

## Metabolites from Roots of *Colubrina greggii* var. *yucatanensis* and Evaluation of their Antiprotozoan, Cytotoxic and Antiproliferative Activities

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A purificação do extrato da raiz de *Colubrina greggii* var. *yucatanensis* levou ao isolamento e identificação do ácido 3-*O*-acetil ceanótico, um novo triterpeno natural, juntamente com os metabólitos já descritos: ácido ceanótico, ácido cenoténico, ácido betulínico, discarina B e crisofaneína. Os produtos naturais e os derivados semi-sintéticos éster de acetil dimetil ceanotato, dimetil ceanotato e peracetato de crisofaneína mostraram moderada a baixa atividade leishmanicida e tripanocida. Nenhum dos metabólitos mostrou ser citotóxicos ou ter atividade antiproliferativa. Os resultados também sugerem que o ácido betulínico contribui para a atividade antiplasmódica inicialmente detectada na raiz do extrato bruto de *C. greggii* var. *yucatanensis*.

Purification of the root extract of *Colubrina greggii* var. *yucatanensis* resulted in the isolation and identification of 3-*O*-acetyl ceanothic acid as a new natural ceanothane triterpene, together with the known metabolites ceanothic acid, ceanothenic acid, betulinic acid, discarine B and chrysophanein. The natural products and the semisynthetic esters acetyl dimethyl ceanothate, dimethyl ceanothate and chrysophanein peracetate showed moderate to low leishmanicidal and trypanocidal activities. None of the metabolites showed cytotoxic or antiproliferative effects. The results also suggested that betulinic acid contributes to the antiplasmodial activity originally detected in the crude root extract of *C. greggii* var. *yucatanensis*.

**Keywords:** *Colubrina greggii* var. *yucatanensis*, Rhamnaceae, antiprotozoan, cytotoxic ceanothane

### Introduction

Leishmaniosis, trypanosomiosis and malaria are a group of protozoan diseases considered of significant importance due to their incidence and rate of mortality in developing countries.<sup>1</sup> The low effectiveness, limited availability and high toxicity of existing treatments for these illnesses emphasize

the importance of continuing the search for new antiprotozoan pharmaceuticals.<sup>2</sup> Plants are considered an important source of biologically active natural products,<sup>3</sup> including a number of them with antiprotozoan activity.<sup>4-6</sup> During the screening of extracts from native Yucatecan medicinal plants as potential sources of bioactive metabolites, the root extract of *Colubrina greggii* S. Watson var. *yucatanensis* M. C. Johnst., a shrub used for the treatment of liver diseases, ulcerations, abscesses, asthma and tuberculosis,<sup>7</sup> showed

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trypanocidal, antimalarial, leishmanicidal and cytostatic activity.<sup>8,9</sup> Previous phytochemical studies of the genus *Colubrina* (Rhamnaceae), which include 31 species,<sup>10</sup> reported the presence of a wide structural diversity of metabolites, including ansa macrolides, saponins, aporphinic alkaloids, phenolic acids, flavones and triterpenoid acids.<sup>11-18</sup> To date, only chrysophanol, an anthraquinone with antimicrobial activity, has been reported as a metabolite from *C. greggii*.<sup>19</sup> In the present study we report the leishmanicidal, trypanocidal, antiplasmodial, cytotoxic and antiproliferative activity of the crude extract, semi-purified fractions and isolated metabolites from the root extract of *C. greggii* var. *yucatanensis*.

## Results and Discussion

Fractionation of the bioactive root extract of *C. greggii* var. *yucatanensis* yielded a low polarity fraction with leishmanicidal, trypanocidal and antiplasmodial activities and a medium-polarity fraction with leishmanicidal activity. Successive purification of the medium-polarity fraction resulted in the isolation of 3-*O*-acetyl-ceanoth-20(30)-en-1,28-dioic acid (3-*O*-acetyl ceanothic acid) (**1**) as a new natural ceanothane triterpene, together with the known metabolites ceanothic acid (**2**), discarine B (**5**) and chrysophanein (**6**). Similarly, purification of the low-polarity fraction led to the isolation and identification of ceanothenic acid (**3**) and betulinic acid (**4**) (Figure 1).

