JOURNAL OF ESSENTIAL OIL RESEARCH, 2016
http://dx.doi.org/10.1080/10412905.2016.1210041

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Chemical composition and antiparasitic activity of essential oils from leaves of *Guatteria friesiana* and *Guatteria pogonopus* (Annonaceae)

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ABSTRACT

Natural products represent a valuable source for discovery of antiparasitic agents. Here, we describe the antiparasitic activity from essential oils extracted from leaves of *Guatteria friesiana* (EOGF) and *Guatteria pogonopus* (EOGP) (Annonaceae). The essential oils were obtained by hydrodistillation and analyzed by GC/MS and GC-FID. The sesquiterpenes are more abundant in both essential oils. *G. friesiana* are dominated by β-eudesmol (51.9%), γ-eudesmol (18.9%) andα-eudesmol (12.6%). The major compounds identified for EOGP were spathulenol (24.8%), γ-amorphene (14.7%) and germacrene D (11.8%). The essential oils demonstrated potent trypanocidal and antimalarial activities with values of IC_{50} lower than 41.3 µg/mL. EOGF also inhibits the proliferation of amastigotes. In addition, we identified significant ultrastructural alterations induced by the essential oils, especially in the cell membrane, Golgi complex, endoplasmatic reticulum and mitochondria. The results presented herein reinforce the potential of other members of this family for search of antiparasitic compounds.

ARTICLE HISTORY
Received 1 December 2015
Accepted 3 July 2016

KEYWORDS
Guatteria friesiana; *Guatteria pogonopus*; essential oil; antimalarial activity; trypanocidal activity

Introduction

Chagas disease and malaria are caused by protozoan parasites and constitute serious public health problems worldwide, mainly in tropical countries (1). Pharmacotherapy of both diseases is limited to a small number of drugs, which are associated with several side effects, variable efficacy and resistance (2, 3). These challenges, combined with the lack of a vaccine for both diseases make the research and development of new antiparasitic agents a matter of great relevance to public health.

The genus *Guatteria* (Annonaceae) comprises 307 species and is distributed from southeastern Mexico to southern Brazil (4). Some species of this genus are known for their aromatic fragrances and their medicinal properties (5). Previous phytochemical and pharmacological investigations on some *Guatteria* species revealed cytotoxic effects, antimicrobial properties and antiparasitic activity (6–9). These bioactivities are attributed to the presence of terpenes and alkaloids in these plant species.

Guatteria friesiana (W.A. Rodrigues) Erkens & Maas (synonym *Guatteriopsis friesiana* W.A. Rodrigues) is a small tree known as both 'envireira' and 'envira' found in the Brazilian and Colombian Amazon Basin (10). Previous phytochemical investigations on this species described the chemical constituents of its essential oils (11–13), as well as the presence of alkaloids (10, 14). The essential oils and alkaloids exhibited antitumor, antimicrobial properties (10, 11, 14), and larvicidal activity against *Aedes aegypti* larvae (12).

Guatteria pogonopus Martius is a tree characterized by very large leaves and short pedicel of flowers and fruits. It was reported to grow in the Brazilian states of Bahia, Espírito Santo, Minas Gerais, and Sergipe (15). Previous phytochemical studies on these species described the isolation of alkaloids, as well as, yours cytotoxic activities and the composition of the essential oil (15, 16).

In the continuous search for antiparasitic compounds from Brazilian Annonaceous plants, herein, the chemical composition and antiparasitic activity of the essential oils

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The supplementary material for this paper is available online at http://dx.doi.org/10.1080/10412905.2016.1210041

from the leaves of *G. friesiana* and *G. pogonopus* were investigated.

Experimental

Plant material

Leaves of *G. friesiana* were collected at the Experimental Farm of the Federal University of Amazonas (UFAM), Manaus, Amazonas, Brazil. The plant sample was identified Annonaceae by specialist Dr. Antonio Carlos Webber from the Federal University of Amazonas. A voucher specimen (No. 9282) was deposited in the Herbarium of the Department of Biology, UFAM. The *G. pogonopus* leaves were collected in the Itabaiana Mountain National Park, Itabaiana, Sergipe, Brazil. The plant material was identified by Dr. Ana Paula do Nascimento Prata, a plant taxonomist from the Department of Biology, Federal University of Sergipe, Brazil, and a voucher specimen (No. 22793) has been deposited with the Herbarium of the Federal University of Sergipe. Leaves were obtained, in March 2013, from flowered plants.

