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ORIGINAL ARTICLE

Brazilian Cerrado *Qualea grandiflora* Mart. Leaves Exhibit Antiplasmodial and Trypanocidal Activities *In vitro*

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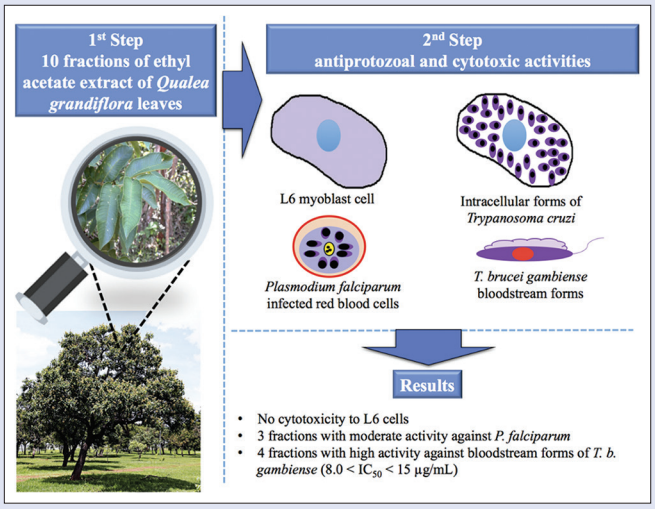
ABSTRACT

Background: The rapid spread of drug-resistant strains of protozoan parasites required the urgent need for new effective drugs. Natural products offer a variety of chemical structures, which make them a valuable source of lead compounds for the development of such new drugs. Cerrado is the second largest biome in Brazil and has the richest flora of all the world savannahs. We selected *Qualea grandiflora*, a plant species known for its proprieties in folk medicine and its antibacterial activity. **Objective:** However, its antiprotozoal activity was not yet explored. **Materials and Methods:** We investigated the activities of fractions from the ethyl acetate extract of *Q. grandiflora* leaves against human life forms of *Plasmodium falciparum*, *Trypanosoma cruzi*, and *Trypanosoma brucei gambiense*, and for its cytotoxicity upon the rat L6-myoblast cell line. Ten fractions were produced by ethyl acetate:hexane chromatography. **Results and Conclusion:** The fractions showed no cytotoxicity against L-6 cells (IC_{50} > 100 µg/mL) and no hemolysis propriety. Three fractions had a moderate activity against *P. falciparum*, anyone was active against *T. cruzi* but four fractions demonstrated a high activity against bloodstream forms of *T. brucei gambiense* (8.0< IC_{50} <15 µg/mL). Identification and characterization of the active compounds are currently under investigation.

Key words: Antiprotozoal activity, Brazilian cerrado, Chagas disease, malaria, *Qualea grandiflora*, sleeping sickness

SUMMARY

- *Qualea grandiflora* is an endemic tree of the Brazilian Cerrado, which presents medicinal properties
- Ten fractions of the ethyl acetate extract of *Q. grandiflora* leaves were assessed against *Plasmodium falciparum*, *Trypanosoma Cruzi*, and *Trypanosoma brucei gambiense*
- No fraction showed relevant cytotoxicity and hemolysis activity
- All the fractions presented antiplasmodial and trypanocidal activities
- Three fractions with moderate antiplasmodial activity (49< IC_{50} <56 µg/mL)
- Four fractions with high activity against bloodstream forms of *T. brucei gambiense* (8.0< IC_{50} <15 µg/mL).