The FTIR spectrum of **1** showed absorption bands at 3073 (CH=C), 1731 (ester) and 1681 cm<sup>-1</sup> (carboxyl). The pseudo molecular ion peak [M + Na]<sup>+</sup> at *m/z* 551.3524 in HRMS (high resolution mass spectrometry) indicated a molecular formula C<sub>32</sub>H<sub>48</sub>O<sub>6</sub>, implying nine degrees of unsaturation. Carbon multiplicity, deduced from HMQC (heteronuclear multiple quantum coherence) and DEPT (distortionless enhancement by polarization transfer) experiments, indicated the presence of seven methyl groups, nine methylene, seven methine, and nine quaternary carbons. The <sup>1</sup>H NMR spectrum of **1** (Table S1, Figure S1) displayed seven three-proton singlets at δ 0.88, 0.98, 0.99, 1.06, 1.16, 1.68 and 2.03 consistent with methyl groups attached to quaternary carbons. The presence of an acetyl group was confirmed by the HMBC (heteronuclear multiple bond coherence) correlation of the methyl at δ 2.03 with the carbonyl at δ 172.5, whereas an isopropenyl group was assigned from NMR signals corresponding to a methyl group (δ<sub>H</sub> 1.68) attached to a *sp*<sup>2</sup>-carbon (δ<sub>C</sub> 152.0) showing HMBC correlations with two vinylic protons at δ<sub>H</sub> 4.58 and δ 4.70. The double bond and three carbonyl groups observed in the <sup>13</sup>C NMR of **1** (δ 172.5, 177.4 and 180.1) accounted for four degrees of unsaturation thus indicating that the five remaining unsaturation sites corresponded to a pentacyclic structure. The spectroscopic data of **1** proved to be very similar to those reported for ceanothic acid (**2**), a ceanothane triterpene also known as emmolic acid, originally isolated from *Ceanothus americanus*<sup>20</sup> and later identified from *Colubrina granulosa*.<sup>13</sup> However, the presence of a

