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ALKALOIDS AND A FLAVONOID FROM AERIAL PARTS (LEAVES AND TWIGS) OF DUGUETIA FURFURACEA - ANNONACEAE

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ABSTRACT

Duguetia furfuracea is a shrub of the Annonaceae family that occurs in several regions in Brazil. In the state of Mato Grosso do Sul this species often becomes an invading plant when the cerrado is turned into pastures. In the local folk medicine, its powdered seeds are mixed with water and used against pediculosis, and the infusion of twigs and leaves is used to treat rheumatism.. The present work led to the isolation and characterization from the aerial parts of the plant (leaves and twigs), of twelve isoquinoline alkaloids of aporphine oxoaporphine, bisbenzyltetrahydro-isoquinoline, benzyltetrahydro-isoquinoline and tetrahydroprotoberberine skeletons. In addition b-sytosterol and the flavonoid isorhamnethin were obtained. The activity of these different patterns of isoquinoline skeleton and the flavonoid was tested against *Trypanosoma cruzi*, and only aporphinoid skeleton with (*R*)-configuration, (-

)-asimilobine, was really effective against trypomastigotes (strain Y), killing approximately 72% of the parasites at a maximal concentration of 128.0 mM (IC_{50} 57.2 mM). The trypanocidal activity of the alkaloids and the flavonoid have been investigated.

Keywords: *Duguetia furfuracea*, Annonaceae, alkaloid, flavonoid, *Trypanosoma cruzi*.

INTRODUCTION

Duguetia furfuracea is a shrub of the Annonaceae family that occurs in several regions in Brazil. In the state of Mato Grosso do Sul this species often becomes an invading plant when the cerrado is turned into pastures[1]. In the local folk medicine, its powdered seeds are mixed with water and used against pediculosis[2,3], and the infusion of twigs and leaves is used to treat rheumatism[4]. Although some chemical data are available for Brazilian native species of this genus, few biological[5,6] and chemical studies have been conducted with *D. furfuracea*. These include reports of the isolation, from the leaves of the plant, of flavonoid glycosides identified by HPLC-MS-ES and UV and visible spectrometric data[7] and of several other sesquiterpenoids obtained from the volatile oil[8]. Antiplasmodial[5] and protozoal[6] activities have also been reported, and capsules containing "*D. furfuracea* material" indicated for renal disorders are currently being patented[9].

The present paper describes the isolation and characterization of 12 alkaloids from aporphine, oxoaporphine, benzyltetrahydro-isoquinoline, bisbenzyltetrahydro-isoquinoline, and tetrahydroprotoberberine skeletons. In addition, b-sytosterol and the flavonoid isorhamnetin were isolated from different extracts obtained from the aerial parts (leaves and twigs) of the plant. The activity of these isoquinoline alkaloids and of the flavonoid was tested against Tripomastigote forms of Y strain protozoan parasite *Trypanosoma cruzi*.

EXPERIMENTAL

General experimental procedures

Melting points were determined on a Uniscience 498 apparatus and remain uncorrected. Optical rotations were run in chloroform on a Perkin Elmer 341 instrument. All NMR experiments were performed on a DPX-300 Brucker instrument (^1H: 300 MHz; ^{13}C: 75 MHz) using $CDCl_3$ as solvent and TMS as the internal standard. Chemical shifts are reported in d units and coupling constants (*J*) in Hz. 60 G and 60 GF_{254} silica gel (Merck) and 70-230 mesh (Aldrich) were used for TLC and CC, respectively. TLC impregnated with 2% or 5% oxalic acid was also performed, using the usual methodology[10]. (Caution! Oxalic acid releases toxic vapors when heated.)

Plant material

The aerial parts (leaves and twigs) of *Duguetia furfuracea* (A. St.-Hil.) Benth. & Hook. f. Annonaceae, were collected on the UFMS campus in Campo Grande, MS, Brazil, and identified by Prof. R. Mello Silva. A voucher specimen (number 023) has been deposited in the CGMS Herbarium (Campo Grande, MS).

Extraction and isolation

Preparation and fractionation of the hexane extract from leaves

Powdered dried leaves of *D. furfuracea* (160 g) were submitted to exhaustive hexane extraction in a soxhlet apparatus and yielded 5.3 g of hexane extract. By submitting 4.5 g of the extract to column chromatography (150 g of silica-gel) and using hexane, methylene chloride and methanol as eluents, b-sytosterol was isolated.

