

# **Croton pulegiodoros Baill and Croton piauhiensis Mull. Arg. (Euphorbiaceae) Essential Oils: Chemical Composition and Anti-Leishmania Activity**

## **Óleos Essenciais de Croton pulegiodoros Baill e Croton piauhiensis Mull. Arg. (Euphorbiaceae): Composição Química e Atividade Anti-Leishmania**

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Recebido em: 16 de Agosto de 2021

Aceito em: 23 de Dezembro de 2021

Publicado online: 28 de Março de 2022

Visceral leishmaniasis is a zoonosis transmitted by sandflies insects infected with *Leishmania infantum*, the etiological agent. It is a serious disease that may evolve to a fatal outcome when patients do not receive treatment for more than two years. Plant essential oils are a promising alternative to control this parasite. Therefore, in the present work *Croton pulegiodoros* and *C. piauhiensis* leaves essential oils were chemically characterized and tested for their anti-*Leishmania infantum* activity. Forty-three constituents were identified in the oils through GC-MS and GC-FID analysis. In *C. pulegiodoros* oil, ascaridole (47.99%), *p*-cymene (10.92%) and camphor (8.42%) were the predominant compounds, while in *C. piauhiensis* the major compounds were (*E*)-caryophyllene (15.22%), caryophyllene oxide (14.87%), *d*-limonene (11.84%), epi- $\alpha$ -muurolol (7.64%) and *p*-cymene (6.09%). Regarding the bioassays, the oils presented activity against *L. infantum* promastigote (IC<sub>50</sub> = 0.05-1.70  $\mu$ g/mL) and amastigote forms (IC<sub>50</sub> = 2.33-13.79  $\mu$ g/mL). *C. pulegiodoros* oil was the most active. Nevertheless, it did not present higher efficacy than routine drugs. Therefore, the tested oils are promising for the development of new leishmanicidal agents, however, further research is needed.

**Keywords:** volatile oils; *Croton pulegiodoros*; *C. piauhiensis*; anti-*Leishmania*.

## **1. Introduction**

Visceral leishmaniasis (VL) is considered one of the most important neglected tropical diseases due to its high incidence and mortality, especially among malnourished children and non-treated patients.<sup>1</sup> In the American continent, VL estimated annual occurrence is of 3,000 cases and around 95% of these occur in Brazil, with the Northeast Region of the country being the most affected.<sup>2,3</sup>

In Brazil, VL is also known as kala-azar, a zoonosis that circulates among wild and domestic animals and human beings, being transmitted by *Lutzomyia longipalpis*, *L. cruzi* and *L. migonei* sandflies infected with *Leishmania infantum*.<sup>4,5</sup> In the environment, dogs (*Canis familiaris*) are the most important reservoirs of the parasite, being involved in the maintenance of the zoonotic cycle in different regions of Brazil.<sup>6</sup> The parasite is found in the dogs' viscera and dermis, although these animals even when infected may not present clinical signals for long periods of time, but still being a source of infection for the insect vectors.<sup>7</sup>

Pentavalent antimonials and amphotericin B are first-line drugs for the treatment of human leishmaniasis in most countries. Nonetheless, they present some limitations like high toxicity, high financial costs, long-term treatment, variable efficacy and emergence of resistant parasite lines.<sup>8</sup> For the treatment of canine VL, the only allowed drug in Brazil is based on miltefosine,<sup>9</sup> in contrast with other countries where pentavalent antimonials and allopurinol among other drugs that are prescribed. However, these drugs present several harmful side effects to the dogs.<sup>10</sup>

Regarding this scenario, the characterization of less toxic, more effective and cheaper bioactive plants-derived compounds against to human and canine VL must be supported. Towards this goal, some studies have reported the efficacy of essential oils from different plants against *L. infantum*.<sup>11,12</sup>

Plants belonging *Croton* genus are found in abundance in the Northeast region of Brazil, where they are utilized in folk medicine for several proposes like treatment against malaria,

fever, gastrointestinal disorders, cancer, diabetes, wounds, hypercholesterolemia, worm infestation, inflammation, dysentery, pain and ulcers.<sup>13</sup> Many *Croton* spp. are source of essential oils rich in mono, sesquiterpenes and phenylpropanoids.<sup>14</sup> Their diverse biological properties experimentally established are antioxidant, anti-inflammatory, antinociceptive, allelopathic, antibacterial, antifungal, larvicidal, acaricidal, anti-*Trypanosoma*<sup>15-23</sup> and also anti-*Leishmania*.<sup>3,24-26</sup>

