SUPPLEMENTARY MATERIAL

In vitro* investigation of Brazilian Cerrado plant extract activity against *Plasmodium falciparum*, *Trypanosoma cruzi* and *T. brucei gambiense

Sébastien Charneau^{a*}, Mariana Laundry de Mesquita^b, Izabela Marques Dourado Bastos^c, Jaime Martins de Santana^c, José Elias de Paula^{d†}, Philippe Grellier^e and Laila Salmen Espindola^b.

^a Laboratório de Bioquímica e Química de proteínas, Departamento de Biologia Celular, Instituto de Ciências Biológicas, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Asa Norte, 70910-900, Brasília, DF, Brazil; ^b Laboratório de Farmacognosia, Faculdade de Ciências da Saúde, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Asa Norte, 70910-900, Brasília, DF, Brazil; ^c Laboratório de Interação Patógeno-Hospedeiro, Departamento de Biologia Celular, Instituto de Ciências Biológicas, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Asa Norte, 70910-900, Brasília, DF, Brazil; ^d Laboratório de Anatomia Vegetal, Instituto de Ciências Biológicas, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Asa Norte, 70910-900, Brasília, DF, Brazil; ^e Muséum National d'Histoire Naturelle, UMR 7245 CNRS - Molécules de Communication et Adaptation des Micro-organismes, CP52, 61 rue Buffon, 75231 Paris Cedex 05, France

* Corresponding author. Email: charneau@unb.br

† Deceased

ABSTRACT

The threatened Brazilian Cerrado biome is an important biodiversity hotspot still few explored that constitutes a potential reservoir of molecules to treat infectious diseases. We selected eight Cerrado plant species for screening against the erythrocytic stages of *Plasmodium falciparum*, human intracellular stages of *Trypanosoma cruzi* and bloodstream forms of *T. brucei gambiense*, and for their cytotoxicity upon the rat L6-myoblast cell line. Bioassays were performed with 37 hexane, ethyl acetate and ethanol extracts prepared from different plant organs. Activities against parasites were observed for 24 extracts: nine with anti-*P. falciparum*, four with anti-*T. cruzi* and eleven with anti-*T. brucei gambiense* activities. High antiprotozoal activity (IC₅₀ values < 10 µg/mL) without obvious cytotoxicity to L6 cells was observed for eight extracts from plants: *Connarus suberosus*, *Blepharocalyx salicifolius*, *Psidium laruotteanum* and *Myrsine guianensis*. Overall, studies of plant extracts will contribute to increase the biodiversity knowledge essential for Cerrado conservation and sustainable development.

Keywords:

antiprotozoal activity; malaria; Chagas disease; sleeping sickness; Brazilian Cerrado; *Connarus suberosus*

1. Experimental

1.1. *Collection of plant materials*

Eight plant species were collected in the Cerrado biome, Federal District of Brazil, in 2010: *Astronium fraxinifolium* Schott ex Spreng., *Chamaecrista desvauxii* (Collad.) Killip, *Vatairea macrocarpa* Ducke, *Connarus suberosus* Planch., *Blepharocalyx salicifolius* (Kunth) O. Berg, *Psidium laruotteanum* Cambess., *Myrsine guianensis* (Aubl.) Kuntze and *Salvertia convallariodora* A. St.-Hil. (Table S1). These species were identified by the botanist Prof. José Elias de Paula, and voucher specimens deposited in the University of Brasília (UB/UnB) Herbarium.

1.2. *Preparation of plant extracts*

Dried and powdered plant materials (stem and root bark and wood, leaves and aerial parts) were submitted to successive exhaustive extractions with 3 different solvents - hexane, ethyl acetate or ethanol, through a maceration process. The crude extracts were obtained following evaporation of solvents under reduced pressure at 40 °C. Extracts were subsequently dissolved in dimethylsulfoxide (DMSO) and homogenized by ultrasonic bath, at a stock concentration of 10 mg/mL and stored at - 80 °C. All extracts were soluble in DMSO at a concentration of 10 mg/mL.

1.3. *In vitro* bioassay of antiparasmodial activity

The chloroquine-resistant strain FcB1/Colombia of *Plasmodium falciparum* was maintained *in vitro* on human erythrocytes in RPMI 1640 medium supplemented by 8% (v/v) heat-inactivated human serum, at 37 °C, under an atmosphere of 3% CO₂, 6% O₂, and 91% N₂ (Trager and Jensen 1976). *In vitro* extract susceptibility was measured by [³H]-hypoxanthine incorporation as described (Desjardins et al. 1979, Guillon et al. 2004). Extracts were serially diluted two-fold with 100 µL culture medium in 96-well plates. Asynchronous parasite cultures (100 µL, 1% parasitaemia and 1% final hematocrit) were then added to each well and incubated for 24 h at 37 °C prior to the addition of 0.5 µCi of [³H]-hypoxanthine (GE Healthcare, France, 1 to 5 Ci·mmol/mL) per well. After a further incubation of 24 h, the plates were frozen and thawed. Cell lysates were then collected onto glass-fiber filters and counted in a liquid scintillation spectrometer. The growth inhibition for each drug concentration was determined by comparison of the radioactivity incorporated in the treated culture with that in the control culture maintained on the same plate. The concentrations

causing 50% (IC₅₀) and 90% growth inhibition (IC₉₀) were determined by linear regression analysis from the extracts and drug control concentration-response curve and the results were expressed as the mean values $\pm$ standard deviations determined from three independent experiments. The highest concentration of DMSO to which the parasites were exposed was 0.4%, which was shown to have no measurable effect on parasite viability. Stock solution of chloroquine was prepared in purified water (milli-Q grade) at 10 mM and was used as drug control.