Abbreviations used: CQ: Chloroquine, DMSO: Dimethyl sulfoxide, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HMI: Modified Iscove's medium, IC_{50}: Concentration inhibiting 50% of parasite growth, IC_{90}: Concentration inhibiting 90% of parasite growth, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, RPMI: Roswell Park Memorial Institute, SD: Standard deviation, SI: Ratio of cytotoxicity to biological activity – TC_{50}/IC_{50}, TC_{50}: Concentration causing 50% of cell growth inhibition, TC_{90}: Concentration causing 90% of cell growth inhibition, TLC: Thin-layer chromatography

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INTRODUCTION

The term "tropical diseases" is often used to refer to the infectious diseases that thrive in hot and humid climates such as malaria, leishmaniasis, schistosomiasis, onchocerciasis, lymphatic filariasis, Chagas disease, sleeping sickness, and dengue. Tropical diseases affect millions of people worldwide and represent a continuous threat.[1]

About 3.4 billion people are at risk of malaria, which is endemic in 106 countries. Malaria is caused by *Plasmodium* spp. protozoa. It is considered to be the most serious protozoal infection in the world with 214 million cases of malaria and 438,000 deaths worldwide in 2015 mainly due to *Plasmodium falciparum*, mostly among children under 5 and pregnant women.[2,3] Chagas disease is caused by *Trypanosoma cruzi* and affects an

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estimated 8 million people worldwide, mostly in Latin America.[4] Sleeping sickness is endemic in Africa and is caused by two different species of trypanosomatids: *Trypanosoma brucei gambiense* currently accounts for over 98% of reported cases of the disease and is responsible for a chronic form; *T. brucei rhodesiense* represents fewer than 2% of reported cases and is responsible for an acute form. Sleeping sickness is found in remote Sub-Saharan areas where health systems are often weak.[5]

Treatments of the three vector-borne parasitic diseases, malaria, Chagas disease, and sleeping sickness are based on chemotherapies. However, there are not many drugs available for the treatment of Chagas disease and sleeping sickness and the drugs used are more effective in the early stages of these diseases and have many serious adverse reactions.[5] In contrast, treatment of malaria is more effective compared to other parasitic diseases. However, due to the rapid emergence of resistant strains of *Plasmodium*, new drugs for the treatment of this disease are considered as a priority.[6] To make matters worse, none of these diseases has an effective vaccine. Consequently, a great importance continues to be given to the screening of biodiversity compounds.

Natural products play a crucial role in the search for molecules with antiparasitic activities. Brazilian biomes are reservoirs of potential molecules with therapeutic activities; the Amazon rainforest (largest rainforest in the world) offers a massive biodiversity, and the Cerrado biome, the richest flora of all savannahs worldwide, is still little explored.[7-10] Medicinal plants may absolutely contain yet undiscovered antimalarial properties, which can serve as a template for production of new contemptible antimalarial drugs.[11,12]

Qualea grandiflora (Vochysiaceae) is an endemic tree of the Brazilian Cerrado, which presents medicinal properties. The oral administration of *Q. grandiflora* bark extract is used in folk medicine to treat gastric ulcers for skin diseases and inflammatory processes. Extracts of this plant demonstrated activity against some bacteria, especially Gram-positive bacteria.[13-17] To our knowledge, antiprotozoal activity was not yet explored for this plant species.

Here, we report the antiparasitic activity of fractions from the ethyl acetate extract of *Q. grandiflora* leaves on the erythrocytic stages of *P. falciparum*, the *T. cruzi* human intracellular stage and the *T. brucei gambiense* bloodstream form, and the cytotoxicity upon the rat L6-myoblast cell line.

MATERIALS AND METHODS

Preparation of extract of *Qualea grandiflora* leaves from ethyl acetate

Q. grandiflora Mart. (Vochysiaceae) is an endemic tree species of widest distribution in the Cerrado Biome.[18] This is very typical of savannah physiognomies, occurring both in Cerrado *sensu stricto* and some grassland types.[14]

Mature, fully expanded leaves were collected in July 2010 from at least ten adult individuals occurring in natural areas of Cerrado *sensu stricto* in the Federal District (Brazil). In the laboratory, the leaves were cleaned, and those presenting signals of infestation or disease were discarded. After, the leaves were dried out in an oven at 40°C for 24 h, powdered, and stored at 6°C until use.