*Croton pulegioidorus* Baill (syn: *Croton regelianus* Müll. Arg.) and *Croton piauhiensis* Mull. Arg., popularly known as “velame rasteiro” and “velame peludo”, respectively, are mainly found in the Northeast of Brazil.<sup>27</sup> *Croton pulegioidorus* is utilized for the treatment of several diseases including tumors.<sup>28</sup> This essential oil presents antitumoral, nematostatic, antibacterial, anti-tick and insecticide effects.<sup>28-35</sup> *Croton piauhiensis* is traditionally utilized for the population for the treatment of diarrhea, nausea, vomiting, stomach aches, cancer and venereal diseases.<sup>36,37</sup> It has been reported that its essential oil presents larvicidal, antibacterial, antibiofilm, antioxidant activities.<sup>37,38</sup>

Therefore, herein we chemically characterized the essential oils found in the leaves of *C. pulegioidorus* and *C. piauhiensis* and investigated their activities against promastigote and axenic amastigote forms of *Leishmania infantum*.

## 2. Material and Methods

### 2.1. Plant material

*Croton pulegioidorus* Baill and *C. piauhiensis* Mull. Arg leaves were collected in 2016, March, at Boa Vista, district of Groaíras city (geographic coordinates: 3°54'48.0"S 40°22'59.9"W) at Ceará state, Northeast of Brazil. The plants' identification was performed by the botany Professor Daniela Santos Carneiro Torres (Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, Bahia, Brasil) and the voucher specimens were deposited at Professor Francisco José de Abreu Matos Herbarium (Curso de Biologia, Universidade Estadual Vale do Acaraú, Sobral, Ceará, Brasil) under the identification numbers of 21374 and 20433 for *C. pulegioidorus* e *C. piauhiensis*, respectively.

### 2.2. Essential oils obtainment

Portions of *C. pulegioidorus* (800 g) and of *C. piauhiensis* (1 kg) fresh leaves were separately submitted to hydrodistillation at Clevenger apparatus for 2 h, with the extraction of yellow oils, being dried with anhydrous sodium ( $\text{Na}_2\text{SO}_4$ ). The products were stored in sealed amber glass recipients at 4 °C for posterior analysis. The oils yield was determined by the relation of the obtained oil and the mass of fresh vegetal material utilized at the extraction (w/w).

### 2.3. Essential oils chemical analyses

Essential oils composition was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS - Shimadzu QP-2010 Plus) equipped with fused-silica capillary column Factor Four/VF-5ms, 30 m long and with an internal diameter of 0.25 mm and a uniform film thickness of 0.25  $\mu\text{m}$ . Helium was the carrying gas with a constant flow rate of 1 mL/min. The initial temperature was 60 °C, being kept for 2 min and then increased at a rate of 3 °C/min until 260 °C, followed by 10 °C/min at 290 °C and a final isothermal of 5 min at 290 °C. The sample injection volume was of 1  $\mu\text{L}$  (*split* mode 1:30). The temperature in the injector and the detector was of 220 °C and 250 °C, respectively. Mass spectra were obtained at a rate of  $m/z$  10-300, acquired by electron impact technique at 70 eV

Quantitative analysis of oils chemical composition was performed by gas chromatography containing flame ionization detector (FID) at HP 5890 Series II equipment, with the same columns and operational conditions utilized for GC-MS, except for the temperatures of the injector and the detector that were of 240 and 300 °C, respectively. The percentage of constituents was calculated using the integral areas of their respective peaks, related to the total area of the constituents of the sample.

