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Eficácia *in vitro* de *Coriandrum sativum*, *Lippia sidoides* e *Copaifera reticulata* sobre *Leishmania chagasi*

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Abstract

The increased incidence of visceral leishmaniasis (VL) in Brazil is due to a lack of effective disease control measures. In addition to that, no effective treatment exists for canine VL in response to synthetic drugs. Thus, the objective of this study was to evaluate the effect of the essential oils of *Coriandrum sativum* and *Lippia sidoides*, and oleoresin from *Copaifera reticulata*, on *Leishmania chagasi* promastigotes and amastigotes. We also examined the toxicity of these treatments on the murine monocyte cell line RAW 264.7. To determine the IC₅₀ a MTT test (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed on promastigotes, and an *in situ* ELISA assay was conducted on amastigotes. Here, we demonstrate that oleoresin from *C. reticulata* was effective against both promastigotes (IC₅₀ of 7.88 µg.mL⁻¹) and amastigotes (IC₅₀ of 0.52 µg.mL⁻¹), and neither of the two treatments differed significantly ($p > 0.05$) from pentamidine (IC₅₀ of 2.149 µg.mL⁻¹) and amphotericin B (IC₅₀ of 9.754 µg.mL⁻¹). Of the three plant oils tested, only oleoresin showed no toxicity toward monocyte, with 78.45% viability after treatment. Inhibition of promastigote and amastigote growth and the lack of cytotoxicity by *C. reticulata* demonstrate that oleoresin may be a viable option for analyzing the *in vivo* therapeutic effects of leishmanicidal plants

Keywords: Leishmanicidal plants, oils, cytotoxicity, amastigotes, promastigotes, *Leishmania chagasi*.

Resumo

O aumento na incidência da Leishmaníase Visceral (LV) no Brasil deve-se à ineficácia das medidas de controle da doença. Além disso, não há tratamento efetivo para LV canina com drogas sintéticas. Assim, o objetivo deste trabalho foi avaliar o efeito dos óleos essenciais de *Coriandrum sativum* e de *Lippia sidoides* e do óleo-resina de *Copaifera reticulata* sobre promastigotas e amastigotas de *Leishmania chagasi* e analisar o grau de toxicidade sobre células monocíticas murinas RAW 264.7. Para determinar a CI₅₀ sobre promastigotas foi usado teste MTT (brometo de 3-[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazólio) e sobre amastigotas foi realizado imunoensaio *in situ* pela técnica de ELISA. Os resultados obtidos comprovaram que o óleo-resina de *C. reticulata* foi o mais eficaz contra as formas promastigotas (CI₅₀ de 7,88 µg.mL⁻¹) e amastigotas (CI₅₀ de 0,52 µg.mL⁻¹) e em nenhum dos dois testes diferiu do controle pentamidina que obteve CI₅₀ de 2,149 µg.mL⁻¹, no teste sobre promastigotas, e anfotericina B que obteve CI₅₀ de 9,754 µg.mL⁻¹, nos testes com amastigotas ($p > 0.05$). Quanto à citotoxicidade apenas o óleo-resina não apresentou toxicidade com 78,45% de monócitos viáveis. Os resultados obtidos sobre promastigotas e amastigotas e a ausência de citotoxicidade do óleo-resina de *C. reticulata* evidenciam que este óleo-resina pode ser viável para a análise de seus efeitos terapêuticos em testes *in vivo*.

Palavras-chave: Plantas leishmanicidas, óleos, citotoxicidade, amastigotas, promastigotas, *Leishmania chagasi*.

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Introduction

The World Health Organization (WHO) considers visceral leishmaniasis (VL) to be a major tropical zoonotic disease (MISHRA et al., 2009). VL, which continues to resist modern control efforts, is most common in northeastern Asia, eastern Africa and northeastern Brazil, but cases also occur in southern Europe and elsewhere. Each year, there are approximately 500,000 new cases and more than 50,000 deaths worldwide; however, because leishmaniasis is not a commonly reported disease in many countries, these values are probably underestimated (WHO, 2010). In Brazil, this disease has expanded throughout the canine and human populations in various regions (RONDON et al., 2008; BRASIL, 2009). In addition, infected dogs have been associated with all human disease outbreaks, representing the main link in the chain of transmission, and, despite the existence of other *Leishmania* reservoirs, such as horses and cats, dogs are the only confirmed domestic reservoir (GRAMICCIA; GRADONI, 2005).