Hydrodistillation of the essential oil

The essential oils from dried leaves (for 72 hours) of the two *Guatteria* species (200 g each) were obtained by hydrodistillation for 3 hours using a Clevenger-type apparatus. The essential oils were dried over anhydrous sodium sulphate and the percentage content was calculated on the basis of the dry weight of plant material [Essential oil% = (volume of the essential oil obtained × density/dried mass of leaves) × 100%]. The extraction was performed in triplicate. The essential oils were stored in a freezer until analysis.

GC-FID and CG-MS analysis of the essential oil

GC-FID and GC-MS analyses were performed on a Shimadzu GC-2010 Plus GCMS-QP2010 Ultra GC-FID, equipped with a Shimadzu AOC-20i auto-injector. The separation of the compounds was achieved on a RTx®-5MS fused capillary chromatography column (30 m × 0.25 mm × 0.25 µm film thickness) coated with 5%-diphenyl-95%-dimethylpolysiloxane. Helium was the carrier gas at 1.2/min flow rate. The column temperature program was 40°C for 4min, at a rate of 4°C/min to 240°C, then at 10°C/min to 280°C, and at 280°C/2min. The injector and detector temperatures were 250°C and 280°C, respectively. Samples (10 mg/mL in CH_2Cl_2) were injected with a 1:30 split ratio. Retention indices were generated with a standard solution of *n*-alkanes (C_8–C_{18}). Peak areas and retention times were measured by an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without a FID response factor correction. MS were taken at 70 eV with scan intervals of 0.5 s and fragments from 40 Da – 550 Da. Essential oil components were identified by comparison of their retention times (t_R) with those of standards and isolated compounds under identical conditions as well as by their retention indices of a series of *n*-alkanes (17), and their mass spectra with those in the NIST (05, 05s, 21 and 107) and Wiley 8 mass spectral libraries, and published data in the literature (18). The analyses of the essential oil were performed in triplicate.

Animals and parasites

BALB/c female mice (8 to 10 weeks old) were maintained in sterilized cages under a controlled environment, receiving a balanced rodent diet and water *ad libitum* at Centro de Pesquisas Gonçalo Moniz (Fundação Oswaldo Cruz, Bahia, Brazil). All animal experiments and procedures were carried out in accordance with the recommendations of the institution's committee on the ethical handling of laboratory animals. Epimastigotes of *Trypanosoma cruzi* (Y strain) were maintained in axenic medium at 26°C, with weekly transfers into LIT medium(Liver Infusion Tryptose) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil), 1% hemin (Sigma, Chemical Co., St. Luis, MO), 1% R9 medium (Sigma), and 50 µg/mL of gentamycin (Novafarma, Anápolis, GO, Brazil) (19). Tissue culture trypomastigotes (Y strain) were obtained from the supernatants of infected LLC-MK2 cells maintained in RPMI-1640 medium supplemented with 10% FBS and 50 µg/mL of gentamycin at 37°C in a 5% humidified CO_2 atmosphere (20). W2 strain *Plasmodium falciparum*(chloroquine-resistant) was maintained in continuous culture of human erythrocytes (blood group O^+) using the RPMI 1640 medium supplemented with 10% of human plasma (21).

Cytotoxicity to mammalian cells

Peritoneal exudate macrophages were obtained by washing, with cold RPMI medium, the peritoneal cavity of BALB/c mice 4–5 days after injection of 3% thioglycolate in saline (1.5 mL per mice). Then, cells were seeded on 96-well plates at a cell density 1×10^5 cells/well in RPMI-1640 medium supplemented with 10% of FBS and 50 µg/mL of gentamycin and incubated for 24 hours at 37°C and 5% CO_2. Then, each essential oil was added at five concentrations ranging from 1.23 µg/mL to 100 µg/mL in triplicate before being incubated for 72 hours. The cytotoxic effect was measured by the Alamarblue assay as described by Meira et al. (22). Gentian violet was used as positive control, at concentrations ranging from 0.04 to 10 µg/mL.