The several constituents of each essential oil were identified by the visual comparison of their mass spectra previously reported<sup>39</sup> and the spectra available at database of the equipment (NIST08), also, by the comparison of the retention indexes previously reported.<sup>39</sup> One single *n*-alkane standard solution ( $\text{C}_8$ - $\text{C}_{20}$ ) was injected at the same chromatographic conditions of the samples and utilized to obtain the retention indexes as described by Van Den Dool and Kratz.<sup>40</sup>

### 2.4. Anti-*Leishmania* assays

*Leishmania infantum* promastigotes (CLIOC 0579 strain) kindly donated by Fundação Oswaldo Cruz from Rio de Janeiro were reared in M199 medium supplemented with 10% fetal bovine serum (Cultilab<sup>®</sup>), HEPES (Sigma-Aldrich<sup>®</sup>), bovine hemin (Inlab<sup>®</sup>), sodium bicarbonate (Sigma-Aldrich<sup>®</sup>) and 40mg/mL gentamicin (Inlab<sup>®</sup>) at pH of 7.2-7.4 and 23.6 °C. Medium was renewed at every 3-4 days. Promastigote forms at logarithmic phase of growth were incubated at 34 °C and  $\text{CO}_2$  atmosphere with pH of 5.8 to 5.9 for 48 h to transform into axenic amastigotes. The transformation rate was of 90 to 95% and the amastigotes remained viable until 7 days in these conditions.

For treatment assays, a total of  $1.25 \times 10^6$  parasites in promastigote or amastigote forms per well were plated in 96 well-plate containing M199 medium (Cultilab<sup>®</sup>). These parasites were treated or not (negative control) in triplicates with different concentrations of the essential oils varying from 0.098 to 100  $\mu\text{g/mL}$  eluted in ethanol p.a. that had been obtained from serial dilution as described by Rondon *et al.*<sup>41</sup>

or with Amphotericin B<sup>®</sup> and Glucantime<sup>®</sup> routine drugs as positive controls at 23.6 °C for 48 h.

MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide) (Sigma<sup>®</sup>) was then added at 20 µL per well followed by 20 µL of 10% SDS (sodium dodecyl sulfate) and incubated at 23.6 °C for 24 h. Finally, hydrochloric acid 4 N (Novaquímica<sup>®</sup>) was added and after shaking for 15 min, the absorbance was measured in a spectrophotometer at a wavelength of 570 nm.<sup>41</sup> The IC<sub>50</sub> was then calculated.

IC<sub>50</sub> values obtained after treatment of *L. infantum* with essential oils were calculated using non-linear regression curve with confidence interval of 95%, logarithmic transformation and subsequent normalization of data percentage, where parasite negative (non-treated) controls optical density were stated as 100% of viability. Comparative analysis was performed by ANOVA *two-way* followed by Bonferroni test using Graph Pad Prism 8.0 statics software.

### 3. Results and Discussion

The oils were obtained by hydrodistillation of *Croton pulegiodorus* Baill and *Croton piauhiensis* Mull. Arg. leaves with yields of 0.27 and 0.04% (w/w), respectively.

In a recent study, after the hydrodistillation of leaves of *C. pulegiodorus*, derived from the same site where the leaves of the specimen investigated in this work were collected, 0.27% (w/w) of essential oil yield was also obtained.<sup>30</sup> In previous studies, yields of 0.8% and 1.1% (w/w) for oils extracted by hydrodistillation of *C. pulegiodorus* leaves were reported in two samples collected in Viçosa (Ceará, Brazil) and São João do Piauí (Piauí, Brazil), respectively.<sup>28,31</sup>

Do Valle *et al.* obtained 0.02% (w/w) yield for the volatile oil of *C. piauhiensis* isolated from hydrodistillation of its leaves.<sup>37</sup>

In the present work, 43 compounds were identified, and they accounted for 94.37 and 98.55% of the total composition of *C. pulegiodorus* and *C. piauhiensis* oils respectively. The retention indices for these compounds in Factor Four/VF-5ms column and percentage of composition are listed in Table 1.

*Croton pulegiodorus* oil presented oxygenated monoterpenes as major constituents (73.16%), while *C. piauhiensis* exhibited monoterpene hydrocarbons (32.93%) and sesquiterpene hydrocarbons (31.92%) followed by oxygenated sesquiterpenes (27.63%). Monoterpene hydrocarbons (32.93%) were present at low concentrations in *C. pulegiodorus* (19.74%). The occurrence of sesquiterpene hydrocarbons and oxygenated sesquiterpenes was restricted to *C. piauhiensis*. Nevertheless, the presence of phenylpropanoid was detected solely in *C. pulegiodorus* (1.47%).