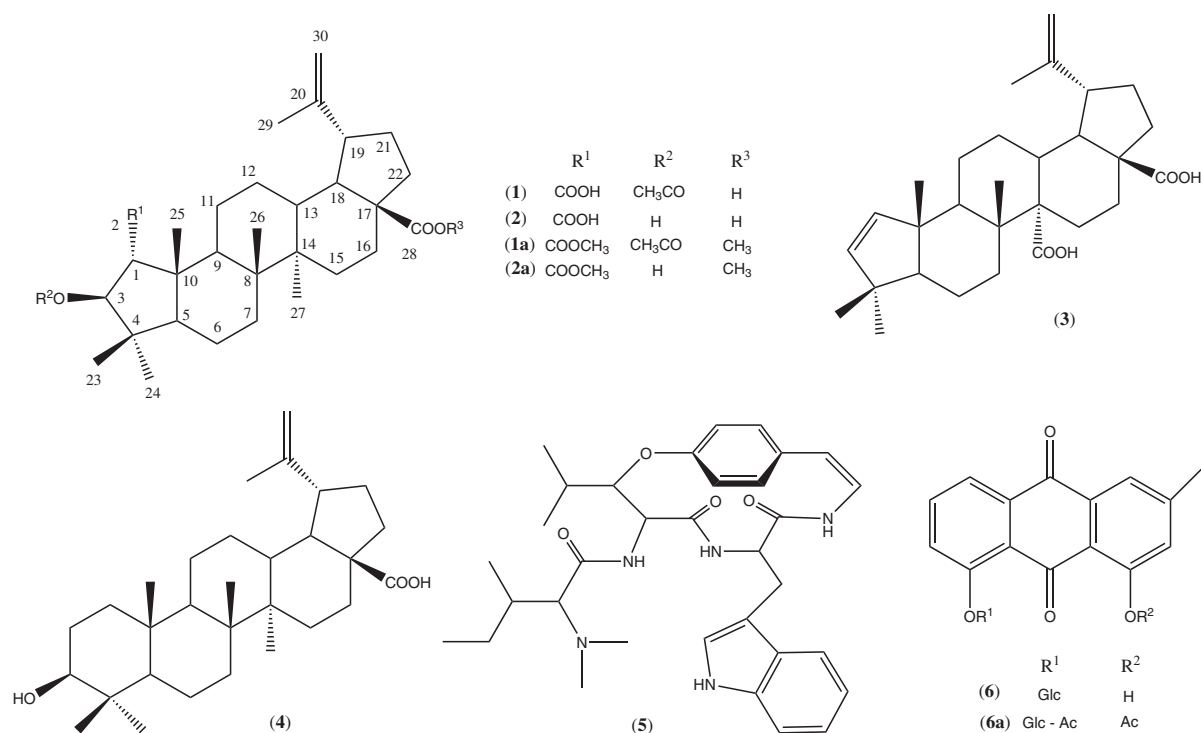


Figure 1. Structures of natural products **1-6** isolated from *C. greggii*, and semisynthetic derivatives **1a**, **2a** and **6a**.

low-field carbinol proton ( $\delta$  5.07) and an acetyl methyl singlet ( $\delta$  2.03) in the  $^1\text{H}$  NMR spectrum of **1**, both showing strong HMBC correlations with the ester carbonyl carbon ( $\delta$  172.5), suggested that **1** was the 3-*O*-acetyl derivative of ceanothic acid, which was confirmed when acetylation of **2** produced **1** as the only product. The presence of **1** in the original root extract of *C. greggii*, as detected by TLC (Figure S2), ruled out its being an artifact of the isolation procedure.

Discarine B (**5**), chrysophanein (**6**) and ceanothenic acid (**3**) were identified from comparison of their spectroscopic data (Tables S1, S2 and S3) with those reported in literature.<sup>21-23</sup> Betulinic acid (**4**) was identified by comparison with an authentic sample.<sup>24</sup>

All of the isolated metabolites and the semisynthetic esters acetyl-dimethyl ceanothate (**1a**), dimethyl ceanothate (**2a**) and chrysophanein peracetate (**6a**) were evaluated for their *in vitro* antiprotozoan (leishmanicidal, trypanocidal, and antiplasmodial), cytotoxic and antiproliferative activities (Table 1). The results showed a moderate leishmanicidal activity ( $\text{IC}_{50}$  values of 20-28  $\mu\text{g mL}^{-1}$ ) for natural ceanothanes **1** and **3** and semisynthetic derivatives **2a** and **6a**, whereas a low trypanocidal activity ( $\text{IC}_{50}$  of 30-70  $\mu\text{g mL}^{-1}$ ) was observed for the natural products **3-5** and the semisynthetic esters **1a** and **6a**. Betulinic acid (**4**) appeared to contribute to the antiplasmodial activity of the crude extract of *C. greggii*, with an  $\text{IC}_{50}$  of 9.7  $\mu\text{g mL}^{-1}$ .<sup>25,26</sup>

Although the crude root extract, together with the low and medium polarity fractions showed cytotoxic activity against HEP-2 cells, none of the isolated metabolites displayed this type of activity (Table S4). Furthermore, none of the metabolites tested, with the exception of the peracetylated chrysophanein (**6a**), showed antiproliferative activity against KB cells (Table S5). It is interesting to point out that the cytotoxic activity of *C. macrocarpa* and *C. texensis* has been attributed to the presence of colubrinol and its acetate,<sup>11,27</sup> however these metabolites were not detected in the root extract of *C. greggii*.