Antimalarial activity

Parasites grown at 1–2% parasitemia and 2.5% hematocrit were plated onto 96-wells plate and incubated with the compounds at different concentrations ranging from 1.23 μg/mL to 100 μg/mL in culture medium (RPMI 1640). After 24 hours, [^{3}H]-hypoxanthine (Perkin Elmer, Waltham, MA) was added, the plate were incubated again and parasites were harvested using a cell harvester to quantify the [^{3}H]-hypoxanthine incorporation in a β-radiation counter. Inhibition of parasite growth was evaluated by comparison with [^{3}H]-hypoxanthine uptake in treated cultures versus untreated culture (23). Mefloquine (Farmaguinhos, Rio de Janeiro, RJ, Brazil) was used as standard drug.

Trypanocidal activity

Epimastigotes (1×10^6 cells/well) were placed in LIT medium in the absence or presence of the essential oils at five concentrations (100 to 1.23 μg/mL). The effect of the treatment on epimastigotes proliferation was observed 5 days after incubation by counting viable forms in a Neubauer chamber. Bloodstream trypomastigotes forms of *T. cruzi* were cultured in 96-well plates (4×10^5 cell/well) in RPMI medium supplemented with 10% FBS and 50 μg/mL of gentamycin in the absence or presence of different concentrations of the essential oils, in triplicate. After 24 hours of incubation, the number of viable parasites was assessed in a Neubauer chamber (24). The percentage of inhibition was calculated in relation to untreated cultures. Benznidazole (LAFEPE, Recife, PE, Brazil) was used as positive control.

In vitro *macrophage infection and treatment with essential oils*

Peritoneal macrophages (2×10^5 cells/well) obtained from BALB/c mice were seeded in a twenty-four well-plate with rounded coverslips on the bottom in RPMI supplemented with 10% FBS and incubated for 24 hours. Cells were then infected with trypomastigotes (1:10) for 2 hours. Free trypomastigotes were removed by successive washes using saline solution. Cultures were incubated for 24 hours to allow full internalization and differentiation of trypomastigotes to amastigotes. Next, cultures were incubated in complete medium alone or with the essential oil EOGF (5 or 10 μg/mL) for 72 hours. Cells were fixed in absolute alcohol and the percentage of infected macrophages and the relative number of amastigotes per 100 macrophages was determined by manual counting after hematoxylin and eosin staining in an optical microscope (Olympus, Tokyo, Japan). The percentage of infected macrophages and the number of amastigotes per 100 macrophages was determined by counting 100 cells per slide. Benznidazole (10 μg/mL) was used as the positive control.

Electron microscopy analysis

Trypomastigotes (3×10^7 cells/well) were incubated for 24 hours at 37°C in complete medium alone or with the essential oil EOGF (10.7 μg/mL or 21.4 μg/mL). After incubation, parasites were fixed for 1 hour at room temperature with 2% formaldehyde and 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in sodium cacodylate buffer (0.1 M, pH 7.2) for 1 hour at room temperature. After fixation, parasites were processed for transmission electron microscopy (TEM) as previously described (22). Images were captured in a JEOL TEM-1230 transmission electron microscope.

Statistical analyses

To determine the cytotoxicity concentration 50% (CC_{50}) and inhibitory concentration 50% (IC_{50}) for epimastigote and trypomastigote forms of *T. cruzi*, we used nonlinear regression on Prism 5.02 GraphPad software (Graph Pad Software, San Diego, CA).The one-way ANOVA and Bonferroni for multiple comparisons were used to determine the statistical significance of the group comparisons. Differences were considered significant when the values of P were < 0.05. All experiments were done at least twice.

Results and discussion

Hydrodistillation of the leaves of *G. friesiana* (EOGF) and *G. pogonopus* (EOGP) gave a solid white and red color rude essential oils, with a yield of 1.17 ± 0.14% and 0.22 ± 0.03% (w/w, based on the dry weight of the plant material), respectively. The sesquiterpenes compounds were the most abundant in the essential oils investigated, comprising 93.0% in EOGF and 88.4% in EOGP, compared to the total chemical composition of each essential oil identified (Table 1). Moreover, it was possible to identify twenty-four compounds; eight in EOGF, and nineteen in EOGP (Table 1).