The extraction from *Q. grandiflora* leaves was performed by ultrasound in three successive solvents with increasing polarity: hexane, dichloromethane, and ethyl acetate. Extraction with hexane and dichloromethane was carried out twice for 1 h and each, with renewal of the solvent. Extraction with ethyl acetate, the extract of interest, was carried out three times using the same methodology. After, each material was vacuum filtered on Buchner funnel using filter paper and further dried on a rotary evaporator.

The ethyl acetate extract was fractionated by chromatography on a silica gel column. The eluents used were ethyl acetate:hexane mixture to increase the polarity (10%–100%), acetone, and methanol. The fractions were grouped based on thin-layer chromatography (TLC) according to similarity and purity of the components revealed.

TLC was carried out on plates (ALUGRAM SIL G/UV254) size 4 cm × 5 cm, using eluent ethyl acetate:hexane mixture (1:1, v:V), revealed with Oleum (sulfuric acid, water, and acetic acid in the ratio 1:4:20 [v: v: v]) and heated at approximately 150°C.

The ten fractions (F1–F10) were dried in a rotary evaporator. The fractions were subsequently dissolved in dimethyl sulfoxide (DMSO) and homogenized by ultrasonic bath, at a stock concentration of 10 mg/mL and stored at −20°C.

In vitro bioassay of antiplasmodial activity

The chloroquine (CQ)-resistant strain K1 and the CQ-sensitive strain 3D7 of *P. falciparum* were maintained *in vitro* on human erythrocytes in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented by 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.0005% hypoxanthine, 100 μg/mL gentamicin, and 8% (v/v) heat-inactivated human serum, at 37°C, under an atmosphere containing 5% CO_2 as described.[19]

[^{3}H]-hypoxanthine incorporation assay (strain K1)

For the [^{3}H] hypoxanthine incorporation assay, *in vitro* extract susceptibility was measured as described by Desjardins and Guillon[20,21] as routinely performed.[22] The results were expressed as the means determined from three independent experiments. Stock solution of CQ was prepared in purified water (Milli-Q grade) at 10 mM and was used as antimalarial drug control.

SYBR Green I-based fluorescence assay (strain 3D7)

For the fluorescence assay, from a concentration of 100 μg/mL, the extracts were serially diluted with culture medium RPMI-1640 and added to the parasite culture (1% parasitemia and 2% hematocrit) in 96-well plates. Similarly, positive (CQ) control was diluted from a starting concentration of 2 μg/mL. The highest concentration of DMSO (negative control) to which the parasites were exposed was 0.4%, which was shown to have no measurable effect on parasite viability. The plates were incubated at 37°C under an atmosphere of 5% CO_2 for 48 h. Then, the plates were frozen at −80°C for 12 h. After this period, 100 μL of SYBR Green I (Invitrogen) prepared at 0.2 μL in 1 mL of lysis buffer (20 mM Tris at pH 7.5, 5 mM EDTA, 0.008% (wt/vol) saponin, and 0.08% (vol/vol) Triton X-100 was added to each well; the plates were incubated at 37°C for 1 h before reading. The fluorescence value was measured in SpectraMax M5 device, with excitation and emission wavelength bands at 497 and 520 nm (515 nm cut-off), respectively. The drug concentration that causes 50% inhibition (IC_{50}) was determined by analysis of dose–response curve of mean values for each triplicate extract tested.[23,24]

In vitro bioassay of anti-*Trypanosoma cruzi* activity

Experiments were performed with *T. cruzi* trypomastigotes and amastigotes of the Tulahuen strain (*lacZ* clone 4) expressing the β-galactosidase maintained in monolayers of rat myoblast L6 cells grown in RPMI medium supplemented with 10% (v/v) fetal calf serum at 37°C under an atmosphere of 5% CO_2 as described.[25]

IC_{50} and IC_{90} values were obtained from the extract concentration–response curve, and the results expressed as the mean values ± standard deviations (SDs) determined from three independent experiments.

Stock solution of nifurtimox was prepared in DMSO at 10 mg/mL and was used as drug control.