Preparation of the ethanol extract from the leaves

4.8 kg of powdered dried leaves were submitted to percolation in 95% ethanol for 72 h, furnishing 0.97 kg of extract after concentration.

Partitioning of the ethanol extract obtained from the leaves

The ethanol extract (0.97 kg) was shaken for 6 h with a 3% HCl solution. The marc was discarded and the resulting acidic layer (1) was partitioned with methylene chloride, furnishing a methylene chloride layer (A) and an acidic (2) layer. Layer A was washed with water and, from the organic solvent,

isorhamnetin (**13**) was obtained. The acidic (2) layer was adjusted to pH 9.0 with ammonium hydroxide and then extracted with methylene chloride, furnishing a fraction (14 g) that was labeled "DfFalk-a", which was then solubilized in chloroform and submitted to partition with 3% HCl, yielding acidic (3) and chloroform layers. The acidic (3) layer was submitted to a 10% $NH_4OH/CHCl_3$ procedure and furnished an alkaloid fraction labeled "DfFalk-b," which was fractionated as described below. The chloroform layer mentioned above was sequentially extracted with 3% HCl, 10% NH_4OH and $CHCl_3$, and the resulting chloroform layer was labeled "DfFalk-c".

Further partitioning of the "DfFalk-b" fraction

This fraction (6 g) was submitted to column chromatography with aluminum oxide and a hexane:chloroform:methanol gradient, yielding an aterospermidine-liriodenine mixture (**10**/**11**), noriscorydine (**2**), xylopine (**3**), an obovanine-anonaine mixture (**4**/**5**), ()-asimilobine (**6**), isochondodendrine (**7**), ()-discretamine (**8**) and (+)-reticuline (**9**). The aterospermidine-liriodenine fraction (15 mg) was submitted to column chromatography on silica gel (25 g) and a chloroform:methanol gradient plus 0.5% conc. HCl, allowing each alkaloid to be separated.

Further partitioning of the "DfFalk-c" fraction

This fraction (2 g) was submitted to a column chromatography on silica gel (200 g) and a chloroform:methanol gradient, yielding *stricto sensu* alkaloid (+)-isocorydine (**1**) and a complex mixture of alkaloid fractions that has been not worked yet .

Preparation and fractionation of the alkaloid extract obtained from the bark of twigs

500 g of powdered dried bark of twigs were macerated with 10% ammonium hydroxide and submitted to exhaustive extraction with methylene chloride for 15 days, yielding 10.5 g of extract. By submitting this methylene chloride extract to the usual alkaloid extraction, 1.4 g of alkaloid extract was obtained, which was then fractionated by column chromatography with aluminum oxide (250 g) and an ethyl acetate:methanol:water gradient, yielding a fraction that contained a mixture of aterospermidine, liriodenine and lanuginosine.

This fraction (52 mg) was submitted to preparative TLC on silica gel impregnated with 2% oxalic acid and eluted four times in chloroform:methanol 90:10, yielding fractions A (12 mg) and B (20 mg). Fraction A contained atherospermidin (**3**) mainly, whereas fraction B included two oxoaporphine alkaloids. The latter fraction was again submitted to preparative TLC impregnated with 5% oxalic acid, using methylene chloride : methanol 83:17 as eluent, and yielded fractions B' and B". In fraction B' (8 mg) it was possible to confirm the presence of liriodenine (**11**), predominantly; from fraction B", lanuginosine (**12**) was isolated.

Identification of the isolated compounds

(+)-Isocorydine (**1**): chloroform soluble crystals, 50 mg (0.001%) from bark alkaloid extract, optical rotation, melting point [19], as well ^1H and ^{13}C MNR data[11] are in agreement with those reported in the literature.

—*Norisocorydine* (**2**): dark brown, amorphous, chloroform soluble, 10 mg (0.00021%) from bark alkaloid extract. ^1H and ^{13}C MNR data are in agreement with those reported in the literature[12].

Xylopine (**3**): light brown, amorphous, chloroform soluble, 5 mg (0.0001%) from bark alkaloid extract. The ^1H NMR spectral data was comparable with those described in the literature[13].