These results of the GC-FID and GC-MS analyses confirm the results reported by Costa et al. (11) that *G. friesiana* are dominated by β-eudesmol (51.9%), γ-eudesmol (18.9%), α-eudesmol (12.6%), and elemol (4.3%). The major compounds identified for essential oil of *G. pogonopus* were spathulenol (24.8%), γ-amorphene (14.7%), germacrene D (11.7%), (*E*)-caryophyllene (4.8%), γ-elemene (4.2%), caryophyllene oxide (4.0%), (*Z*)-caryophyllene (3.9%), alloaromadendrene epoxide (3.6%), γ-muurolene (3.5%), and germacrene B (3.0%). These results differ

Table 1. Essential oil composition of *Guatteria* species.

Compounds	RI[a]	RI[b]	Leaf oil %	
			G. friesiana	*G. pogonopus*
α-Ylangene	1369	1373	-	1.05 ± 0.58
α-Copaene	1379	1374	-	1.19 ± 0.34
β-Elemene	1393	1389	-	1.04 ± 0.19
(*Z*)-Caryophyllene	1407	1408	-	3.87 ± 0.08
(*E*)-Caryophyllene	1422	1417	-	4.82 ± 0.30
γ-Elemene	1430	1434	-	4.24 ± 0.20
cis-Muurola-3,5-diene	1447	1448	-	1.78 ± 0.25
Spirolepechinene	1453	1449	-	2.66 ± 0.20
γ-Muurolene	1473	1478	-	3.53 ± 1.60
Germacrene D	1484	1484	-	11.75 ± 6.33
γ-Amorphene	1494	1495	-	14.72 ± 3.37
Bicyclogermacrene	1500	1500	-	0.24 ± 0.05
γ-Patchoulene	1506	1502	-	0.51 ± 0.07
δ-Cadinene	1517	1522	-	0.15 ± 0.02
Elemol	1548	1548	4.31 ± 1.33	-
Germacrene B	1563	1559	-	3.01 ± 0.61
Spathulenol	1576	1577	2.76 ± 0.78	24.80 ± 11.38
Caryophyllene oxide	1580	1582	0.41 ± 0.24	3.97 ± 2.08
Globulol	1596	1590	-	1.43 ± 0.49
10-Epi-γ-eudesmol	1620	1622	0.99 ± 0.40	-
γ-Eudesmol	1633	1630	18.91 ± 5.41	-
Alloaromadendrene Epoxide	1639	1639	1.13 ± 0.27	3.62 ± 1.03
β-Eudesmol	1648	1649	51.92 ± 9.15	-
α-Eudesmol	1651	1652	12.56 ± 2.80	-
Sesquiterpenes identified			93.0 ± 1.7	88.4 ± 2.8
Total identified			93.0 ± 1.7	88.4 ± 2.8

Note: Data are expressed as mean ± SD of three analyses. RI (retention indices): [a]calculated on RTx®-5MS column according to VanDen Dool and Kratz (17), based on a homologous series of normal alkanes; [b]according to Adams (18).

Table 2. Cytotoxicity against macrophages, effect on erythrocitic stages of *Plasmodium falciparum* and trypanocidal action against bloodstream trypomastigotes and epimastigotes forms of *T. cruzi* (Y strain) of the essential oils of *G. friesiana* (EOGF) and *G. pogonopus* (EOGP).

Sample	CC_{50} (μg/mL)	IC_{50} (μg/mL) Pal.	IC_{50} (μg/mL) Try.	IC_{50} (μg/mL) Epi.
EOGF	37.7 (±3.6)	0.53 (±0.1)	10.7 (±1.3)	11.9 (±1.2)
EOGP	> 100	6.8 (±1.7)	41.3 (±0.6)	28.0 (±0.6)
Benznidazole[a]	-	-	2.7 (±0.5)	2.7 (±0.7)
Mefloquine[b]	-	0.02 (±0.0)	-	-
Gentian violet[c]	0.19 (± 0.0)	-	-	-

Note: Values are means (±SD) of three independent experiments. [a]Positive control against epimastigote (Epi.) and trypomastigote (Try.) forms of *T. cruzi*; [b]Positive control against *P. falciparum* (Pla.); [c]Positive control against mammalian cells.

somewhat from study by Fontes et al. (15), especially for the absence of monoterpene compounds. These variations or detector sensitivity or sample dilution in the composition of the major constituents of *G. pogonopus*, as well as the contents of all components, can be related to soil and climate conditions, water stress, collection place, nutrition and other biotic factors (24).