The oil of *C. pulegiodorus* presented ascaridole (47.99%), *p*-cymene (10.92%) and camphor (8.42%) (Figure 1) as major constituents and in the oil of *C. piauhiensis*,

(*E*)-caryophyllene (15.22%), caryophyllene oxide (14.87%), *d*-limonene (11.84%), epi- $\alpha$ -muurolol (7.64%) and *p*-cymene (6.09%) (Figure 1) were predominant.

Torres *et al.*<sup>29</sup> obtained two essential oils from the leaves of *C. pulegiodorus* by hydrodistillation collect at two cities in the state of Ceará, Brazil. In the city of Viçosa, the major constituents were *p*-cymene, ascaridole, camphor and  $\alpha$ -phellandrene with contents in the respective proportions of 22.3, 17.0, 13.0 and 7.1%. In the city of Acarape, the *C. pulegiodorus* oil presented as predominant compounds ascaridole, *p*-cymene,  $\alpha$ -terpinene and  $\gamma$ -terpinene, with percentages of 33.9, 21.6, 9.6 and 6.8%, respectively. On the other hand, leaves of this species collected in Groaíras (Ceará, Brazil) after being hydrodistilled provided volatile oil rich in *trans*-chrysanthenyl acetate (27.05%),  $\alpha$ -terpinene (19.21), and *p*-cymene (12.27%).<sup>30</sup> Thus, although the leaves were obtained in the same county and belong to the same species, the chemical profile of the *C. pulegiodorus* oil in the present study differs of that presented in the literature.<sup>30</sup> In another report, hydrodistillation-extracted oils from the leaves of *C. pulegiodorus* collected in the state of Sergipe, Brazil,  $\beta$ -caryophyllene (20.96%), bicyclogermacrene (16.89%), germacrene-D (10.55%),  $\tau$ -cadinol (4.56%) and  $\beta$ -copaen-4- $\alpha$ -ol (4.35%) were the major components.<sup>32</sup>

Variations in the chemical composition of *C. piauhiensis* oil was also reported. Silva *et al.*<sup>38</sup> compared the chemical profile of the essential oils from leaves of plants collect at different hours of the day. The predominant compounds of the oil at 8 a.m., 12 p.m. and 5 p.m. were  $\beta$ -caryophyllene (21.58, 34.69 and 21.01%), *d*-limonene (13.47, 13.75 and 16.35%),  $\gamma$ -terpinene (10.08, 8.00 and 9.60%) and germacrene D (9.56, 10.42 and 8.71%) respectively.

Essential oils of vegetable origin may present a high qualitative and quantitative variability regarding yield and composition. Innumerable factors contribute to this variability such as genetic factors, interaction with microorganisms or other plants, development stage, light, temperature, climate, soil, nutrition, seasons, collect hour in the day, geographical origin and oil extraction method.<sup>42</sup> Therefore, these variations interfere with the biological activity of the essential oil.

Tests against *L. infantum* promastigote and axenic amastigote forms were performed as described above. IC<sub>50</sub> values (Table 2) were obtained by non-linear regression analysis.

The essential oils investigated at the present work exhibited activity against *Leishmania infantum* with better results for *C. pulegiodorus* oil. In the assays against promastigotes, the oil of *C. pulegiodorus* had a leishmanicidal effect with a IC<sub>50</sub> of 0.05 µg/mL (CI95% of 0.03 - 0.09) similar to IC<sub>50</sub> (CI95% of 0.03- 0.10) presented by Amphotericin B<sup>®</sup>, routine drug for the treatment of leishmaniasis. Regarding the amastigote forms, this oil was less effective (IC<sub>50</sub> = 2.33 µg/mL, CI95% of 0.86-6.33) than positive controls Amphotericin B<sup>®</sup> (IC<sub>50</sub> = 0.75 µg/mL, CI95% of 0.53-1.70) and Glucantime<sup>®</sup> (IC<sub>50</sub> = 0.12 µg/mL,