To date, ceanothane triterpenes have only been reported to occur in species of the Rhamnaceae family,<sup>20,23,28-36</sup> and particularly in those belonging to the ziziphoids in the tribal classification reported by Richardson *et al.*<sup>38</sup> These results, together with our finding of ceanothanes in the root extract of *C. greggii*, support the possible use of this class of triterpenes as chemotaxonomic markers for a classification of Rhamnaceae based on a phylogenetic analysis.

## Experimental

### General

Analytical TLC (thin layer chromatography) was carried out on aluminum-backed silica gel (60F<sub>254</sub>) plates

**Table 1.** Leishmanicidal, trypanocidal, antiplasmodial and cytotoxic activity [ $\text{IC}_{50}$  ( $\mu\text{g mL}^{-1}$ )] of crude extract, semipurified fractions, natural products **1-6** and semisynthetic derivatives **1a**, **2a** and **6a**

Extract/ Compound	Antiprotozoan activity					
	<i>L. amazonensis</i>		<i>T. cruzi tulahuén</i>		<i>P. falciparum</i>	VERO
	$\text{IC}_{50}$	SI	$\text{IC}_{50}$	SI	$\text{IC}_{50}$	$\text{IC}_{50}$
CG-1	32.4	-	> 100	-	8.0	NT
CG-2A	< 25	-	73.9	-	4.5	NT
CG-2B	< 25	-	> 100	-	> 10	NT
<b>1</b>	28.2 ± 2.7	3.6	> 100	-	> 10	103.1 ± 1.8
<b>2</b>	46.4 ± 8.4	2.8	> 100	-	NT	131.2 ± 3.2
<b>1a</b>	40.2 ± 9.8	-	56.2 ± 5.2	-	NT	NT
<b>2a</b>	20.6 ± 6.8	-	> 100	-	NT	NT
<b>3</b>	22.1 ± 2.7	4.4	64.0 ± 4.3	1.5	NT	98.6 ± 1.2
<b>4</b>	> 100	-	34.2 ± 11.1	4.2	9.7	145.0 ± 2.9
<b>5</b>	> 100	-	56.2 ± 3.2	3.5	> 10	199.1 ± 2.7
<b>6</b>	> 100	-	56.7 ± 19.5	9.1	> 10	521.0 ± 6.3
<b>6a</b>	12.7 ± 1.1	9.7	65.3 ± 9.0	1.8	> 10	123.8 ± 4.2
PTM	10.0 ± 0.8	-	-	-	-	NT
BZD	-	-	7.4 ± 0.5	-	-	NT
CLQ	-	-	-	-	0.1 ± 0.02	NT

CG-1: crude extract of *C. greggii*; CG-2A: low polarity fraction; CG-2B: medium polarity fraction; PTM: pentamidine; BZD: benznidazole; CLQ: chloroquine; SI: selectivity index were calculated as the ratio  $\text{IC}_{50}$  of cytotoxic activity in VERO cells/ $\text{IC}_{50}$  of antiprotozoan activity.

(E.M. Merck, 0.2 mm thickness) and the chromatograms visualized using a solution of phosphomolybdic acid (20 g) and ceric sulfate (2.5 g) in 500 mL of sulfuric acid (5%). Flash chromatography purifications were performed using silica gel (Aldrich, 200-400 mesh), while TLC-grade silica gel 60GF<sub>254</sub> (E.M. Merck) was used for vacuum liquid chromatography (VLC). Prep-TLC purifications were carried out using glass plates impregnated with silica gel 60 F<sub>254</sub> (E.M. Merck, 0.25 mm thickness, 20 × 20 cm). Melting points (uncorrected) were determined from a Mel-Temp II apparatus (Laboratory Devices Inc.). The optical rotations were measured in CHCl<sub>3</sub> using a Perkin Elmer 341 polarimeter. FTIR (Fourier transform infrared) spectra were recorded in CHCl<sub>3</sub> or MeOH (film) using an FT-Nicolet Magna Protégé 460 spectrophotometer. <sup>1</sup>H NMR (400 and 600 MHz) and <sup>13</sup>C NMR (100 and 150 MHz) spectra were acquired on a Bruker Avance 400 spectrometer or a Bruker Avance 600 spectrometer with CHN cryoprobe, using the residual solvent resonances as internal references, calibrated to TMS. Electrospray high-resolution mass spectra (ESI-HRMS) were determined by direct injection on a Waters Q-TOF microsystem (using 0.1% phosphoric acid in a 1:1 water/acetonitrile mixture as reference), or using an Orbitrap MS (Thermo) connected to a Surveyor HPLC (high-performance liquid chromatography, Thermo) for sample injection.

