

## 1 SUPPLEMENTARY MATERIAL

### 3 **Anti-*Leishmania* and cytotoxic activities of perillaldehyde epoxide** 4 **synthetic positional isomers**

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17 **Abstract** Leishmaniasis belongs to a complex of zoonotic disease caused by protozoa  
18 of the genus *Leishmania* and is considered a major public health problem. Several  
19 essential oil chemical components have inhibitory effect against protozoa, including  
20 *Leishmania donovani*. Thus, the aim of this study was to evaluate for the first time the  
21 anti-*Leishmania* activity of two *p*-menthane monoterpene isomers (EPER-1:  
22 perillaldehyde 1,2-epoxide and EPER-2: perillaldehyde 8,9-epoxide) against *L.*  
23 *donovani* promastigotes as well as evaluating cytotoxic effect on mononuclear  
24 peripheral blood cells. Results of anti-*Leishmania* assay revealed that EPER-2 (IC<sub>50</sub> =  
25 3.8 µg.ml<sup>-1</sup>) was 16-fold more potent than its isomer EPER-1 (IC<sub>50</sub> = 64.6 µg.ml<sup>-1</sup>). In  
26 contrast to PBMC cells, EPER-2 was not cytotoxic (IC<sub>50</sub> > 400 µg.ml<sup>-1</sup>) when compared  
27 to positive control. These data suggest that the disposition of epoxide group into the *p*-  
28 menthane skeleton affects the anti-*Leishmania* activity, being that the presence of the  
29 exocyclic epoxide group considerably increased potency. Thus, it was possible to  
30 observe that the location of the epoxide group into the *p*-menthane skeleton resulted in  
31 different potencies.

32 **Keywords:** Monoterpene; natural products; *Leishmania*; antiparasitic activity.

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## **Experimental**

### ***Drugs***

The compounds **EPER-1** (Tantanak et al., 1998) and **EPER-2** (Kido et al., 1992) were prepared in our laboratory according to the literature. Their chemical structures were confirmed by spectral data analysis of infrared, <sup>1</sup>H- and <sup>13</sup>C- NMR and comparison of their spectra with those published in the literature. The chemical structure of the evaluated compounds is shown in **Figure 1**.

### ***Parasite culture***

*Leishmania donovani* (MHOM/ET/1967/HU3) promastigote forms were cultivated at 26 °C (pH 7.0) in Schneider's medium supplemented with 20% fetal bovine serum (FBS), 2% male human urine and 1% penicillin (200 U.ml<sup>-1</sup>) (Sigma Chemical -t. Louis, MO, USA) in a BOD incubator until arrive at logarithmic phase of cell growth (Rodrigues *et al.*, 2015).

### ***Promastigote assay***

Anti-*Leishmania* activity on promastigote forms of *L. donovani* was evaluated using the MTT method [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Amresco, Ohio, USA] (Soares et al., 2007). After parasite cultivation as described above, promastigotes forms (1 x 10<sup>6</sup> cells) were maintained in contact with serially diluted of test substances (400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg.ml<sup>-1</sup>) in Schneider's medium for 72 h. At the end of incubation time, was added 10 µl of the MTT reagent (diluted in PBS to a final concentration of 5 mg.ml<sup>-1</sup>) and the plates were incubated for 4 h. Then, was added 50 µl of a solution of Dodecyl Sulphate Sodium (10%) (Sigma Chemical-t. Louis, MO, USA) for dissolution of the formazan crystals. Finally, the absorbance reading was performed in a spectrophotometer at 540 nm (Biotek model Elx800). Amphotericin B and Meglumine Antimoniate were used as positive control. DMSO 1% was used as negative control (Pirttimaa et al., 2015). The assays were conducted in triplicate.

### ***Method Red Blood Cells Assay***

The hemolytic activity of the compound **EPER-2** was performed on red blood cells of human peripheral blood samples (about 5 ml) from adult and healthy volunteers