**Table 1.** Chemical composition, calculated retention index (RI<sub>C</sub>), percentages of identified components and their chemical classes (%) in essential oils from fresh leaves of *Croton pulegioidorus* and *Croton piauhiensis*

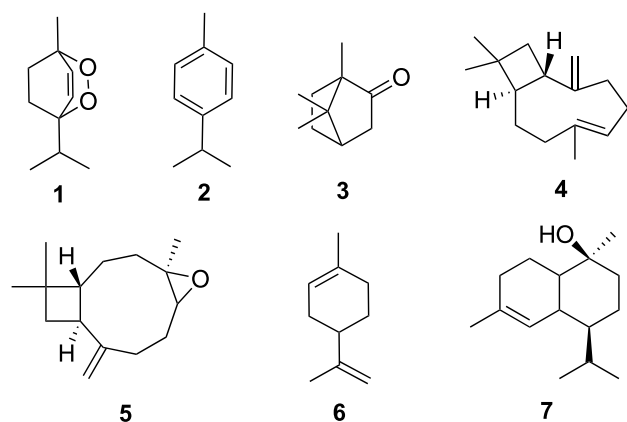
Compounds	RI <sub>C</sub>	<i>Croton pulegioidorus</i> (%)	<i>Croton piauhiensis</i> (%)
<i>Monoterpene hydrocarbons</i>	-	19.74	32.93
Tricyclene	926	0.11	-
α-Thujene	930*/932	0.36	0.83
α-Pinene	938*/940	0.66	3.94
Camphene	953	0.38	-
Sabinene	977*/979	0.38	2.48
β-Pinene	980*/982	0.27	0.39
Myrcene	992*/994	1.61	4.04
α-Phellandrene	1006*/1008	0.23	0.63
α-Terpinene	1020	2.32	-
<i>p</i> -Cymene	1028*/1030	10.92	6.09
<i>d</i> -Limonene	1032*/1035	0.77	11.84
( <i>E</i> )- <i>p</i> -Ocimene	1053	-	0.39
γ-Terpinene	1063*/1064	1.73	1.52
Terpinolene	1092	-	0.78
<i>Oxygenated monoterpenes</i>	-	73.16	6.22
1,8-Cineole	1035*/1038	4.44	3.07
Camphor	1149	8.42	-
β-Linalool	1102	-	1.82
Terpinen-4-ol	1181*/1182	3.57	1.23
<i>p</i> -Cymen-8-ol	1189	-	0.1
α-Terpineol	1193	1.68	-
Ascaridole	1245	47.99	-
( <i>E</i> )-Ascaridole glycol	1273	1.93	-
Isoascaridole	1308	5.13	-
<i>Aliphatic hydrocarbon ester</i>	-	-	0.13
(3 <i>Z</i> )-Hexenyl isobutanoate	1151	-	0.13
<i>Phenylpropanoid</i>	-	1.47	-
Methylchavicol	1199	1.47	-
<i>Monoterpene esters</i>	-	-	0.42
Bornyl acetate	1290	-	0.14
Terpinyl acetate	1355	-	0.28
<i>Sesquiterpene hydrocarbons</i>	-	-	31.22
δ-Elemene	1344	-	0.33
α-Ylangene	1382	-	0.53
β-Elemene	1397	-	0.91
( <i>E</i> )-Caryophyllene	1429	-	15.22
α-Humulene	1462	-	2.39
Alloaromadendrene	1469	-	0.64
α-Amorphene	1489	-	4.04
Bicyclogermacrene	1504	-	5.45
( <i>E</i> )-Cadina-1,4-diene	1531	-	1.20
Germacrene D	1566	-	0.51
<i>Oxygenated sesquiterpenes</i>	-	-	27.63
Palustrol	1577	-	0.40
Caryophyllene oxide	1588	-	14.87
Viridiflorol	1601	-	1.48
Ledol	1613	-	2.30
Epi-α-muurolol	1653	-	7.64
α-Cadinol	1665	-	0.94
Total		94.37	98.55

\* RI<sub>C</sub> value for component of essential oil of fresh leaves of *Croton pulegioidorus*.