Obovanine-anonaine mixture (**4/5**): Amorphous, chloroform soluble, 30 mg (0.00063%) from bark alkaloid extract. The ^1H and ^{13}C NMR spectral data from the mixture were comparable with those found in the literature for each substance separately[14,15].

(—)-Asimilobine (**6**): Dark brown, amorphous, chloroform soluble, 40 mg (0.00083%) from bark alkaloid extract. The ^1H and ^{13}C NMR spectral data and optical rotation were comparable with those described in the literature[16,17].

Isochondodendrine (**7**)[18]: brown, amorphous, slightly soluble in chloroform, soluble in chloroform: methanol 9:1 and chloroform: DMSO 1:1. 32 mg (0.00067%) from bark alkaloid extract. MS-ESI(+) *m/z* (rel. int.): 596 $[M + H]^+$ (75), 299 $[M/2 + H]^+$ (100). ^1H MNR (300 MHz, $CHCl_3$:DMSO-d_6 (1:1), δ): 2.39

(dd, J: 11.6; 12.4 Hz, H-α/H-α'), 2.39 (s, N-Me), 2.70-3.30 (m, H-4/H-4'/H-3/H-3'), 3.05 (br d, J: 12.8 Hz, H-α/H-α'), 3.73 (s, O-Me), 4.10 (br d, f: 10.3 Hz, H-1/H-1'), 5.63 (dd, f: 2.5; 8.5 Hz, H-11/H-11'), 6.17 (dd, f: 1.6; 8.3 Hz, H-10/H-10'), 6.43 (dd, f: 2.5; 8.2 Hz, H-13/H-13), 6.53 (s, H-5/H-5'), 6.91 (dd, f: 1.6; 8.3 Hz, H-14/H-14'), 8.08 (s, OH-7). ^{13}C NMR (75 MHz, $CHCl_3$:DMSO-d_6 (1:1), δ): 23.6 (C-4/C-4'), 38.3 (C-α/C-α'), 42.0 (N-CH_3), 44.2 (C-3/C-3'), 55.7 (OMe), 59.0 (C-1/C1'), 108.4 (C-5/C-5'), 113.6 (C-11/C-11'), 117.1 (C-13/C-13'), 123.9 (C-4a/C-4a'), 124.7 (C-8a/C-8a'), 127.7 (C-10/C-10'), 128.3 (C-14/C-14'), 129.7 (C-9/C-9'), 135.6 (C-7/C-7'), 137.9 (C-8/C-8'), 147.0 (C-6/C-6'), 153.9 (C-12/C-12').

Obtaining a diacetylated derivative of isochondodendrine (7a)

12 mg of isochondodendrine were mixed with 1 mL of acetic anhydride and 1 mL of pyridine and maintained at room temperature overnight. The usual workup afforded 10 mg of a diacetylated derivative of isochondodendrine. Dark brown, amorphous, soluble in chloroform. ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.57 (s, Ac-Me), 2.47 (s, N-Me), 2.57 (dd, f: 12.0 Hz, H-α/H-'), 2.90-3.49 (m, H-4/H-4' and H-3/H-3'), 3.37 (br d, f: 12.7 Hz, H-α/H-α), 3.77 (s, O-Me), 4.42 (br d, f: 10.3 Hz, H-1/H-1'), 5.78 (dd, J: 2.5; 8.3 Hz, H-11/H-11'), 6.28 (br d, f: 7.4 Hz, H-10/H-10'), 6.56 (dd, f: 2.5; 8.5 Hz, H-13/H-13), 6.60 (s, H-5/H-5'), 7.13 (br d, f: 7.4 Hz, H-14/H-14'). ^{13}C NMR (75 MHz, $CDCl_3$, d): 19.8 (Ac-Me), 23.2 (C-4/C-4'), 40.1 (C-α/C-α'), 41.0 (N-CH_3), 43.2(C-3/C-3'), 56.0 (OMe), 59.4(C-1/C1'), 108.7(C-5/C-5'), 114.0 (C-11/C-11'), 117.9 (C-13/C-13'), 121.9 (C-8a/C-8a'), 128.3 (C-10/C-10'), 128.8 (C-14/C-14'), 129.8 (C-7/C-7'), 130.0 (C-9/C-9'), 131.5 (C-4a/C-4a'), 143.6 (C-8/C-8'), 151.5(C-6/C-6'), 153.7 (C-12/C-12'), 167.2 (C=O).