1 O<sup>+</sup> (n=3) using sterile material (BD Plastipak<sup>®</sup> needles and syringes BD Biosciences,  
2 San Jose, CA, USA) and vacuum tubes (Greiner bione, Vacuette, Americana, SP,  
3 Brazil) containing the anticoagulant EDTA (Jain et al., 2015). After, the blood was  
4 diluted to a final volume of 80 µl and was added 70 µl of PBS (Sigma Chemical -t.  
5 Louis, MO, USA) until 5% of red blood cells concentration. Subsequently, pre-  
6 established concentrations of **EPER-2** (400, 200, 100, 50, 25, 12.5, 6.25 and 3.125  
7 µg.ml<sup>-1</sup>) and Amphotericin B were added at the same concentrations used in MTT test,  
8 diluted for a final volume of 20 ml with PBS and incubated for 1 h at 37 °C. Reaction  
9 was stopped by the addition of 200 µl of PBS. Then, the suspensions were centrifuged  
10 at 2,000 rpm for 10 min at 25 °C. In sequence, 100 µl of the supernatant was collected  
11 and haemolysis was measured spectrophotometrically (Biotek model Elx800) at 550  
12 nm. The absence of lysis (negative control) and total lysis of red blood cells (positive  
13 control) were determined, replacing the sample solution tested with equal volume of  
14 PBS and Milli-Q water (hypotonic shock lysis), respectively.

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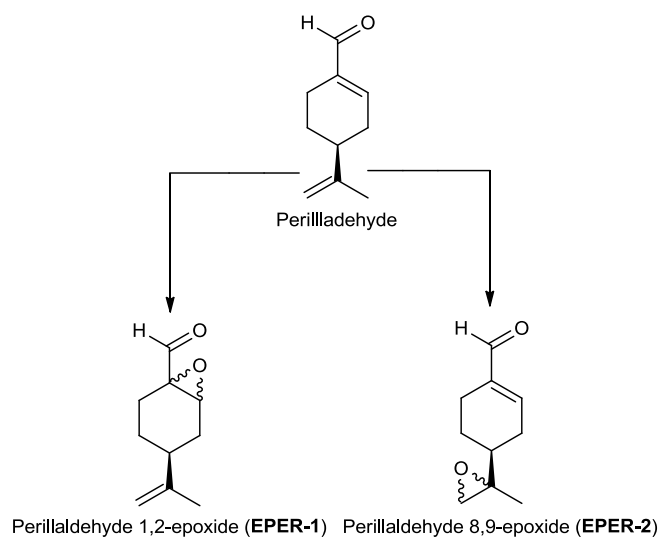
#### 16 *Cytotoxicity on Mononuclear Peripheral Blood Cells (PBMC)*

17 Human peripheral blood samples (15 ml) were collected from 3 healthy adult  
18 volunteers using sterile material (BD Plastipak<sup>®</sup> - BD Biosciences, San Jose, CA, USA)  
19 and vacuum tubes (Greiner bione, Vacuette, Americana, SP, Brazil) containing the  
20 anticoagulant heparin. In sequence, the blood was transferred to sterile falcon tube (50  
21 ml) and diluted at the same volume of 1 x PBS. In sequence, 15 ml of ficoll was added  
22 and the falcon tube was centrifuged (1,300 rpm) for 40 min at 25 °C. At the end of each  
23 centrifugation, the buffy coat containing leucocytes and monocytes cells was collected  
24 and transferred to a falcon tube (50 ml) and the volume was completed to 45 ml with  
25 PBS followed by centrifugation (1,300 rpm) for 10 min at 4 °C. The supernatant was  
26 discarded and cells were resuspended in 1 ml of RPMI. Viable macrophages were  
27 quantified in Neubauer haemocytometer chamber in presence of 0.4% Trypan blue vital  
28 stain (190 µl cell + 10 µl trypan blue). After quantification, an approximate  
29 concentration of 2.5 x 10<sup>5</sup> per well of viable cells were transferred to 96-well bottom  
30 plate and were added concentrations equivalent of IC<sub>50</sub> and 4 x IC<sub>50</sub> values of **EPER-2**.  
31 Plates were incubated for 24 h at 37 °C in a 5% CO<sub>2</sub> oven. The cell viability was  
32 evaluated by the colorimetric MTT method at the **EPER-2** concentration capable to  
33 reduce 50% number of cell. Optical density was read at 540 nm (Biotek model Elx800).

1 RPMI 1640 medium (Cultilab - São Paulo, SP, Brazil) supplemented with 0.5% DMSO  
2 (Sigma Chemical-t. Louis, MO, USA) was used as negative control and considered as  
3 100% viability of monocytes and lymphocytes (Parisi et al., 2014).