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

Experiments were performed with bloodstream trypomastigote forms of *T. brucei gambiense* strain Feo, which were cultured in HMI-9 medium supplemented with 10% fetal calf serum, at 37°C, in an atmosphere of 5% CO_2. Extract assays were based on the conversion of a redox-sensitive dye (Resazurin sodium salt, SIGMA) to a fluorescent product by viable cells.[22,25,26]

IC_{50} and IC_{90} values were determined from the dose–response curves with extract concentrations ranging from 100 to 0.05 µg/mL as described.[22] The results were the mean values ± the SDs. Stock solution of pentamidine was prepared in DMSO at 10 mM and used as drug control.

Biological activity scale

The antiprotozoal activities of fractions from the ethyl acetate extract of *Q. grandiflora* leaves were considered: high (<15 µg/mL), significant (15–30 µg/mL), moderate (30–60 µg/mL), weak (60–100 µg/mL), and no activity (>100 µg/mL).

Cytotoxic activity on L-6 cells

Monolayers of rat L-6 myoblasts, at 5×10^3 per well of 96-wells plates in 200 µL of RPMI medium containing 10% fetal calf serum, were maintained with different concentrations of extract for 5 days, at 37°C under a 5% CO_2 atmosphere. Cytotoxicity was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,[27] and the reduction percentages were measured by absorbance at 540 nm as described.[22] The concentrations causing 50% (TC_{50}) and 90% (TC_{90}) of cell growth inhibition were obtained from the drug concentration–response curves. The results were expressed as the mean values ± SDs determined from three independent experiments.

In vitro hemolysis assay

The extracts were serially diluted with the culture medium (RPMI + 8% human serum) in 96-well plates. Human erythrocytes resuspended in the same medium were added to each well for 1% final hematocrit. The plates were incubated for 48 h at 37°C under CO_2 atmosphere. The percentage hemolysis was defined by measuring the hemoglobin absorbance at 540 nm. Lysis of 0% was defined as the absorbance measured in culture in the absence of extracts. The 100% lysis was defined as the absorbance measured in totally lysed erythrocytes culture by freezing/thawing. Final concentrations of the extracts were within a range of 100–0.1 µg/mL.[28]

RESULTS AND DISCUSSION

In the present study, ten fractions of ethyl acetate extract of *Q. grandiflora* leaves were assessed against *P. falciparum*, *T. cruzi,* and *T. brucei gambiense.* The extractions were performed with ethyl acetate and then fractionated by chromatography on a silica gel column using eluent ethyl acetate:hexane in order of increasing polarity (10%–100%). The dry weight of leaves was 352 g and the yield of 3.98 g extract from ethyl acetate. The yield of each fraction was F1-42.8 mg, F2-33.3 mg, F3-14.6 mg, F4-8.1 mg, F5-4.3 mg, F6-15.6 mg, F7-7.8 mg, F8-3.0 mg, F9-1.0 mg, and F10-10.7 mg. All fractions were soluble in DMSO at a concentration of 10 mg/mL and were tested against: (1) the CQ-resistant *P. falciparum* K1 strain by monitoring the inhibition of the uptake of [^{3}H]-hypoxanthine and the CQ-sensitive *P. falciparum* 3D7 strain by monitoring the inhibition of fluorescence assay [Table 1]; (2) the development of intracellular amastigote forms of the Tulahuen *T. cruzi* strain expressing β-galactosidase [Table 2]; and (3) *T. brucei gambiense* bloodstream forms by measuring cell metabolic activity by the redox indicator resazurin [Table 2]. Cytotoxic activity was subsequently investigated upon the rat L6 myoblast cell line [Table 3].

Remarkably, no fraction showed relevant cytotoxicity [Table 3] and hemolysis activity. Consequently, the selectivity indexes (SI = ratio of cytotoxicity to biological activity – TC_{50}/IC_{50}) were determined for all fractions with antiprotozoal activity. Hence, the interest in the study of this plant as chemotherapy agent is relevant because it is nontoxic to mammalian cells.