(+)-Discretamine (**8**): colorless crystals, slightly soluble in chloroform, soluble in DMSO, ethanol, 15 mg (0.00031%) from bark alkaloid extract. Optical rotation)[19], melting point[19,20], and ^{1}H and ^{13}C NMR data were comparable with those described in the literature.

(+)-Reticuline (**9**): green, amorphous, soluble in chloroform, 96 mg (0.002%) from bark alkaloid extract. $[a]_D^{20}$: +70($CHCl_3$, *c*: 0.0035); literature: +90 ($CHCl_3$, *c*: 0.04)[12]. ^{1}H and ^{13}C MNR data are in agreement with those reported in the literature[21].

*Aterospermidin*e (**10**): orange crystals, slightly soluble in chloroform, soluble in chloroform-methanol mixture, 12 mg, 0.001% from bark alkaloid extract, 3 mg, 0.000063% from leaf alkaloid extract. ^{1}H and ^{13}C MNR data are in agreement with those reported in the literature[22].

Liriodenine (**11**): Yellow crystals, slightly soluble in chloroform, soluble in methanol; obtained from the bark alkaloid extract, 8 mg, 0.00067%, and from leaf alkaloid extract, 7 mg, 0.00015%. The spectral data were comparable with those described in the literature[23].

Lanuginosine (**12**): Dark yellow crystals, slightly soluble in chloroform, soluble in methanol, 4 mg, 0.00033% from bark alkaloid extract. The spectral data were comparable to those described in the literature[??].

Isorhamnetin (**13**): Light yellow, amorphous, slightly soluble in chloroform, soluble in DMSO, 30 mg (0.00063%) from leaf ethanolic extract. ^{1}H MNR (300 MHz, DMSO-d_6, δ)[24]: 3.83 (s, OMe-3'), 6.19 (br s, H-6). 6.47 (br s, H-8), 6.93 (d, f: 8.2 Hz, H-5'), 7.67 (br d, f: 8.2 Hz, H-6'), 7.74 (br s, H-2'), 9.41 (s, OH-3), 9.75 (s, OH-4'), 10.81 (s, OH-7), 12.44 (s, OH-5). ^{13}C MNR (75 MHz, DMSO-d_6, d): 55.8 (O-Me-3'), 93.6 (C-8), 98.2 (C-6), 103.0 (C-10), 111.8 (C-2'), 115.6 (C-5'), 121.7 (C-6'), 122.0 (C-1'), 135.8 (C-3), 146.7 (C-2), 147.4 (C-3'), 148.8 (C-4'), 156.2 (C-9), 160.7 (C-5), 163.9 (C-7), 175.9 (C-4).

***In vitro* anti-Trypanosoma activity**

Parasite

For the trypanocidal assay, trypomastigote forms of the Y strain of *T. cruzi* were cultivated in LLC-MK2 cells. The cells were cultivated in RPMI medium supplemented with 2mM of L-glutamine, 10mM of $NaHCO_3$, 100U/mL of penicillin, 100 µg/mL of streptomycin and 5% of inactivated, at 37°C in an environment containing 5% of CO_2 and 95% of humidity. The trypomastigote forms were added into the culture at 3:1 proportion. After seven days, the supernatant was removed and centrifuged furnishing free

trypomastigote forms of the parasite for the bioassay.

Anti-trypanosoma activity

Stock solutions were prepared by dissolving the compounds in pure dimethylsulfoxide (DMSO), to obtain a final concentration of 20 mM of each compound. Aliquots of the stock solution were added to the parasite suspension, to furnish final concentrations of 8, 32 and 128 mM, respectively.

Bioassay

In a 96 well microtitle plate about 106 forms of the parasite were added, plus the compounds to be assayed. After 24 hours of incubation, the activity was evaluated by using a colorimetric technique at 595nm by the addition of a tetrazolium salt [MTT; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Also, negative controls (solvent plus culture medium) and positive control (gentian violet at 250 µg/mL) were run in parallel. All the assays were undertaken in triplicate.