**Table 2.** Values of 50% inhibitory concentration (IC<sub>50</sub>) of the essential oils of *Croton pulegiodor* and *C. piauiensis* against *Leishmania infantum* promastigote and axenic amastigote forms

Products	Promastigotes IC <sub>50</sub> (µg/mL)	Confidence Interval of 95% (CI95%)	Axenic amastigotes IC <sub>50</sub> (µg/mL)	Confidence Interval of 95% (CI95%)
<i>Essential Oils</i>				
<i>C. pulegiodor</i>	0.05 <sup>a</sup>	0.03 – 0.09	2.33 <sup>ab</sup>	0.86 – 6.33
<i>C. piauiensis</i>	1.70 <sup>a</sup>	0.77 – 3.76	13.79 <sup>b</sup>	3.54 – 53.70
<i>Reference Drugs</i>				
Amphotericin B <sup>®</sup>	0.05 <sup>a</sup>	0.03–0.10	0.75 <sup>a</sup>	0.53 – 1.70
Glucantime <sup>®</sup>	-	-	0.12 <sup>a</sup>	0.07 – 0.21

Identical lower-case letters represent no statistical differences compared to positive control (s) and between each oil in each column (P>0.05, P = 0.2044).



**Figure 1.** Chemical structures of the major compounds of *Croton pulegiodor* and *C. piauiensis* essential oils: ascaridole **1**, *p*-cymene **2**, camphor **3**, (*E*)-caryophyllene **4**, caryophyllene oxide **5**, *d*-limonene **6** and epi- $\alpha$ -muurolol **7**.

CI95% of 0.07–0.21) (Table 2). The activity of *C. piauiensis* oil was lower than positive controls, Amphotericin B<sup>®</sup> and Glucantime<sup>®</sup>, against *L. infantum* promastigotes (IC<sub>50</sub> = 1.70 µg/mL, CI95% of 0.77-3.76) as well as amastigotes (IC<sub>50</sub> = 13.79 µg/mL, CI95% of 3.54-53.70) (Table 2).

Considering the anti-*Leishmania* activity, essential oils as well as their isolates are classified as highly active (IC<sub>50</sub> < 1 µg/mL), active (1 µg/mL < IC<sub>50</sub> < 10 µg/mL), moderately active (10 µg/mL < IC<sub>50</sub> < 50 µg/mL), weakly active (50 µg/mL < IC<sub>50</sub> < 100 µg/mL) or inactive (IC<sub>50</sub> > 100 µg/mL).<sup>43</sup> Therefore, the oils of *C. pulegiodor* and *C. piauiensis* were respectively highly active and active against *L. infantum* promastigotes and active and moderately active against *L. infantum* amastigotes.

The oils' major isolated constituents also present described activity against parasite protozoa including *Leishmania* spp. Ascaridole, the most abundant component from *C. pulegiodor* oil, exhibited activity against *Plasmodium falciparum*, *Trypanosoma cruzi*, *Entamoeba histolytica* and *L. amazonensis*.<sup>44</sup> Assays with *Dysphania ambrosioides* (L.) Mosyakin & Clemants (syn: *Chenopodium ambrosioides* L.) essential oil and its major components (ascaridole, carvacrol and caryophyllene oxide), showed that ascaridole was the most toxic against *L. amazonensis*

with IC<sub>50</sub> of 0.1 and 0.3 µg/mL for promastigotes and amastigotes, respectively. The authors hypothesized that this monoterpene could be the main responsible for the anti-*Leishmania* activity of the *D. ambrosioides* essential oil.<sup>45</sup> The second most abundant constituent of *C. pulegiodor* and fifth of *C. piauiensis* oils, *p*-cymene, presented activity against *Trypanosoma brucei* and *T. cruzi*. However, it presented no effect against *L. amazonensis* and *L. chagasi* (syn: *L. infantum*) promastigote and amastigotes forms. Nevertheless, in the oils' mixture of compounds, *p*-cymene could potentialize the bioactivity of other constituents.<sup>46</sup> Tests with camphor, another major monoterpene component of *C. pulegiodor* oil, showed that it presented activity against *L. major* (IC<sub>50</sub> = 7.90 µg/mL) and *L. infantum* promastigotes (IC<sub>50</sub> = 5.55 µg/mL).<sup>43</sup>