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5 ***Statistical Analysis***

6 All assays were performed in triplicate using three independent experiments.  
7 GraphPad Prism 6.0 software (San Diego, CA, USA) was used to calculate the  
8 inhibitory concentration 50% (IC<sub>50</sub>) using dose-response curves, and 95% confidence  
9 interval was included. Values of IC<sub>50</sub>, HC<sub>50</sub> and CC<sub>50</sub> (drug concentration to inhibit 50%  
10 of parasites, erythrocytes and PBMC cell growth, respectively) were calculated using  
11 Probit Regression, assuming a 95% confidence level ( $p < 0.05$ ). Variance analysis  
12 (ANOVA) was performed followed by Tukey test, taking  $p < 0.05$  as the minimum  
13 level required for statistical significance.



**Figure S1.** Chemical structures of **EPER-1** and **EPER-2**.

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1 **Table S1.** Anti-*Leishmania* activity (IC<sub>50</sub>) of compounds **EPER-1**, **EPER-2**, Amphotericin B  
2 and Meglumine Antimoniate against *L. donovani* promastigotes. The assays were performed in  
3 triplicate.

<b>Compounds</b>	<b>IC<sub>50</sub> (µg.ml<sup>-1</sup>; CI 95%) <i>L. donovani</i> promastigotes</b>
EPER-1	64.6 (62.9-66.24)
EPER-2	3.87 (3.02-3.9)
Amphotericin B	0.722 (0.692-0.752)
Meglumine Antimoniate	9916 (9087-10745)

4 Data are presented as IC<sub>50</sub> values (µg.ml<sup>-1</sup>) and their 95% confidence interval obtained by non-  
5 linear regression from three independent experiments performed in triplicate, measured by the  
6 MTT assay after 72 h of incubation. Amphotericin B and Meglumine Antimoniate were used as  
7 the positive controls.

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1 **Table S2.** Evaluation of cytotoxicity ( $HC_{50}$ ) of compound **EPER-2**, Amphotericin C and  
2 Meglumine Antimoniate on erythrocytes from human peripheral blood samples ( $HC_{50}$ ).

Compounds	Red blood cells $HC_{50}$ ( $\mu\text{g}\cdot\text{ml}^{-1}$ )
EPER-2	> 400
Amphotericin B	10.73
Meglumine Antimoniate	429.5

3 Data are presented as  $HC_{50}$  values ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) and their 95% confidence interval obtained by non-  
4 linear regression from three independent experiments performed in triplicate, measured by the  
5 red blood cells assay after 72 h of incubation. Amphotericin B and Meglumine Antimoniate  
6 were used as the positive control.

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1 **Table S3.** Cytotoxicity (IC<sub>50</sub>) of compound **EPER-2** and DMSO on mononuclear peripheral  
2 blood cells.

<b>Compounds (µg.ml<sup>-1</sup>)</b>	<b>PBMC IC<sub>50</sub> (µg.ml<sup>-1</sup>)</b>
<b>EPER-2 (3.87)</b>	> 400
<b>EPER-2 (15.48)</b>	10.73
<b>Amfotericin B (0.722)</b>	0.166 ± 0.06
<b>Antimoniato de meglumina (9926)</b>	1035 ± 71.6

3 Data are presented as IC<sub>50</sub> values (µg/ml<sup>-1</sup>) and their 95% confidence interval obtained by non-  
4 linear regression from three independent experiments performed in triplicate, measured by the  
5 human peripheral blood samples after 72 h of incubation. DMSO was used as diluent of drugs.

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1 **Table S4.** HC<sub>50</sub> values of the **EPER-2** and Amphotericin B for red blood cells, CC<sub>50</sub> for PBMC,  
 2 IC<sub>50</sub> for *L. donovani* promastigotes and their respective selectivity index (SI).

	<b>EPER-2</b>	<b>Amphotericin B</b>
Red blood cells HC <sub>50</sub> (µg/ml <sup>-1</sup> )	> 400	10.73
PBMC CC <sub>50</sub> (µg/ml <sup>-1</sup> )	> 16	0.166
Promastigotes IC <sub>50</sub> (µg/ml <sup>-1</sup> )	3.87	0.77
SI (HC <sub>50</sub> /IC <sub>50</sub> )	103.5	13.9
SI (CC <sub>50</sub> /IC <sub>50</sub> )	> 4.13	> 4.81

3 HC<sub>50</sub> = Cytotoxic concentration for 50% of red blood cells.

4 CC<sub>50</sub> = Cytotoxic concentration for 50% of PMBC.

5 IC<sub>50</sub> = Inhibitory concentration 50%.

6 IS = CC<sub>50</sub> PMBC / IC<sub>50</sub> promastigotes

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