#### Plant material

Roots of *Colubrina greggii* S. Watson var. *yucatanensis* M. C. Johnst. were collected in Abalá, Yucatán, México. A voucher specimen (P. Simá-D. Domínguez 2916) was deposited in the Herbarium of Centro de Investigación Científica de Yucatán.

#### Extraction of plant material and purification of crude extract

Dry roots of *C. greggii* (365 g) were extracted three times with ethanol (4 L) at room temperature. Evaporation of the solvent yielded the corresponding crude extract (CG-1, 54.5 g, 14.9%), which was suspended in 1.8 L of a H<sub>2</sub>O/MeOH (3:2, v/v) mixture. The suspension was fractionated by successive liquid-liquid partition with hexane (three times; 2:1, 1:1, 1:1; v:v of solvent:aqueous suspension), ethylacetate (three times; 2:1, 1:1, 1:1) and water-saturated butanol (1:2; v:v solvent:aqueous suspension) to yield the corresponding low (CG-2A), medium (CG-2B) and high (CG-2C) polarity fractions. Purification of the medium polarity fraction (CG-2B, 9.6 g) by VLC, using a gradient elution with CH<sub>2</sub>Cl<sub>2</sub>/Me<sub>2</sub>CO (99:1 to 94:6) followed by CHCl<sub>3</sub>/hexane/MeOH (70:25:5), yielded fractions CG-5A-N. Fraction CG-5E (550 mg) was purified by flash

chromatography using an isocratic elution with hexane/Me<sub>2</sub>CO 8:2, to produce pure 3-*O*-acetyl-ceanothic acid (**1**, 172.1 mg), and fraction CG-6I (50 mg) which was further purified by column chromatography [ether/hexane (1:1)] to yield ceanothic acid (**2**, 16.5 mg). Additional purification of fraction CG-5C (414 mg) by flash chromatography, using a gradient elution with hexane/Me<sub>2</sub>CO (8:2-7:3), resulted in the isolation of discarine B (**5**, 108 mg). Finally, chrysophanein (**6**, 168.5 mg) was collected as a yellow precipitate by filtration from fraction CG-5G. Purification of the low polarity fraction (CG-2A, 1.5 g) by VLC, using a gradient elution with mixtures of hexane/Me<sub>2</sub>CO/MeOH (95:3:2 to 60:38:2), produced eleven fractions (CG-3A-K). Fractions CG-3E-F were combined (500 mg) and purified by flash chromatography (hexane/ether 7:3), to yield ceanothenic acid (**3**, 20.8 mg) and betulinic acid (**4**, 20.4 mg).

#### 3-*O*-acetyl-ceanothic acid (**1**)

White amorphous solid; mp: 271.5-273.1 °C;  $[\alpha]_D^{20} +33.7^\circ$  (c 0.01, Me<sub>2</sub>CO); FTIR (film)  $\nu_{\max}$  cm<sup>-1</sup>: 3073 (CH=C), 1731 (ester), 1685 (carboxyl); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz) and <sup>13</sup>C NMR (CD<sub>3</sub>OD, 100 MHz) data: see Table S1; ESI-HRMS  $m/z$  551.3524 [M + Na]<sup>+</sup> (calc. for C<sub>32</sub>H<sub>48</sub>NaO<sub>6</sub>: 551.3584).

