of compound **1** applied on the HPTLC plate (see **A** and **B**). The curve represents the fit of the experimental data to a rectangular hyperbola.

a broad spectrum of biological activities, including antibacterial [15], antifungal [14], anthelmintic, amoebicidal, antiulcer [16], antiviral, insecticidal, antiprotozoal [17], anticancer [18], cytotoxic, and immunosuppressive activities [19]. Regarding *T. cruzi*, it is interesting to note that compound **1** showed better trypanocidal activity (IC_{50} of 9.5 µM; ▶ **Table 1**) than the unsubstituted chalcone, which was reported by Lunardi et al. [20] as the best of a series of synthetic chalcones (IC_{50} of 24.8 µM). Regarding antifungal activity, compound **1** was inactive when tested, in contrast with the unsubstituted chalcone, which was the most active molecule, as shown previously [13, 14]. These results show the importance of substituents in chalcones, which certainly determine bioactivity and could suggest some selectivity of compound **1** as a trypanocidal agent. Regarding *T. brucei* inhibition by chalcones, there is scarce information. Some synthetic analogs were tested on *T. brucei brucei*, though, nevertheless, none of them had the typical skeleton of natural chalcones bearing –OH or $–OCH_3$ as substituents [21]. Taking into account that compound **1** is a natural metabolite from plant origin with interesting trypanocidal potential, it was quantified in the EHB extract, and a study about its mode of action was carried out. The content of flavokawin B in the EHB extract was calculated by HPTLC scanning densitometry, as described in Material and Methods, and was 13.6% (▶ **Fig. 3**).



▶ **Fig. 4** Agarose gel (1.5%) stained with ethidium bromide showing the semiquantitative RT-PCR products after treated and non-treated *T. brucei* parasites. PCR primers: 7 SL:7SL1/7SL2; Pre-mRNA α-tubulin : TS3/TS4; mature RNA α-tubulin : TS1/TS2. ↑: RNA accumulation; ↓: RNA decrease. Growth control: parasites with no drug treatment. Inhibition control: pentamidine.

In order to gain information about the mode of action, compound **1** was evaluated for its capacity to generate changes in the production of mature mRNA after the *trans*-splicing of parasitic immature mRNA molecules of *T. brucei*. A semiquantitative RT-PCR experiment was carried out with the EHB extract and compound **1** in comparison with pentamidine (▶ **Fig. 4**).

The presence of bands from both processed and non-processed forms of α-tubulin (α-tub) were observed in the growth control, as expected. Pentamidine produced a low intensity of all bands (7 SL, pre-α-tub, and α-tub). Since it bonds to kinetoplastid DNA, it caused the disintegration and breakdown of the mitochondrial membrane and cell death, which is in agreement with previous literature [22]. After EHB treatment, parasites demonstrated a slight decrease on the expression of α-tubulin non-processed form (pre-α-tub), with no clear changes on the processed form (α-tub) compared with growth control. This result suggests some interference of EHB at pre-mRNA processing, but the *trans*-splicing still present forming mature RNA. On the other hand, after treatment with **1**, a strong decrease on the processing of pre-α-tub and the accumulation of both the α-tub and 7 SL was observed. These results demonstrate that compound **1** effectively interferes with the *trans*-splicing reaction on *T. brucei* (▶ **Fig. 4**).

The trypanocidal activity of *P. ferrugineum* was studied and the EHB was selected for further studies because its promising IC_{50} values on *T. brucei* strains (IC_{50} of 8.6 µg/mL for strain 427 and 9.2 µg/mL for strain 29–13, respectively). The fractionation of EHB led to the isolation of four previously reported compounds, flavokawin B (**1**), pinostrobin (**2**), pashanone (**3**), and cardamonine (**4**). Our results demonstrate that compound **1** showed higher trypanocidal activity than pentamidine and benznidazole, with SI values better than the control drugs, indicating that it is an interesting starting point for further studies using *T. brucei* as a model, especially its bloodstream infecting forms. In addition, its quantification by HPTLC methodology was performed.

Further experiments of RT-PCR demonstrated that EHB and compound **1** affected the mRNA process by interference of the *trans*-splicing reaction on *T. brucei*. Consequently, mature mRNA and protein synthesis may be affected. Although deeper and more conclusive studies are necessary to understand how compound **1** causes *T. brucei* inhibition, to our knowledge, this is the first time that a *trans*-splicing mechanism is suggested using this approach. Although procyclic forms are not infective to humans, the screening could be considered a first step in the approach for discovery of natural trypanocidal agents, especially *T. brucei* inhibitors. A search for new candidates that are cheaper and less toxic than the available drugs to treat neglected diseases is an urgent necessity of our globalized world, as was recognized by the 2015 edition of the Nobel Prize in Medicine, which awarded three scientists who developed therapies against parasitic infections with natural products.

