SUPPLEMENTARY MATERIAL

Antileishmanial Anthraquinones from the Rhyzomes of *Rumex abyssinicus* Jacq (Polygonaceae)

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ABSTRACT

Phytochemical investigation of the rhyzomes of *Rumex abyssinicus* (Polygonaceae) afforded six anthraquinones viz chrysophanol (**1**), physcion (**2**), emodin (**3**), mixture of physcion-8-*O*-*β*,D-glucopyranoside (**4**) and chrypsophanol-8-*O*-*β*,D-glucopyranoside (**5**), and emodin-8-*O*-*β*,D-glucopyranoside (**6**). All the compounds were characterised and identified by comparison of their MS and NMR data with available literature data. The isolated compounds were evaluated for their antileishmanial activity. Emodin (**3**) was the most active compounds with IC_{50} 13.82 and 0.26 µg/mL against *Leishmania donovani* amastigotes and promastigotes, respectively. Emodin-8-*O*-*β*,D-glucopyranoside (**6**) also showed a moderate activity with IC_{50} 27.53 and 37.08 µg/mL. This is the first report of antileishmanial compounds from *R. abyssinicus* and the antileishmanial activities of compounds **2**, **4**, **5** and **6** are here reported for the first time.

Experimental

General experimental procedures

Melting points of the isolated compounds were determined using an Electrothermal IA9000 Series digital melting point apparatus (Bibby scientific, Great Britain). MS and NMR data were recorded on a Bruker micrOTOF mass spectrometer and Varian Mercury-300 NMR instrument, respectively. Analytical TLC was performed on precoated Si gel 60 F254 (Merck. 1.05735, Hohenbrunn, Germany) plates. After development (*n*-hexane/EtOAc at the different polarity), the dried plates were examined under short-wave (254 nm) or long-wave (366 nm) UV light and sprayed with sulfuric vanillin followed by heating at 110 °C. Silica gel 60-200 µm was used for column chromatography with step gradients of *n*-hexane-EtOAc and EtOAc-MeOH as eluents. All solvents were distilled before using.

Plant material

R. abyssinicus were collected in Bamenda, North West region, Cameroon. The plant was identified at the Cameroon National Herbarium in comparison with an authentic sample with voucher N° 27289 SRFcam. The rhizomes were cut into small pieces, dried at room temperature and pulverised. The obtained powders were then extracted.

Extraction and isolation of compounds from R. abyssinicus rhizomes

The powder of *R. abyssinicus* rhizomes (2.6 Kg) were extracted by maceration using CH_2Cl_2/MeOH (1:1) for 72 h at room temperature to give 531 g of crude extract after filtration and evaporation under reduced pressure. The obtained extract (382 g) was suspended in water and then extracted again successively with ethyl acetate (3 x 1 L) and *n*-butanol (3 x 1 L) through liquid-liquid partition to give 82 g and 210 g of ethyl acetate and *n*-butanol, respectively.

A portion of the ethyl acetate extract (70 g) was subjected to silica gel (200-400 mesh) column chromatography and eluted with a gradient mixture of hexane-acetone starting from 100%-0% hexane-acetone to 0% -100% hexane-acetone. Seventy two (72) fractions of 600 mL each were collected and combined into four sub-fractions (A-D) based on TLC profiles. From subfraction A (4 g), an orange precipitate (15 mg) identified as chrypsophanol **(1)** was obtained at *n*-hexane-acetone (95:5). Subfraction B (30 g) was re-subjected to silica gel (100−200 mesh) column chromatography eluted with a gradient mixture of *n*-hexane-EtOAc to yield more chrysophanol (**1**, 15 mg) at *n*-hexane-acetone (95:5) in addition to physcion (**2**,