#### Ceanoctic acid (**2**)

White amorphous solid;  $[\alpha]_D^{20} + 30.8^\circ$  (c 0.003, MeOH); FTIR (film)  $\nu_{\max}$  cm<sup>-1</sup>: 3411 (OH), 1697 (>C=O); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz):  $\delta$  0.88 (s, 3H, H-24), 0.90 (s, 3H, H-26), 0.97 (s, 3H, H-27), 1.01 (s, 3H, H-25), 1.35 (s, 3H, H-23), 1.67 (s, 3H, H-29), 2.45 (s, 1H, H-1), 3.06 (m, 1H, H-19), 4.06 (s, 1H, H-3), 4.57 (br s, 1H, H-30a), 4.69 (br s, 1H, H-30-b); ESI-HRMS  $m/z$  487.3418 [M + H]<sup>+</sup> (calc. for C<sub>30</sub>H<sub>47</sub>O<sub>5</sub>: 487.3423).

#### 3-*O*-acetyl-dimethyl-ceanothate (**1a**)

A mixture of **1** (5.1 mg), K<sub>2</sub>CO<sub>3</sub> (80 mg), CH<sub>3</sub>I (300  $\mu$ L) and acetone (1 mL) was stirred for 72 h at room temperature. The reaction mixture was poured over distilled water (14 mL) and the resulting suspension was extracted twice with EtOAc (4:1, v/v). The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated to produce 4.6 mg of the crude esterified product, which was purified by column chromatography (hexane/Me<sub>2</sub>CO 9:1) to give **1a** (4.2 mg, 91.3% yield) as a white powder. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 600 MHz) and <sup>13</sup>C NMR (CDCl<sub>3</sub>, 150 MHz) data: see Table S1; LR-MS  $m/z$  557 [M + H]<sup>+</sup>.

#### Dimethyl-ceanothate (**2a**)

A fraction containing ceanothic acid as the main product (8.5 mg), was mixed with K<sub>2</sub>CO<sub>3</sub> (220 mg), CH<sub>3</sub>I

(800  $\mu\text{L}$ ) and acetone (1 mL), and then stirred for 72 h at room temperature. The reaction mixture was poured over distilled water (13 mL) and the resulting suspension was extracted twice with EtOAc (4:1, v/v). The organic phase was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and evaporated to produce 9.6 mg of the crude esterified product, which was purified by multiple-elution (5  $\times$ ) prep-TLC (hexane/ether 7:3) to give 5.4 mg of **4** (56.2%) as a white solid.  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ , 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) data: see Table S1; ESI-HRMS  $m/z$  515.3731  $[\text{M} + \text{H}]^+$  (calc. for  $\text{C}_{32}\text{H}_{51}\text{O}_5$ : 515.3736).

#### Ceanothenic acid (**3**)

White powder; FTIR (film)  $\nu_{\text{max}}/\text{cm}^{-1}$ : 3067 (CH=C), 1721 (carboxyl), 1685 (carboxyl);  $^1\text{H}$  NMR ( $\text{CDCl}_3/\text{CD}_3\text{OD}$  9:1, 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3/\text{CD}_3\text{OD}$  9:1, 100 MHz) data: see Table S1; ESI-HRMS  $m/z$  455.3156  $[\text{M} + \text{H}]^+$  (calc. for  $\text{C}_{29}\text{H}_{43}\text{O}_4$ : 455.3161).

#### Betulinic acid (**4**)

Colorless needles; ESI-HRMS  $m/z$  440.3690  $[\text{M}-\text{H}_2\text{O} + 2\text{H}]^+$  (calc. for  $\text{C}_{30}\text{H}_{48}\text{O}_2$ : 440.3654).