After elucidated the chemical composition of the essential oils EOGF and EOGP their antiparasitic activity against *P. falciparum* and different forms of *T. cruzi* was investigated. The essential oils displayed potent antimalarial activity against erythrocitic stages of *P. falciparum* and trypanocidal action against bloodstream trypomastigotes and epimastigotes forms of *T. cruzi*. As revealed in Table 2, EOGF and EOGP presented an IC_{50} of 0.5 μg/mL and 6.8 μg/mL, respectively against *P. falciparum*, an IC_{50} of 11.9 μg/mL and 28 μg/mL respectively against epimastigotes forms of *T. cruzi* and an IC_{50} of 10.7 μg/mL and 41.3 μg/mL, respectively against trypomastigotes forms of *T. cruzi*. The cytotoxicity of essential oils against mammalian cells was also determined. EOGF showed a CC_{50} value of 37.7 μg/mL and EOGP showed no toxicity in this assay, having a CC_{50} higher than 100 μg/mL (Table 2), therefore being selective antiparasitic compounds.

To understand how the essential oils affect intracellular forms of *T. cruzi*, an *in vitro* model of mouse macrophages infected with Y strain was performed. As revealed in Figure 1, both concentrations of EOGF significantly reduced the number of infected macrophages ($P < 0.05$) and the higher concentration was able to decrease the

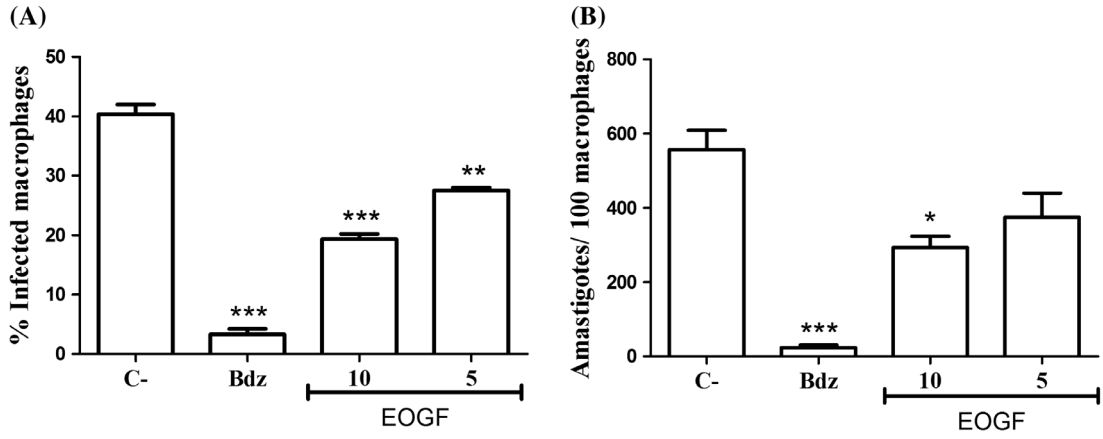

Figure 1. Effect of essential oil from *Guatteria friesiana* on *T. cruzi* amastigotes proliferation in macrophages. Mouse peritoneal macrophages were infected with *T. cruzi* and treated with the essential oil EOGF (5 or 10 µg/mL) or benznidazole (5 µg/mL), a standard drug, for 72 hours. Number of infected cells (A) and amastigotes (B) were determined by couting hematoxylin and eosin-stained cultures. C- is negative control. Bdz is benznidazole. Values represent the mean ± SEM of triplicates. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to infected and untreated cultures.