According to Kamte *et al.*<sup>47</sup>, (*E*)-caryophyllene identified in high concentrations in the oil of *C. piauiensis*, presented important inhibitory activity against *T. brucei*. This sesquiterpene also had effect against *L. infantum* (IC<sub>50</sub> = 1.06 µg/mL) and *L. major* (IC<sub>50</sub> = 1.33 µg/mL) promastigotes.<sup>43</sup> Another component of the *C. piauiensis* oil, caryophyllene oxide sesquiterpene, had activity against *L. amazonensis* promastigotes (IC<sub>50</sub> = 4.90 µg/mL) and amastigotes (IC<sub>50</sub> = 4.04 µg/mL).<sup>45</sup> Previous investigations with *d*-limonene monoterpene, the third more abundant compound in *C. piauiensis* oil, showed activity against *T. b. brucei*, *T. cruzi* and *Plasmodium falciparum*.<sup>48-50</sup> Additionally, Arruda *et al.*<sup>51</sup> reported moderate activity of *d*-limonene against *L. amazonensis* promastigote and amastigote forms (IC<sub>50</sub> = 34.3 and 20.0 µg/mL, respectively) as well as promastigotes of *L. braziliensis* (IC<sub>50</sub> = 25.2 µg/mL), *L. chagasi* (IC<sub>50</sub> = 27.4 µg/mL) and *L. major* (IC<sub>50</sub> = 48.2 µg/mL). To our knowledge, there are no reports concerning epi- $\alpha$ -muurolol from *C. piauiensis* oil activity against parasite protozoa.

Based in these descriptions, it is quite probable that leishmanicidal activities presented by *C. pulegiodor* and *C. piauiensis* essential oils could be attributed to their major components. Nonetheless, synergism with other minor components cannot be excluded.

Once they are composed by different bioactive substances, essential oils target different structures and present diverse anti-*Leishmania* mechanisms of action. Some major

compounds of *C. pulegiodoros* and *C. piauihensis* had their mode of action partially or totally elucidated. Monzote *et al.*<sup>52</sup> observed that ascaridole leishmanicidal effect is associated with significant increase in superoxide radical production and consequent impairment of mitochondrial coupling. The authors also showed that caryophyllene oxide also acts in the mitochondria of the parasite causing impairment of the electron chain transport in complex III.

Due to its hydrophobic nature, *p*-cymene possesses high affinity to biological membranes, being incorporated into de lipid bilayer and favoring the entrance of other bioactive compounds<sup>53</sup>, which in the case of the present investigation would be the other constituents of the *C. pulegiodoros* and *C. piauihensis* essential oils. According to Díaz *et al.*<sup>25</sup>, the toxicity of (*E*)-caryophyllene on *Leishmania* spp. has been attributed to the inhibition of cell isoprenoid biosynthesis. In the case of *d*-limonene the leishmanicidal mechanism of action is attack of the parasite plasma membrane, limited to the lipid component of this structure and causing increase in its fluidity.<sup>54</sup>

In the work developed by Rondon *et al.*<sup>41</sup>, volatile oils from three plant species were tested against the promastigote and amastigote forms of *L. chagasi* (syn: *L. infantum*). As results, it was found that the most effective were *Lippia sidoides* oil (IC<sub>50</sub> = 19.76 µg/mL - CI95% = 11.00-38.98 and IC<sub>50</sub> = 5.07 µg/mL - CI95% = 0, 47-54.33, respectively) and the resin oil of *Copaifera reticulata* (IC<sub>50</sub> = 7.88 µg/mL - CI95% = 1.52-40.86 and IC<sub>50</sub> = 0.528 µg/mL - CI95% = 0.05-5.39, respectively), both performances were comparable to those of routine drugs used as positive controls pentamidine (IC<sub>50</sub> = 2.15 µg/mL - CI95% = 0.07-58.14) and amphotericin B® (IC<sub>50</sub> = 9.75 µg/mL - CI95% = 0.01-263.40). *Coriandrum sativum* oil was the least effective of the three oils, with IC<sub>50</sub> values of 181.00 µg/mL (CI95% = 57.53 - 269.60) and 1.51 µg/mL (CI95% = 0.06 - 37.64), against *L. chagasi* promastigotes and amastigotes, respectively. The comparison of these data with the findings of the present research shows that the three oils tested by Rondon *et al.*<sup>41</sup> were less active on parasite promastigotes than the two *Croton* oils, however, they were more effective against amastigotes than *C. piauihensis* oil.