#### Discarine B (**5**)

White amorphous solid;  $\nu_{\text{max}}/\text{cm}^{-1}$ : 3267 (NH), 3027 (CH=C), 1634 (>C=O);  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ , 30  $^\circ\text{C}$ , 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ , 30  $^\circ\text{C}$ , 100 MHz) data: see Table S3; ESI-HRMS  $m/z$  574.3393  $[\text{M} + \text{H}]^+$  (calc. for  $\text{C}_{33}\text{H}_{44}\text{N}_5\text{O}_4$ : 574.7336).

#### Chrysophanein (**6**)

Yellow powder; FTIR (film)  $\nu_{\text{max}}/\text{cm}^{-1}$ : 3344 (OH), 1634 (>C=O);  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ , 100 MHz) data: see Table S2; ESI-HRMS  $m/z$  255.2302  $[\text{M}-\text{C}_6\text{H}_{10}\text{O}_5 + \text{H}]^+$  (calc. for  $\text{C}_{15}\text{H}_{11}\text{O}_4$ : 255.0657).

#### Chrysophanein peracetate (**6a**)

A mixture of **6** (10 mg), acetic anhydride (1 mL) and pyridine (0.5 mL) was stirred at room temperature for 72 h. The reaction mixture was poured over distilled water (20 mL) and the resulting suspension was extracted twice with ethylacetate (2:1 v/v). The organic layer was washed successively with equal volumes of HCl (5%), NaOH (3%),  $\text{H}_2\text{O}$ , and NaCl saturated, and then dried over anhydrous  $\text{MgSO}_4$ . Filtration and evaporation of the solvent yielded 13.9 mg (92.4%) of crude acetylated product obtained as a yellow powder; FTIR (film)  $\nu_{\text{max}}/\text{cm}^{-1}$ : 3021 (CH=C), 1757 (ester), 1680 (>C=O);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) data: see Table S2; ESI-HRMS  $m/z$  649.1499  $[\text{M} + \text{Na}]^+$  (calc. for  $\text{C}_{31}\text{H}_{30}\text{NaO}_{14}$ : 649.1533).

## Bioassays

### Leishmanicidal assay

The growth inhibition of promastigotes was carried out following the procedure previously reported by Muñoz *et al.*,<sup>39</sup> and Inchausti *et al.*<sup>40</sup> Briefly, a strain of *L. amazonensis* (IFLA/BR/75/PH8) was grown in Schneider culture medium with 10% fetal bovine serum (FBS), penicillin (100 IU  $\text{mL}^{-1}$ ) and streptomycin (100 mg  $\text{mL}^{-1}$ ) at 25  $^\circ\text{C}$ ; parasites in the log phase of their growth cycle were then transferred to a microplate (96 wells;  $1 \times 10^5$  parasites/well). Stock solutions of DMSO (blank), pentamidine (positive control), crude extract, semipurified fractions and pure metabolites were diluted in Schneider medium at  $\leq 100 \mu\text{g mL}^{-1}$ , added to the plate, and incubated for 72 h. The percentages of inhibition were obtained by directed observation of each well with an inverted phase microscope. All the assays were carried out in triplicate.

### Trypanocidal assay

Epimastigotes of *Trypanosoma cruzi* strain Tulahuen parasites were maintained in liver infusion tryptose (LIT) medium supplemented with 5% FBS, following the procedure modified by Chataing *et al.*<sup>41</sup> Briefly, parasites in the log phase of growth cycle were transferred to a microplate (96 wells;  $1 \times 10^6$  parasites/well) together with stock solutions of benznidazole, DMSO (positive control and blank respectively), extract, semipurified fractions or pure metabolites prepared at different concentrations ( $\leq 100 \mu\text{g mL}^{-1}$ ). The microplates were incubated at 26  $^\circ\text{C}$  for 72 h.