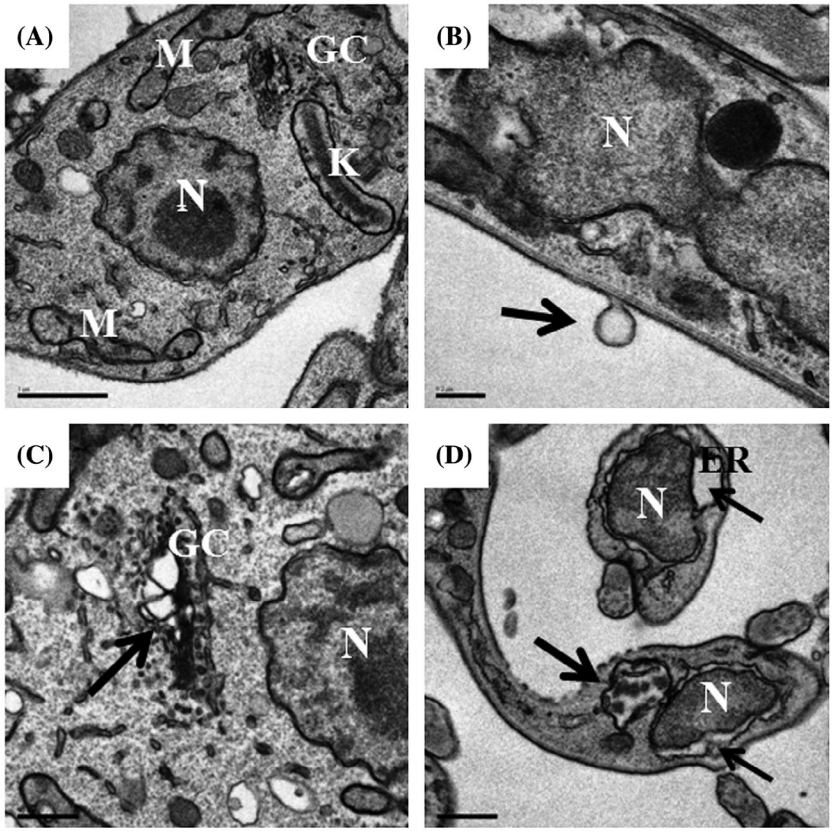

Figure 2. Transmission electron micrographs of trypomastigotes treated or not with EOGF for 24 hours. (A) shows an image of untreated trypomastigotes presenting a typical morphology of the nucleus (N), kinetoplast (K), mitochondria (M) and Golgi complex (GC). Treatment with EOGF at 10.7 µg/mL (B-C) and 21.4 µg/mL (D) causes alterations in parasite plasma membrane (B), Golgi complex disorganization (C), mitochondrial disruption and alteration in ER membrane (D). Black arrows indicate changes in organelles. Scale bars: A = 1 µm; B and D = 0.5 µM; C = 0.2 µm.

number of amastigotes. Benznidazole, under the same conditions, was more effective than the essential oil.

Finally, after confirming that essential oils were able to kill parasitic cells, we performed electron microscopy analysis to understand how they affect parasite cells. For this purpose we used transmission electron microscopy (TEM) to examine the ultrastructural morphology of trypomastigotes (Y strain) treated with EOGF (10.7 or 21.4 µg/mL)

for 24 hours. As we can see in Figure 2, thin sections of untreated trypomastigotes observed by TEM revealed normal appearance of organelles, intact plasma membrane and parasite cytoplasm without alterations (Figure 2A). However, the treatment with EOGF (10.7 μg/mL) caused plasma membrane alterations (Figure 2B) and disorganization of Golgi complex (Figure 2C). Degeneration of mitochondria and alteration in membranes of endoplasmic reticulum were also observed on parasites treated with 21.4 μg/mL of EOGF (Figure 2D). Structural alterations in Golgi complex and endoplasmic reticulum are common on parasites treated with cruzain inhibitors, the major cysteine protease of parasite (25). On the other hand, alterations on plasma membrane and on mitochondrial morphology indicate depletion on parasite metabolism (26). Thus, further investigations are necessary to clarify how the essential oils from *Guatteria* species promote parasite cell death.

In conclusion, the low cytotoxicity and the higher antiparasitic activity of essential oils from *Guatteria* species reinforce the potential of Annonaceae species for development of safer antiparasitic drugs.

Acknowledgments

The authors are grateful to CNPq, CAPES, FAPITEC/SE, and FAPESB for financial support.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico [grant number 301837/2015-5]; Fundação de Amparo à Pesquisa do Estado da Bahia [grant number PET0042/2013]; Programa de Apoio a Núcleos de Excelência [grant number PNX0002/2014].

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