18 mg), and emodin (**3**, 150 mg) at *n*-hexane-EtOAc (90:10) and at *n*-hexane-EtOAc (90:10), respectively. Sub-fraction D (10 g) was also re-subjected to silica gel (100−200 mesh) column chromatography eluted with a gradient mixture of CH_2Cl_2-MeOH. 38 fractions of 100 mL were collected and grouped into four (4) subfractions (D1-D4) based on their TLC profiles. From D3 (400 mg), a mixture of physcion-8-*O*-*β*,D-glucopyranoside and chrypsophanol-8-*O*-*β*,D-glucopyranoside (**4-5**, 70 mg) which precipitate in CH_2Cl_2-MeOH (90:10) was obtained. Emodin-8-*O*-*β*,D-glucopyranoside (**6**, 5 mg) was obtained from D4 (20 mg) after preparative TLC eluted with CH_2Cl_2-MeOH (92 :8). All the compounds were characterised and identified either by TLC comparison with authentic sample available in the laboratory (chrysophanol and physcion) or by comparison of their MS data with available literature data. ^{1}H NMR and MS spectra of compounds **3-6** are shown below (Figures S1-S9)

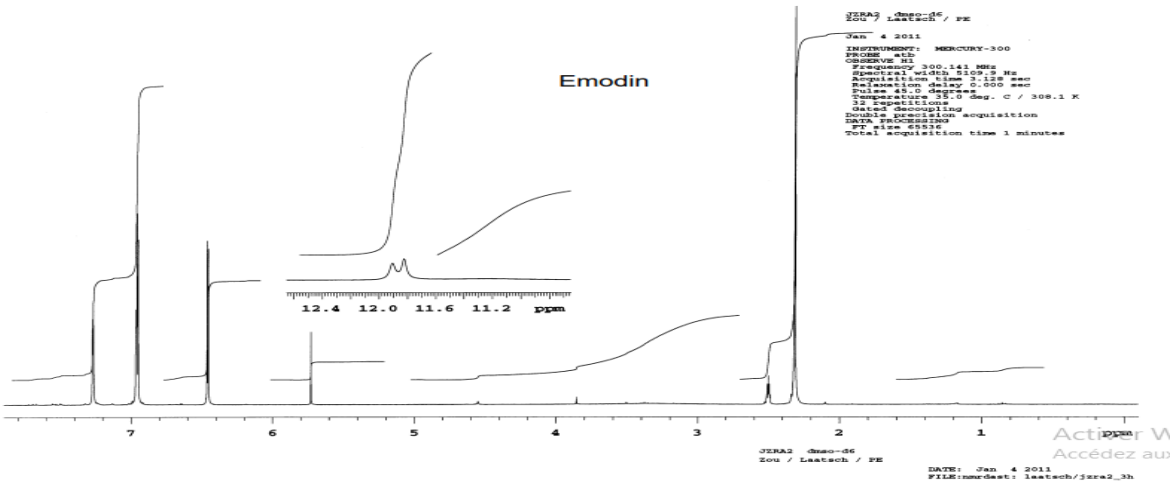

Figure S1 ^{1}H NMR (DMSO-d6, 300 MHz) of compound **3**

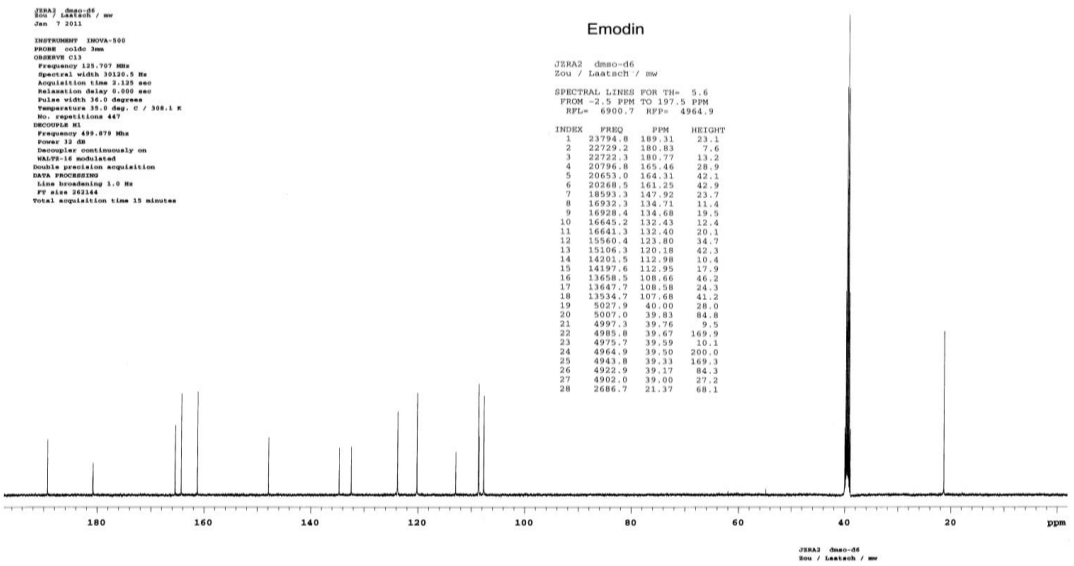

Figure S2 ^{13}C NMR (DMSO-d6, 125 MHz) of compound **3**

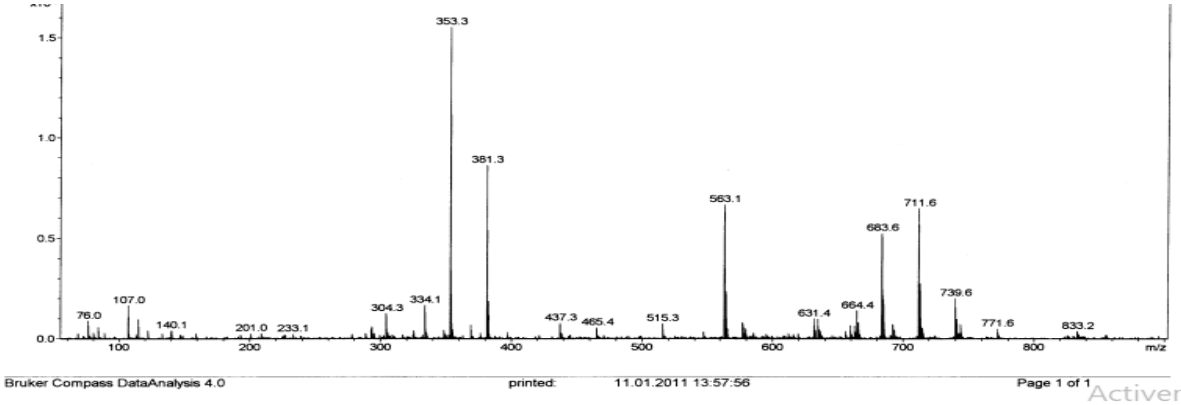

Figure S3 ESI-MS spectrum of compound **3**

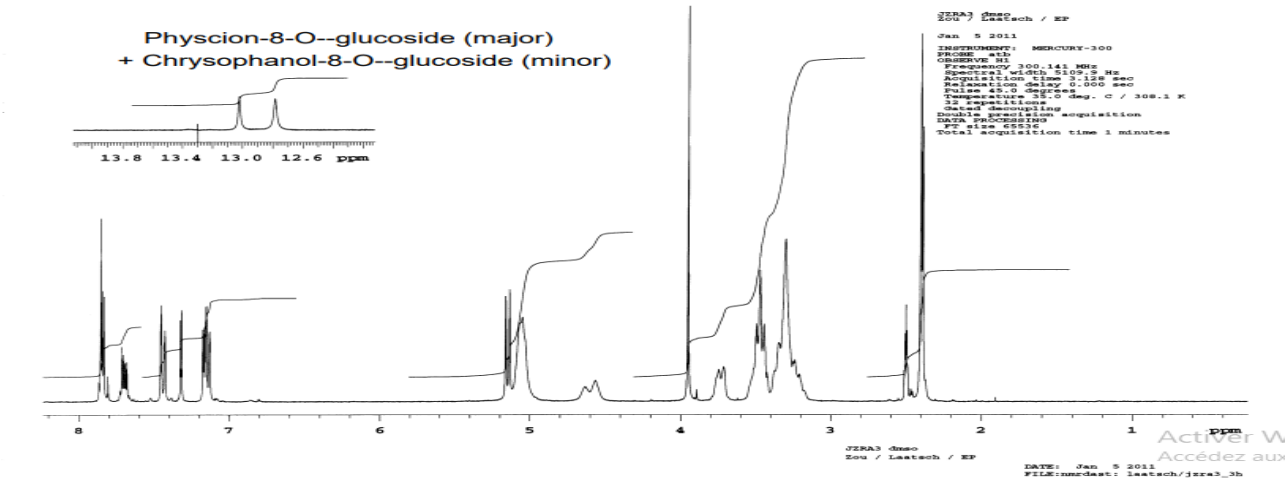

Figure S4 ^{1}H NMR (DMSO-d6, 300 MHz) of mixture of **4-5**

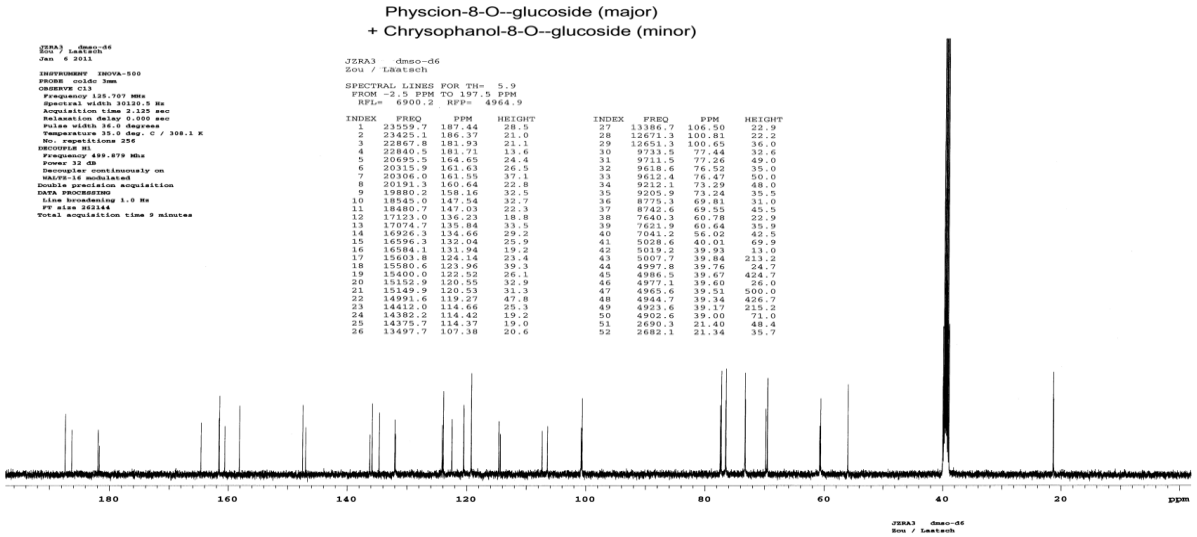

Figure S5 ^{13}C NMR (DMSO-d6, 125 MHz) of mixture of **4-5**

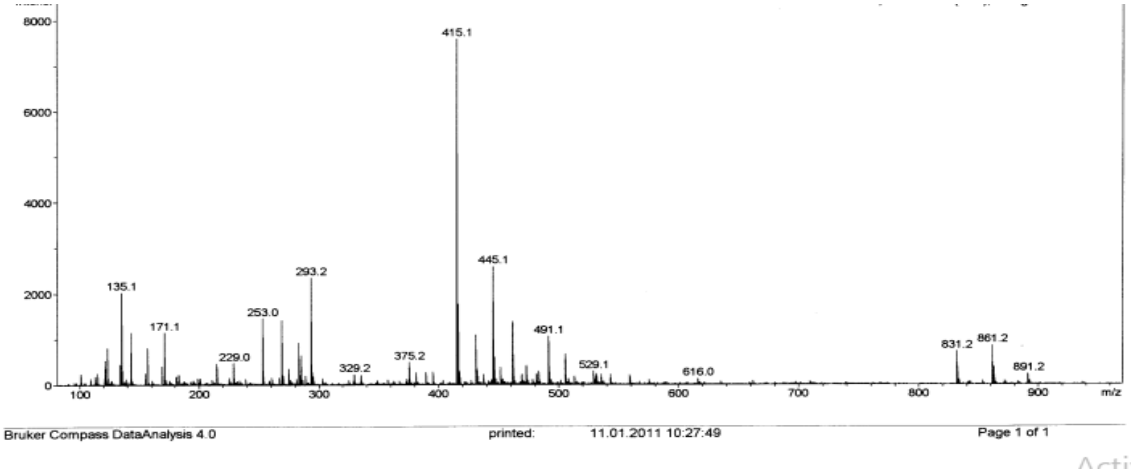

Figure S6 ESI-MS spectrum of mixture of **4-5**

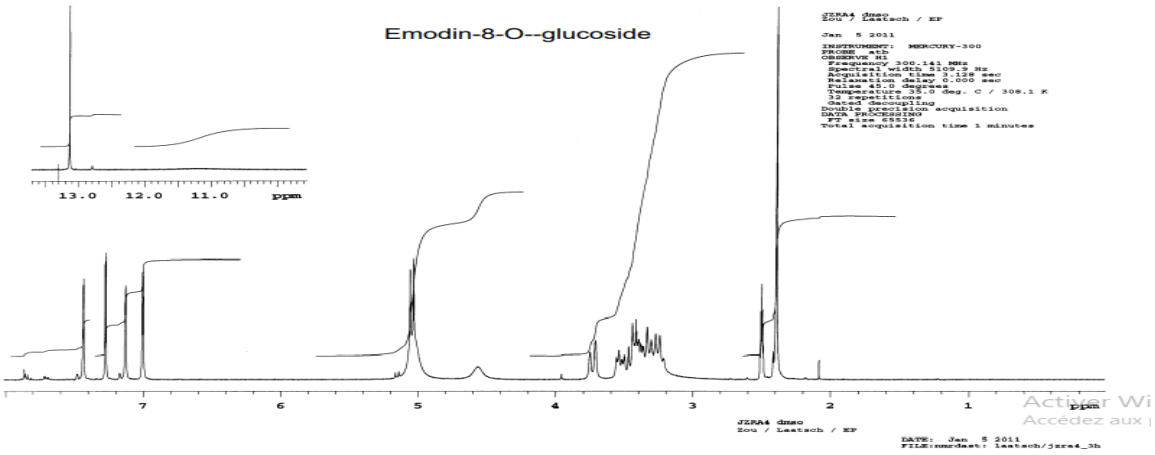

Figure S7 ^{1}H NMR (DMSO-d6, 300 MHz) of compound **6**

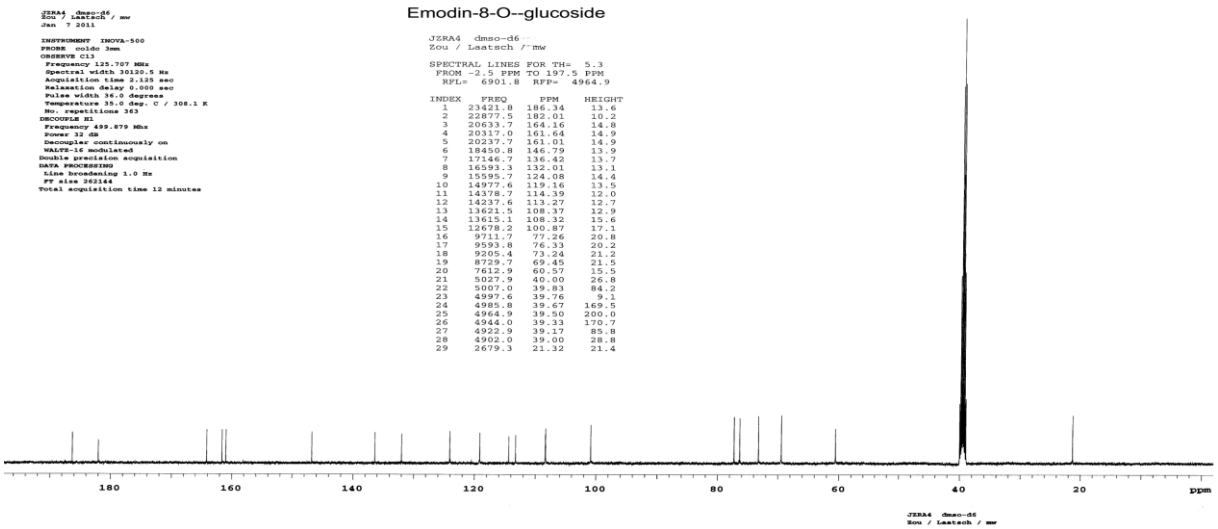

Figure S8 ^{13}C NMR (DMSO-d6, 125 MHz) of compound **6**

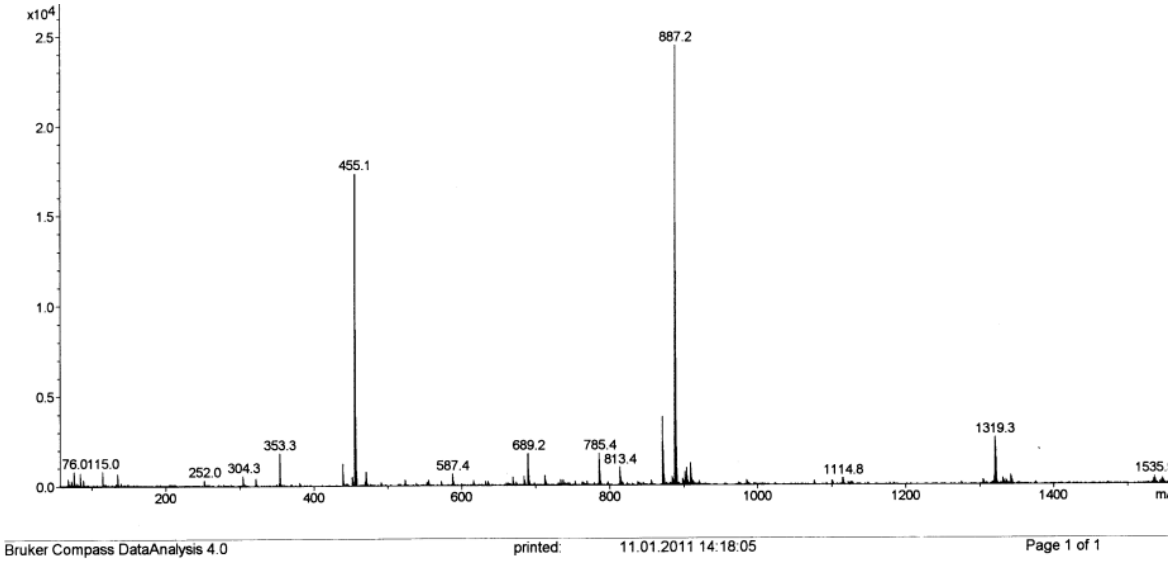

Figure S9 ESI-MS spectrum of compound **6**