1.4. *In vitro* bioassay of anti-*Trypanosoma cruzi* activity

The β -galactosidase-expressing *T. cruzi* trypomastigotes and amastigotes of the Tulahuen strain (*lacZ* clone 4) (Buckner et al. 1996) were maintained in monolayers of L6 cells grown in RPMI medium supplemented with 10% (v/v) fetal calf serum at 37 °C in 5% CO₂ (Grellier et al. 2001). Inhibition assays of intracellular parasite multiplication were performed in 96-well plates as described (Bosc et al. 2013). Briefly, L6 myoblasts were seeded at 5×10^3 cells per well in culture medium. After an overnight incubation, 5×10^4 trypomastigotes were added per well for 18 h. Cells were then washed twice to remove extracellular trypomastigotes and incubated with two-fold dilutions of extracts, at 37 °C, under a 5% CO₂ atmosphere for 5 days. The assays were developed by addition of the substrate chlorophenolred- β -D-galactopyranoside at 100 μ M final concentration and Nonidet P-40 (0.1% final concentration) (Buckner, Verlinde, La Flamme and Van Voorhis 1996). Plates were incubated for 4 h at 37 °C. Wells with β -galactosidase activity turned the media from yellow to red and were quantitated at 570 nm by an automated microplate reader spectrophotometer. The growth inhibition for each extract concentration was determined by comparison of the absorbance of control cultures processed in the same way, but receiving an equivalent amount of DMSO instead of extract. IC₅₀ and IC₉₀ values were obtained from the extract concentration-response curve and the results expressed as the mean values $\pm$ standard deviations determined from three independent experiments. Stock solution of nifurtimox was prepared in DMSO at 10 mg/mL and was used as drug control.

1.5. *In vitro* bioassay of anti-*Trypanosoma brucei gambiense* activity

Bloodstream trypomastigote forms of *T. b. gambiense* strain Feo were cultured in HMI9 medium supplemented with 10% fetal calf serum, at 37 °C, in an atmosphere of 5% CO₂ (Hirumi and Hirumi 1994, Loiseau et al. 1997). In all experiments, log-phase cell cultures

were harvested by centrifugation at $3,000 \times g$ and used immediately. Extract assays were based on the conversion of a redox-sensitive dye (resazurin sodium salt, SIGMA) to a fluorescent product by viable cells (Raz et al. 1997). *T. b. gambiense* bloodstream forms were transferred in 96-well plates (at 1×10^4 parasites per well in 200 μ L of culture medium) either in the absence or in the presence of different concentrations of extracts and with a final DMSO concentration that did not exceed 1%. After a 72 h incubation period, resazurin solution was added to each well at the final concentration of 45 μ M. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelengths after further 4 h incubation. Each extract concentration was tested in triplicate and the experiment repeated twice. The percentage of inhibition of the parasite growth was calculated by comparing the fluorescence of parasites maintained in the presence or absence of extract. DMSO was used as a control. IC_{50} and IC_{90} values were determined from the dose-response curves with extract concentrations ranging from 100 to 0.05 μ g/mL. The results were the mean values $\pm$ the standard deviations. Stock solution of pentamidine was prepared in DMSO at 10 mM and used as drug control.

1.6. Cytotoxic activity upon L-6 cells

Monolayers of rat L6 myoblasts, at 5×10^3 per well of 96-well plates in 200 μ L of RPMI medium containing 10% fetal calf serum, were maintained with different concentrations of extract for 5 days, at 37 °C in a 5% CO₂ atmosphere. Cytotoxicity was determined using the colorimetric MTT assay (Mosmann 1983), by measuring absorbance at 540 nm. Percentage of growth inhibition was determined by comparison with the untreated control culture. The concentrations causing 50% (TC₅₀) and 90% (TC₉₀) of cell growth inhibition were obtained from the extract concentration–response curves. The results were determined as the mean values $\pm$ the standard deviations from three independent experiments.

Supplementary Table

Table S1. Plants, parts of plants and extracts used for the *in vitro* antiprotozoal assays.