Material and Methods

Plant material

P. ferrugineum Wedd. (Polygonaceae) was collected at Puerto Gaboto, Santa Fe province, Argentina in January 2011. Botanical identification was performed by Dr. Susana Gattuso and a voucher specimen was deposited in the Herbarium of the Vegetal Biology Area of the National University of Rosario and identified as Gattuso, S (899) UNR 2028.

General methods

Chromatographic analyses were performed using Merck silica gel 60 (230 mesh) for column separations and precoated silica gel 60 PF_{254} for TLC qualitative analysis. Glass-backed silica gel 60 F_{254} HPTLC plates (10 × 10 cm) were from Merck. 1D (^1H, ^{13}C, and DEPT 135°) and 2D (gHMQC, gHMBC, and ^1H-^1H gCOSY) NMR experiments were recorded on a Bruker spectrometer operating at 300 MHz (^1H) and 125 MHz (^{13}C), respectively, with $CDCl_3$ and/or TMS as the internal reference. Negative ion high-resolution mass spectra were acquired on an MS ultrOTOFQ-ESI-TOF instrument (Bruker Daltonics) using MeOH-H_2O (1 : 1) as the solvent, and a cone voltage of 40 V. Automatized HPTLCs were performed in a Camag system constituted by an automatic TLC Sampler 4, an automatic development chamber ADC2, a TLC Visualizer, and a TLC Scanner 4. Plates were analyzed with Vision CATS software (Camag).

Fractionation of plant material and isolation of compounds 1–4

Air-dried powdered aerial parts of *P. ferrugineum* (211.2 g) were sequentially macerated with solvents with increasing polarity, hexane (3 × 24 h), dichloromethane (5 × 24 h), ethyl acetate (3 × 24 h), and methanol (3 × 24 h). Solvents were eliminated by evaporation under reduced pressure to obtain EHB (16.0 g), EDB (8.2 g), EAB (1.8 g), and EMB (18.1 g).

An aliquot of EHB (0.6 g) was submitted to column chromatography on silica gel and was eluted with mixtures of hexane and ethyl acetate with increasing polarity as the mobile phases (from Hex 100 % to ethyl acetate 100 %, increasing 10 % each) to give 15 fractions. Fractions 4 and 5 were submitted to recrystallization overnight at − 20 °C in Hex : ethyl acetate (90 : 15) and (90 : 10) mixtures to give compounds **2** (5-hydroxy-7-methoxyflavanone, pinostrobin) [23] and **1** (2′-hydroxy-4′,6′-dimethoxychalcone, flavokawin B) [24], respectively. Compounds **3** (2′,6′-dihydroxy-3′,4′-dimethoxychalcone, pashanone) [25] and **4** (2′,4′-dihydroxy-6′-methoxychalcone, cardamonin or alpinetin chalcone) [26] were purified by gradient column chromatography on fractions 7 and 8 (eluted with Hex : ethyl acetate 85 : 15 to 75 : 25). Copies of the original spectra are obtainable from the corresponding author.

HPTLC densitometric quantification of compound 1 in the hexanic extract

A stock solution of purified compound **1** (1 mg/mL; > 98 % purity determined by densitometric analysis on TLC after spraying with H_2SO_4 ethanolic solution followed by heating) was prepared by dissolving 1.9 mg in 1.9 mL of ethyl acetate. Aliquots of stock solution were diluted with ethyl acetate to obtain standard solutions containing 0.05 and 0.5 mg/mL. Different amounts of substance (0.125 to 1 µg) were automatically applied (band width 8 mm, distance between tracks 5 mm) on a 10 × 10 cm HPTLC plate (Merck 60 F_{254} ng) using the ATS4 (Camag). After developing the

plate twice [Hex : ethyl acetate (80 : 20), distance 6 cm, temperature 24 °C, humidity 33 % ADC2, Camag], the plate was air-dried and scanned (TLC Scanner 4, Camag) at 366 nm. The peak areas (Rf = 0.53) were recorded, and a calibration curve was obtained by plotting the peak area versus the applied amount of compound **1** (SigmaPlot software v 10.0®). Calibration data were fitted to a rectangular hyperbola. In order to quantify compound **1** in the EHB, 2 and 4 µL of a solution of the EHB extract (0.5 mg/mL in Hex) were applied on the same HPTLC plate as described above, and the area of the peak observed at Rf = 0.53 (366 nm) was extrapolated into the calibration curve.