Considering other species of the genus *Croton*, previous reports described leishmanicidal activity in this group. For instance, the essential oil extracted from leaves of *C. cajucara* and its major component 7-hydroxycalamenene presented effects against *L. chagasi* promastigotes with values of IC<sub>50</sub> de 250 and 15.6 µg/mL, respectively.<sup>3</sup> The essential oils present in the leaves of *C. linearis*, which is rich in guaiol, eudesma-4(15),7-dien-1β-ol and guai-3,10(14)-dien-11-ol inhibited the growth of *L. amazonensis* promastigotes and amastigotes with IC<sub>50</sub> values of 20.0 ± 4.9 and 13.8 ± 4.3 µg/mL.<sup>25</sup> Essential oils from the leaves of *C. pedicellatus* and *C. leptostachyus* are rich in borneol, γ-terpinene, germacrene D and (*E*)-β-caryophyllene and presented effect against *L. (Viannia) panamensis* in its promastigotes (IC<sub>50</sub> = 7.14 ± 2.34 and 7.39 ± 1.71 µg/mL,

respectively) and amastigote forms (IC<sub>50</sub> = 46.68 ± 8.38 and 84.59 ± 1.60 µg/mL, respectively).<sup>55</sup> The authors also investigated the activity of *C. pedicellatus* and *C. leptostachyus* oils against *L. (V.) braziliensis* in its promastigote (IC<sub>50</sub> = 19.65 ± 11.64 and 22.74 ± 0.26 µg/mL) and amastigote forms (IC<sub>50</sub> = 19.77 ± 6.86 and 36.74 ± 18.10 µg/mL). More recently, Morais *et al.*<sup>26</sup> tested the activity of *C. argyrophyllodes*, *C. jacobinensis*, *C. nepetifolius* and *C. sincorensis* leaves essential oils against promastigotes of *L. amazonensis* (IC<sub>50</sub> = 15.50 ± 2.48, 23.79 ± 2.11, 9.87 ± 2.21 and 27.03 ± 1.61 µg/mL, respectively), *L. braziliensis* (IC<sub>50</sub> = 16.71 ± 1.35, 22.06 ± 4.98, 9.08 ± 2.59 and 14.16 ± 3.49 µg/mL, respectively) and *L. chagasi* (syn: *L. infantum*) (IC<sub>50</sub> = 16.41 ± 1.98, 17.69 ± 1.19, 14.80 ± 3.34 and 13.05 ± 3.60 µg/mL, respectively). They also identified methyl eugenol, β-caryophyllene and 1,8-cineole as major constituents of *C. nepetifolius* oil. For the other *Croton* species, the most abundant compounds were spathulenol and caryophyllene oxide.

As recently discussed, the high diversity in essential oils composition leads to diverse effects on *Leishmania* spp. parasites<sup>56</sup>. Nonetheless, overall pro-oxidant actions with mitochondrial damage and reactive oxygen species production triggered by plant volatile oils are usual

#### 4. Conclusion

The present work reported for the first time the *in vitro* anti-*Leishmania* action of essential oils extracted from *Croton pulegiodoros* and *C. piauihensis* leaves. Among these, the most effective against promastigote and amastigote forms of *L. infantum* was *C. pulegiodoros* oil. The bioactivity presented by these oils may be related to their prevalent constituents, which are known for their proven leishmanicidal properties. The main components of *C. pulegiodoros* were ascaridole, *p*-cymene and camphor, while the compounds that occur in greater quantity in *C. piauihensis* were caryophyllene, caryophyllene oxide, *d*-limonene, epi-α-murolol and *p*-cymene. Therefore, based on our encouraging results, these oils are promising phytochemicals for the development of new formulations of therapeutic agents for the treatment of visceral leishmaniasis. However, it would be important to analyze anti-*L. infantum* effects of the isolated major compounds alone or in combinations with other essential oils constituents or routine utilized drugs in order to minimize side effects and emergence of resistant parasites.

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