### Antiplasmodial assay

*Plasmodium falciparum* strain F32 was grown at 37  $^\circ\text{C}$  in RPMI medium with 10% of human serum and 4% of hematocrit (O, Rh+), under anaerobic conditions, according to a reported method.<sup>41</sup> Cultures with 1% parasitemic and 2% hematocrite (100  $\mu\text{L}$ ) were transferred to a 96 well plate. Stock solutions of chloroquine (positive control), DMSO (blank), extract, semipurified fractions or pure metabolites were diluted in RPMI medium to a concentration of  $< 10 \mu\text{g mL}^{-1}$  and added to each well. The plate was then incubated at 37  $^\circ\text{C}$  for 48 h.

### Cytotoxicity assay

Human laryngeal carcinoma (HEp-2), human cervical adenocarcinoma (HeLa), human nasopharyngeal carcinoma (KB), and green monkey Vero kidney cells (VERO) were grown in DMEM (Gibco) media supplemented with 10% (v/v) FBS (Gibco), penicillin (100 IU  $\text{mL}^{-1}$ ), and streptomycin (100 mg  $\text{mL}^{-1}$ ). All the cell lines were maintained at 37  $^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere with 95%

humidity. The cytotoxicity assay was performed according to a method described by Rahman *et al.*<sup>43</sup> Briefly, cell lines were transferred to a microplate ( $1.5 \times 10^4$  viable cells of each cell line) and incubated at 37 °C, with 95% humidity and 5% CO<sub>2</sub> in DMEM medium supplemented with 10% of FBS, penicillin (10000 IU), streptomycin (10 mg mL<sup>-1</sup>), and amphotericin B (5 mg mL<sup>-1</sup>). After 24 h, the medium was replaced by fresh medium with 0.05% DMSO (blank) or different concentrations of docetaxel (positive control, Sigma), crude extract, semipurified fractions or pure metabolites dissolved in DMSO (100, 50, 25, 12.5 and 6.25 µg mL<sup>-1</sup>), and the cells were incubated for 72 h under the conditions already described. The medium was removed and 200 µL of a 0.5% MTT (Sigma) solution in PBS (pH 7.2) were added to each well, and left to stand for 4 h at 37 °C. Then 100 µL of acidified isopropanol (0.4 mol L<sup>-1</sup> HCl) were added to each well and the optical density (OD) measured at 540 nm using a Bioassay reader (Bio-Rad). The experiment was carried out in triplicate and each concentration was tested in duplicate.

#### Antiproliferative assay

The sulforhodamine B (SRB) assay was carried out according to the method reported by Rahman *et al.*,<sup>43</sup> using DMEM medium with 10% FBS to induce cell proliferation. After 48 h of incubation, the medium was discarded and 100 µL of ice-cold 40% trichloroacetic acid (TCA, Aldrich) were added to fix the cells, incubating for 1 h at 4 °C. The cells were washed five times with water, left to dry, and then 50 µL of SRB stain (10 mg 1% acetic acid, Sigma) were added to each well and left to stand for 30 min. Finally, the cells were washed with 50 mL 1% acetic acid, and rinsed four times with water. The OD was measured at 540 nm using an ELISA reader (Bio-Rad model 450). The experiment was carried out in triplicate.

#### Statistical analysis

Data were analyzed with commercial software (GraphPad 4.0, Software Inc., San Diego, CA). The dose-response curves (variable slope) to obtain the inhibitory concentration (in µg mL<sup>-1</sup>) of 50% of parasites (IC<sub>50</sub>), the growth inhibition of 50% of cells (IG<sub>50</sub>), and the cytotoxic concentration of 50% of cells (CC<sub>50</sub>), were fitted to the algorithm:  $Y = E_{\min} + [(E_{\max} - E_{\min}) / (1 + 10(\log ED_{50} - \log D) \text{ hill slope})]$ .

### Supplementary Information

Supplementary data (Figures S1-S2, Tables S1-S5) are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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