RESULTS AND DISCUSSION

In order to obtain an alkaloid extract from the ethanol extract of the leaves, acid-base extraction was performed. During this procedure, precipitation of isorhamnetin (**13**), a flavonoid, occurred in the supernatant (organic phase). This flavonoid might be an artifact, since it had previously been isolated[7] from hydrolyzed isorhamnetin glycosides present in the extract. This possibility, however, was discarded on the basis of evidence provided by TLC of the initial extract.

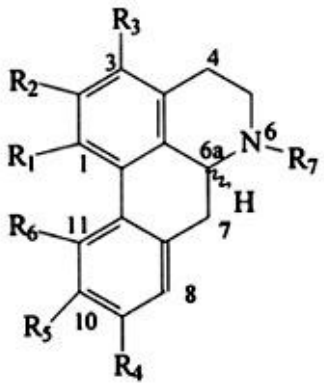
For this compound, ^{1}H and ^{13}C NMR spectral data were used to confirm the presence of a flavonoid skeleton and, together with 2D NMR (HMQC and HMBC) experiments, they provided support for the determination of the methoxyl group position. A singlet signal at δ 3.83 (δ_C 55.8) indicated the presence of a methoxyl group, corresponding to a group linked to C-4' in ring B.

Spectral methods in combination with optical activity data provide an useful tool to help elucidate aporphine *sensu stricto* alkaloid skeletons[12,13]. Six already known aporphine *sensu stricto* alkaloids obtained from the bark of twigs namely, (+)-isocorydine (**1**), norisocorydine (**2**), xylopine (**3**), obovanine (**4**), anonaine (**5**), and ()-asimilobine (**6**) were thus characterized by comparing their ^{1}H and ^{13}C spectral data with those available in the literature[18].

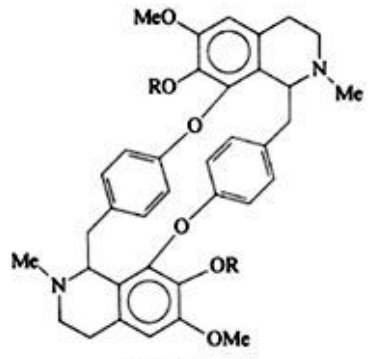
Isochondodendrine (**7**), a dimeric aporphinoid alkaloid with a bisbenzyltetrahydro-isoquinoline skeleton, was also isolated. MS-ESI(+) revealed a pseudomolecular ion at *m/z* 596 ($[M + H]^+$) and a fragment ion at *m/z* 299 ($[M/2 + H]^+$). The ^{1}H and ^{13}C NMR spectral data for **7**, as well as those of its diacetylated derivative, were in accordance with those described in the literature[13].

Compound **8** was isolated as colorless crystals (acetone) and showed positive reaction to Dragendorff's reagent. After uni- and bidimensional analysis of the NMR spectral data and a negative specific rotation value it was possible to confirm **8** as ()-discretamine, a tetrahydro-protoberberinic alkaloid. By comparison of its ^{1}H and ^{13}C NMR spectral data with those described in the literature, some assignments of carbon resonance were corrected.

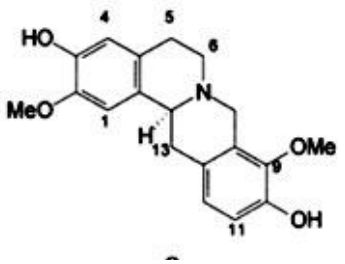
Compound **9**, on the basis of its specific rotation and spectral (^{1}H and ^{13}C NMR) data, which were in accordance with those available in the literature, was identified as being (+)-reticuline, a benzyl-tetrahydroisoquinolinic alkaloid.