For *P. falciparum,* two methods were employed to determine the antiplasmodial activity of these fractions: [^{3}H]-hypoxanthine

Table 1: Antiplasmodial activity of Brazilian Cerrado *Qualea grandiflora* leaves ethyl acetate fractions

Fraction number of *Q. grandiflora*[a]	Antiplasmodial activity (µg/mL)			
	[^{3}H]-hypoxanthine incorporation assay (strain K1)		SYBR Green I-based fluorescence assay (strain 3D7)	
	IC_{50}±SD	IC_{50}±SD	IC_{50}±SD	IC_{50}±SD
F1	55.2±7.8	117.1±45.6	61.4±10.1	130.9±33.7
F2	49.5±14.7	85.3±28.0	54.6±12.6	98.9±19.6
F3	75.0±12.0	125.7±20.1	91.2±4.6	202.5±18.6
F4	89.8±2.5	149.3±7.2	86.9±2.0	171.7±20.4
F5	53.7±6.7	94.1±11.3	79.0±4.1	154.2±12.5
F6	69.3±10.3	125.4±17.8	80.9±2.5	167.1±13.9
F7	>100		>100	
F8	80.4±10.1	146.8±14.6	74.0±2.4	146.7±14.2
F9	60.7±6.7	112.2±10.5	88.8±10.2	174.2±10.9
F10	66.8±1.6	123.5±2.5	90.7±9.5	195.6±27.5
Chloroquine (ng/mL)	10.6±0.2	19.8±0.5	17.3	31.0

Values of IC_{50} and IC_{90} are represented by means of three replicates±SDs. [a]Botanical name of Brazilian Cerrado plant species. The Chloroquine-resistant *P. falciparum* strain: K1; The Chloroquine-sensitive *P. falciparum* strain: 3D7. SDs: Standard deviations; *P. falciparum*: *Plasmodium falciparum*; *Q. grandiflora*: *Qualea grandiflora*; IC_{50}: Concentration inhibiting 50% of parasite growth; IC_{90}: Concentration inhibiting 90% of parasite growth

Table 2: Antitrypanosomal activities of Brazilian Cerrado *Qualea grandiflora* leaf ethyl acetate fractions

Fraction number of *Q. grandiflora*[a]	Anti-*T. cruzi* activity (µg/mL)		Anti-*T. brucei gambiense* activity (µg/mL)	
	IC_{50}±SD	IC_{90}±SD	IC_{50}±SD	IC_{90}±SD
F1	>100		14.4±0.7	25.2±0.8
F2	>100		27.5±1.7	50.6±1.0
F3	>100		26.0±0.5	48.7±1.2
F4	>100		20.0±4.3	34.0±11.6
F5	>100		13.9±2.2	25.0±1.7
F6	>100		8.0±0.6	13.4±0.5
F7	>100		17.7±4.9	32.8±1.0
F8	>100		12.2±2.6	22.0±5.8
F9	>100		22.8±4.7	40.6±10.8
F10	>100		21.3±4.0	38.7±9.8
Nifurtimox (µg/mL)	0.6±0.1	1.1±0.1		
Pentamidine (ng/mL)			1.7±0.0	3.3±0.2

Values of IC_{50} and IC_{90} are represented by means of three replicates±SDs. [a]Botanical name of Brazilian Cerrado plant species. Bloodstream forms of *T. brucei gambiense* strain Feo. The β-galactosidase-expressing *T. cruzi* trypomastigotes of the Tulahuen strain. *T. cruzi*: *Trypanosoma cruzi*; SDs: Standard deviations; *Q. grandiflora*: *Qualea grandiflora*; *T. brucei*: *Trypanosoma brucei*; IC_{50}: Concentration inhibiting 50% of parasite growth; IC_{90}: Concentration inhibiting 90% of parasite growth

Table 3: Cytotoxic activity of Brazilian Cerrado *Qualea grandiflora* leaf ethyl acetate fractions on L-6 cells