Screening of compounds for biological activity

Parasite culture and maintenance

The cryopreserved promastigote form of *L. donovani* (1S (MHOM/SD/62/1S) was obtained from Bei Resources (https://www.beiresources.org/) and is routinely cultured at the Antimicrobial and Biocontrol Agents Unit, University of Yaoundé I, in Medium 199 (Sigma, Darmstadt, Germany) supplemented with 10% Heat-Inactivated fetal Bovine Serum (HIFBS) (Sigma, Darmstadt, Germany) and 100 IU/mL penicillin and 100 µg/mL streptomycin. The culture was maintained in 75 Cm^2 cell culture flask at 28 °C and checked for growth daily and sub-cultured everyday 72 h (Khanjani et al. 2015).

Determination of the antileishmanial activity of isolated compounds

Inhibitory assay against Leishmaniosis donovani promastigotes

The determination of the antileishmanial activity of compounds against cultured *L. donovani* promastigotes was performed using the resazurin colorimetric assay as previously described by Kowa et al. (2020). For each sample, growth percentages were calculated and dose–response curves were constructed to determine the 50% inhibitory concentration (IC_{50}) using the GraphPad Prism-version 5.0 software (San Diego, California, USA).

Inhibitory assay against L. donovani amastigotes

The effect of plant isolates against the intracellular amastigote form of *L. donovani* was evaluated essentially as described by Jain et al. (2012) with some modifications (Njanpa et al. 2021). Inhibition percentages were calculated using Microsoft Excel Software and median inhibitory concentration (IC_{50}) obtained from dose-response curves using GraphPad Prism 5.0. Software.

Cytotoxicity assay

The cytotoxicity profile of compounds was investigated against Raw 264.7 cells lines using the resazurin assay [18] according to the procedure described by Kowa et al. (2020). The percent growth inhibition was calculated from