Cultures of *Trypanosoma brucei* and *Trypanosoma cruzi*

Procyclic forms of *T. brucei* (427 and 29–13 strains) [27, 28] were grown at 28 °C in SDM-79 medium [29] containing 10 % fetal bovine serum (Gibco), penicillin (Sigma-Aldrich), and streptomycin (Sigma-Aldrich). Cultures were carried out to obtain the exponential growth phase (1×10^6 parasites/mL). Log phase procyclic parasites were frozen at − 80 °C with 10 % glycerol for storage.

Epimastigote forms of *T. cruzi,* Y strain [30] were cultured at 28 °C in liver infusion tryptose (LIT) medium supplemented with 10 % fetal bovine serum (Gibco). Epimastigote form cultures were carried out until to obtaining them in the exponential growth phase (1×10^7 parasites/mL).

MTT colorimetric assay of *Trypanosoma brucei* strains

Procyclic forms of *T. brucei* (427 and 29–13 strains) were incubated for 24 h with each extract or with pure compounds for determination of the cytotoxicity index (CI_{50}) accessed by the MTT colorimetric assay. Assays were performed in 96-well plates, according to the methodology described by Cotinguiba et al. [31]. Each test was made in duplicate. The extracts and isolated compounds were solubilized in DMSO (Synth), and were diluted to give different concentrations using SDM-79 medium. The final concentration of the solutions reached up to 3 % DMSO, which does not affect the viability of the parasites. Pentamidine (98 % purity, Sigma) in SDM-79 and the sole SDM-79 medium were used as positive and negative controls, respectively.

MTT colorimetric assay of *Trypanosoma cruzi* Y strain

Some authors mention the positive correlation between activity against epimastigotes *in vitro* and *in vivo* activity against trypomastigotes. In our study, we decided to use the epimastigote form [32–34]. Epimastigotes of *T. cruzi* Y strain were treated with either extract in accordance with the method of Muelas-Serrano et al. with modifications [31]. Benznidazol (97 % purity, Sigma) was used as a positive control. The IC_{50} values were obtained from the duplicate average [31].

From both parasites tests, the cytotoxicity index (IC_{50}) was calculated with the Origin 7.0 program [35] using the probit analysis for the statistics [31, 36].

MTT colorimetric assay of HepG2 cells [37]

Cytotoxicity assays were performed using human hepatoma cells (HepG2) [38]. The cells were cultured in minimum essential medium (MEM) supplemented with 10 % fetal bovine serum and antibiotics [penicillin (Sigma-Aldrich) 100 U/mL, streptomycin (Sigma-Aldrich) 0.1 mg/mL]. Cultures were maintained at 37 ± 2 °C in an atmosphere of 5 % CO_2. For cytotoxicity assays, 1×10^6 cells/mL were used. The plates were incubated for 24 h to complete the adhesion of the cells in the 96-well plates [39].

In each assay, a negative control [MEM with respective concentration of DMSO (Sigma-Aldrich) per treatment] and a positive control (DMSO MEM 10 %) were tested. All compounds were tested at different concentrations (0, 5.0, 31.2, 62.5, 125.0, 250.0, and 500 µg/mL) and solubilized in medium MEM after prior dissolution in DMSO. After 24 h, the treatment was removed and the plates were gently washed with PBS (Sigma-Aldrich), and 100 µL of MTT (Sigma-Aldrich) (1 mg/mL in PBS) were added to each well [40]. The microplates were incubated at 37 ± 2 °C for 4 h, and protected from light to observe the presence of formazan crystals [39]. For the solubilization of the formazan crystals, 100 µL of isopropyl alcohol (Synth) were added to each well and the absorbance was read at 595 nm. The IC_{50} values were obtained from the duplicates and all the calculations were made according Leone et al. [41].

Semiquantitative RT-PCR to evaluate *Trypanosoma brucei* mRNA processing

Total RNA of the treated and non-treated parasites was extracted using Trizol (Invitrogen) to perform RT-PCR (reverse transcription-polymerase chain reaction) and detect the messenger RNA present in these cells according to the protocol already described [1, 8, 42]. Five µg of total RNA were used in the RT-PCR reaction. For this purpose, the kit 3 'RACE – System for Rapid Amplification of cDNA Ends (Invitrogen) was used according to the manufacturer's specifications. The PCR primers are described in ▶ **Fig. 4**.

Acknowledgements

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support. S. N. L. thanks Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina (PIP 112–201 201–00 695 CONICET), FONCyT (PICT E 2014–0175) and Universidad Nacional de Rosario (UNR, Grant BIO442) for funding. M. V. C. and S. N. L. are members of CONICET.

Conflict of Interest

The authors declare no conflict of interest.

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