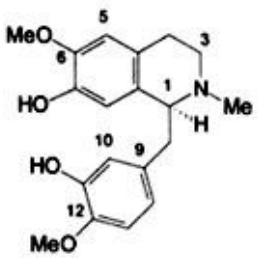


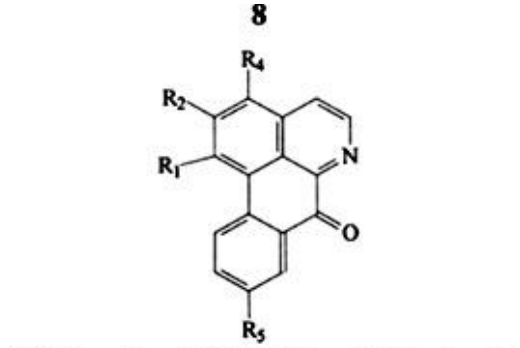
(1) $R_1 = R_2 = R_5 = OCH_3$, $R_3 = R_4 = H$, $R_6 = OH$, $R_7 = CH_3$, and 6a α;
(2) $R_1 = R_2 = R_5 = OCH_3$, $R_3 = R_4 = H$, $R_6 = OH$, $R_7 = H$, and 6a α;
(3) $R_1 + R_2 = OCH_2O$, $R_4 = OCH_3$, $R_3 = R_5 = R_6 = R_7 = H$, and 6a α;
(4) $R_1 + R_2 = OCH_2O$, $R_6 = OH$, $R_3 = R_4 = R_5 = R_7 = H$, and 6a α;
(5) $R_1 + R_2 = OCH_2O$, $R_3 = R_4 = R_5 = R_6 = R_7 = H$, and 6a α;
(6) $R_1 = OCH_3$, $R_2 = OH$, $R_3 = R_4 = R_5 = R_6 = R_7 = H$, and 6a β.



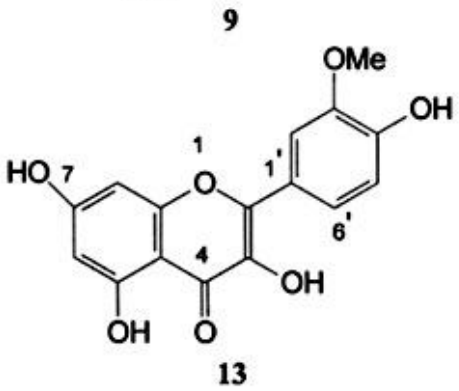
(7) R = H
(7a) R = $COCH_3$







(10) $R_1 + R_2 = OCH_2O$, $R_3 = OCH_3$, $R_4 = H$
(11) $R_1 + R_2 = OCH_2O$, $R_3 = R_4 = H$
(12) $R_1 + R_2 = OCH_2O$, $R_3 = H$, $R_4 = OCH_3$



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Anti-trypanosoma activity

Alkaloidal and non-alkaloidal extracts and isolated compounds from *Duguetia* genus, as well as another species of Annonaceae family were previously tested for *in vitro* antiplasmodial and antiprotozoal activities. In general, the alkaloid extracts show more activity than others[6)] and those activity has been related with the presence of isoquinoline alkaloid-type. While the antiplasmodial activity of some alkaloids isolated from *D. vallicola* belong to oxoaporphinoids, (-)-7-hydroxy-aporphinoids and aza-antraquinone (cleistopholine), only the isoquinoline alkaloid, (-)-oliveroline was really effective, followed by cleistopholine[25]. In the present work, as reported on Table 1, the lysis percentages of *T. cruzi* trypomastigote forms, represents the trypanocidal activity of the compounds isolated **1**, **6**, **7**, **8**, **9**, **10/11** mixture and **13**. Only aporphinoid skeleton with (*R*)-configuration, (-)-asimilobine, (**6**) was also effective, killing approximately 72% of the parasites at maximal concetration of 128.0 mM (IC_{50} 57.2 mM). The present result could, at least, provide supports for the structure activity relationship between this kind of aporphinoid alkaloids and the activity in subject.

Table 1. Percentual of lysis activity against T. cruzy trypomastigote form (strain Y)

Compound	Concentration in µM and percentual of parasitary lise (± SD)			IC_{50} (µM)
	8.0	32.0	128.0	
1	4.9 ± 0	16.1 ± 0	40.6 ± 4.9	193.5
6	0.7 ± 0.3	35.7 ± 3.9	72.0 ± 7.9	57.2
7	12.6 ± 4.9	19.6 ± 4.9	35.7 ± 1.9	418.4
9	7.0 ± 3.0	9.1 ± 5.9	34.3 ± 5.9	252.8
8	19.6 ± 3.0	24.5 ± 4.0	35.7 ± 3.9	945.2
10/11 mixture	2.1 ± 0	18.9 ± 3.9	19.6 ± 0	-
13	4.2 ± 1.9	4.9 ± 0	12.6 ± 3.0	4.635,0

Positive control gentian Violet to 250 µg/mL (IC_{50} = 31µM);
Negative control Mice infected blood + DMSO;

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