the absorbances relative to the negative control, and the concentration of extract that inhibited 50% cell (CC_{50} values) were determined. The selectivity index (SI) ratio (CC_{50} for macrophages/IC_{50} for amastigotes) was used to compare the toxicity of the compounds against the macrophages and their activity against the parasites.

Data Analysis

All the activity data represent mean ± standard deviation (SD) from three independent experiments. The IC_{50} and CC_{50} values were determined using GraphPad Prism 5.0 Software with data fitted by non-linear regression.

The results of the assays are showed in Table S1.

Table S1: Antipromastigote, anti-amastigote and cytotoxicity activities of chemical constituents from *R. abyssinicus*

Compounds	**IC_{50} on intracellular form amastigotes**	**IC_{50} on *L.donovani* promastigotes**	**CC_{50} on RAW cell lines macrophages**	**SI = CC_{50}/CI_{50} (Promastigotes)**	**SI = CC_{50}/CI_{50} (Amastigotes)**	**$SP_{PRO/AMA}$**
1	> 50	> 50	ND	ND	ND	> 50
2	> 50	0.57± 0.24	34.94±1.54	61.29	ND	> 0.0114
3	13.82 ± 0.05	0.26 ± 0.06	32.41 ± 4.58	122.53	2.34	0.0188
4+5	25.03 ± 0.05	28.09 ± 1.44	42.85 ± 3.59	1.52	1.55	1.122
6	27.53 ± 0.03	37.08 ± 0.01	ND	ND	ND	1.34
Curcumin	ND	ND	16.76 ± 0.01	ND	ND	ND
Amphotericin B	0.39 ± 0.09	0.2434 ± 0.1075	ND	ND	ND	0.624

IC_{50}: 50% Inhibitory Concentration in µg/mL; CC_{50}: 50% Cytotoxic Concentration in µg/mL; Results are expressed as mean ± standard deviation; SI (Selective Index) of bioactive compounds was determined as a measure of their toxicity against RAW cells lines macrophages. SI = CC_{50} against macrophages/IC_{50} against promastigotes, SP: specificity index (ratio between promastigotes IC_{50} and intracellular amastigotes IC_{50}), ND: Not Determined. Amphotericin B was used as reference drug.

References

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