Family	Plant species / Vernacular names	Parts of plant tested (Solvents) ^a / % yield ^b	Voucher number
Anacardiaceae	<i>Astronium fraxinifolium</i> Schott ex Spreng. gonçalo-alves, aroeira-vermelha	SB (H)/ 0.51 SB (EA)/ 0.74 SB (E)/ 12.05 RW (H)/ 0.25 RW (E)/ 7.80 RB (EA)/ 1.14 RB (E)/ 19.88	(UB) 3814
Fabaceae	<i>Chamaecrista desvauxii</i> (Collad.) Killip Sene	AP (E)/ 5.70	(UB) 3800
	<i>Vatairea macrocarpa</i> Ducke angelim-do-Cerrado, maleiteira	RB (H)/ 4.50 RB (EA)/ 2.20 RW (H)/ 0.90	(UB) 3815
Connaraceae	<i>Connarus suberosus</i> Planch. bico-de-papagaio, galinha-choca	RW (H)/ 4.50 RB (H)/ 2.80	(UB) 3820
Myrtaceae	<i>Blepharocalyx salicifolius</i> (Kunth) O. Berg maria-preta	L (H)/ 3.23 L (E)/ 27.66 SW (H)/ 0.26 SB (H)/ 0.05 SB (EA)/ 0.73 L (EA)/ 4.18	(UB) 3798
	<i>Psidium laruotteanum</i> Cambess. araçá-cascudo	SW (EA)/ 2.38 L (H)/ 6.20 L (EA)/ 8.58	(UB) 3810
Myrsinaceae	<i>Myrsine guianensis</i> (Aubl.) Kuntze capororoca-comum	SB (H)/ 0.40 SB (EA)/ 0.90 SB (E)/ 28.23 L (H)/ 3.00 L (EA)/ 6.78 SW (H)/ 0.27 SW (E)/ 3.76 RW (H)/ 0.28 RW (EA)/ 0.69 RW (E)/ 6.62	(UB) 3795
Vochysiaceae	<i>Salvertia convallariodora</i> A. St.-Hil. colher de vaqueiro	SW (H)/ 0.25 SW (EA)/ 2.40 SW (E)/ 4.02 L (H)/ 0.69 L (EA)/ 3.50	(UB) 3777

Note: ^aAP: aerial parts; L: leaves; RB: root bark; RW: root wood; SB: stem bark; SW: stem wood. Solvents used in extraction: H: hexane. EA: ethyl acetate. E: ethanol. ^bPercentage yield (% w/w) of plant extracts.

References

Bosc D, Mouray E, Grellier P, Cojean S, Loiseau PM, Dubois J. 2013. Introduction of methionine mimics on 3-arylthiophene: influence on protein farnesyltransferase inhibition and on antiparasitic activity. *Med Chem Commun.*4:1034-1041.

- Buckner FS, Verlinde CL, La Flamme AC, Van Voorhis WC. 1996. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-galactosidase. *Antimicrob Agents Chemother.* Nov;40:2592-2597. Epub 1996/11/01.
- Desjardins RE, Canfield CJ, Haynes JD, Chulay DJ. 1979. Quantitative Assessment of Antimalarial Activity in Vitro by a Semiautomated Microdilution Technique. *Antimicrob Agents Chemother.* 16:710-718.
- Grellier P, Vendeville S, Joyeau R, Bastos IM, Drobecq H, Frappier F, Teixeira AR, Schrevel J, Davioud-Charvet E, Sergheraert C, et al. 2001. *Trypanosoma cruzi* prolyl oligopeptidase Tc80 is involved in nonphagocytic mammalian cell invasion by trypomastigotes. *J Biol Chem.* Dec 14;276:47078-47086. Epub 2001/10/13.
- Guillon J, Grellier P, Labaied M, Sonnet P, Leger JM, Deprez-Poulain R, Forfar-Bares I, Dallemagne P, Lemaitre N, Pehourcq F, et al. 2004. Synthesis, antimalarial activity, and molecular modeling of new pyrrolo[1,2-a]quinoxalines, bispyrrolo[1,2-a]quinoxalines, bispyrido[3,2-e]pyrrolo[1,2-a]pyrazines, and bispyrrolo[1,2-a]thieno[3,2-e]pyrazines. *Journal of medicinal chemistry.* Apr 8;47:1997-2009. Epub 2004/04/02.
- Hirumi H, Hirumi K. 1994. Axenic culture of African trypanosome bloodstream forms. *Parasitol Today.* Feb;10:80-84. Epub 1994/01/01.
- Loiseau PM, Dreyfuss G, Daulouede S, Lachatre G, Vincendeau P, Craciunescu DG. 1997. Trypanocidal effect of Ir-(COD)-pentamidine tetraphenylborate on *Trypanosoma brucei* and *T. b. gambiense* rodent models and serum kinetics in sheep. *Tropical medicine & international health : TM & IH.* Jan;2:19-27. Epub 1997/01/01.
- Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* Dec 16;65:55-63. Epub 1983/12/16.
- Raz B, Iten M, Grether-Buhler Y, Kaminsky R, Brun R. 1997. The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) in vitro. *Acta tropica.* Nov;68:139-147. Epub 1997/12/05.
- Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. *Science.* Aug 20;193:673-675.