Fraction number of *Q. grandiflora*[a]	Cytotoxicity against L6 cell (μg/mL)		Cytotoxicity index[b] (TC50/IC50)			
	TC_{50}±SD	TC_{90}±SD	*P. falciparum* strain K1	*P. falciparum* strain 3D7	*T. cruzi*	*T. brucei gambiense*
F1	>100		>1.8	>1.6		>7.0
F2	>100		>2.0	>1.8		>3.6
F3	>100		>1.3	>1.1		>3.9
F4	>100		>1.1	>1.2		>5.0
F5	>100		>1.9	>1.3		>7.2
F6	>100		>1.4	>1.2		>12.4
F7	>100					>5.7
F8	>100		>1.2	>1.4		>8.2
F9	>100		>1.6	>1.1		>4.4
F10	>100		>1.5	>1.1		>4.7
Chloroquine (ng/mL)	>44.1					
Nifurtimox (μg/mL)	22.3±8.0	44.5±16.0			37.2	
Pentamidine (ng/mL)	>44.1					>11

Values of IC_{50} and IC_{90} are represented by means of three replicates±SDs. [a]Botanical name of Brazilian Cerrado plant species. [b]Cytotoxicity index defined as the ratio of the TC_{50} value determined on the mammalian L6 cell line on the IC_{50} value determined on each parasite. *T. cruzi*: *Trypanosoma cruzi*; SDs: Standard deviations; *Q. grandiflora*: *Qualea grandiflora*; *T. brucei*: *Trypanosoma brucei*; *P. falciparum*: *Plasmodium falciparum*; IC_{50}: Concentration inhibiting 50% of parasite growth; IC_{90}: Concentration inhibiting 90% of parasite growth; TC_{50}: Concentration causing 50% of cell growth inhibition; TC_{90}: Concentration causing 90% of cell growth inhibition

incorporation assay, wherein the CQ-resistant *P. falciparum* K1 strain was used, and SYBR Green I-based fluorescence assay, wherein the CQ-sensitive *P. falciparum* 3D7 strain was used. The results from both methods used were similar [Table 1] although the strain used was different in each test. Except the F7, all the fractions were active for both strains K1 and 3D7, considering IC_{50} <100 μg/mL. The best antiplasmodial activity was found for the fraction F2 with IC_{50} values of 49.5 ± 14.7 and 54.6 ± 12.6 μg/mL against K1 and 3D7, respectively.

Concerning the trypanosomatids, no fraction presented any activity against *T. cruzi* [Table 2]. However, all fractions showed a significant activity against *T. brucei gambiense* (with IC_{50} <28 μg/mL), especially the fractions F1, F5, F6, and F8 that showed the highest activity with IC_{50} values of 14.3, 13.9, 8, and 12.2 μg/mL, respectively [Table 2].

Studies with the ethanol extracts of *Q. grandiflo*ra bark have shown inhibitory activity against strains of Gram-positive bacteria, being efficient both for sensitive and resistant strains to existing antibiotics such as the methicillin-resistant strains of *Staphylococcus aureus* and *S. epidermidis*. It also displayed significant activity on Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*.[15] Phytochemical analysis of the ethanolic extract revealed the presence of saponins, triterpenes, steroids, tannins, phenolic compounds, and catechin.[16]

CONCLUSION

In the present study, we showed for the first time that extracts of leaves of *Q. grandiflora* have antiprotozoal activity, and thus we increased the range of known pharmacological proprieties of this plant. Three fractions of the ethyl acetate extract of *Q. grandiflora* leaves from the ten tested had a moderate activity against *P. falciparum*; all fractions were active against *T. brucei gambiense*, with four fractions found to exhibit high activity against this protozoa (8.0< IC50 <15 μg/mL). Fractions displayed any obvious cytotoxicity upon mammalian cells. Sleeping disease is a neglected tropical disease, for which an urgent need of new active molecules is required. Deeper phytochemical investigations are in progress to isolate and identify the active compounds. Likewise, we expect that the investigation of this plant will contribute to Cerrado conservation, which is considered to be one of the most threatened regions of the Earth.

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Conflicts of interest

There are no conflicts of interest.

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