Until now, canine visceral leishmaniasis (CVL) has proven to be resistant to available synthetic antiparasitic drugs, thus reinforcing the need for alternatives in disease control. CVL therapies aim to reduce the parasite load, minimize organ damage by the parasites, and redirect the immune response to improve animal health and prevent relapse (OLIVA et al., 2010).

Alternative methods of parasite control, including deltamethrin-impregnated collars or spot-on permethrin-based topical insecticides, are being employed in an attempt to block transmission (MAROLI et al., 2010). Furthermore, the use of plants for new medicines has increased, both to combat multidrug-resistant parasites and to maximize disease control (CROFT; COOMBS, 2003; SHARIEF et al., 2006).

Essential oils are natural products of great medical importance because they already possess proven anti-fungi, antimicrobial, and antileishmanial activities (ANTHONY et al., 2005; OLIVEIRA et al., 2009).

The essential oil of *L. sidoides*, popularly called alecrim-pimenta, belongs to the family Verbenaceae. It has been previously tested against *Aedes aegypti* (CARVALHO et al., 2003; CAVALCANTI et al., 2004), *Haemonchus contortus*, *Syphacia obvelata* and *Aspiculuris tetraptera* (CAMURÇA-VASCONCELOS et al., 2007) and has proven anti-inflammatory, gastroprotective, antioxidant (MONTEIRO et al., 2007) and anti-fungi activities (FONTENELLE et al., 2007). *C. reticulata* oleoresin, commonly called, óleo de Copaíba in Portuguese, belongs to the family Fabaceae. It has been evaluated against *Culex quinquefasciatus* as well as *A. aegypti* (SILVA et al., 2003; GERIS et al., 2008) and has proven activity anti-*Trypanosoma* and anti-*Leishmania* (MACIEL et al., 2002; VEIGA JUNIOR; PINTO, 2002). *C. sativum*, called coentro in Portuguese, belongs to the family Umbelliferae and has antimutagenic (CORTÉS-ESLAVA et al., 2004), antidiabetic (GALLAGHER et al., 2003), diuretic (AISSAOUI et al., 2008), gastroprotective (AL-MOFLEH et al., 2006), anti-fungi (ATANDA et al., 2007), as well as anxiolytic effects (EMAMGHOREISHI et al., 2005). This herbal is also effective against *H. contortus* (EGUALE et al., 2007).

The objective of this study was to evaluate the effect of the essentials oils of *Coriandrum sativum* and *Lippia sidoides* and

oleoresin from *Copaifera reticulata* on *L. chagasi* promastigotes and amastigotes, as well as their cytotoxicities in the murine monocyte cell line RAW 264.7.

Materials and Methods

1. Extraction of essential oils and oleoresins

The essential oil of *L. sidoides* was purchased from PRONAT (Produtos Naturais LTDA). *C. reticulata* oleoresin and *C. sativum* seeds were purchased at a street fair in the state of Pará and the state of Ceará, Brazil, respectively. *C. sativum* seeds were dried for one week, and the essential oil was extracted by steam distillation.

2. Phytochemical tests

Qualitative phytochemical tests of phenols, tannins, catechins, leucoanthocyanidins, flavonoids, steroids, terpenes, alkaloids, and saponins were performed according to Matos (2009) and Siddiqui et al. (2009). These tests are based on the visual observation of colorimetric changes or on the formation of a precipitate after the addition of specific reagents.

3. Chemical analysis

The oils were chemically analyzed at the Laboratório de Análise de Alimentos, EMBRAPA/Agroindústria using gas chromatography/mass spectrometry (GC/MS) on a 5971 GC/MS (Hewlett-Packard®) at a temperature of 270 °C. Constituent molecules were identified by their retention indices and mass spectra compared to a database of known chemical signatures (ALENCAR et al., 1984; ADAMS, 1989).

4. Cultivation of *Leishmania chagasi*

Promastigotes of *L. chagasi* strain MHOM46/LC/HZ1 (Laboratório de Protozoologia/USP) were grown in M199 (Cultilab®) supplemented with 10% fetal calf serum (FCS) (Cultilab®), HEPES (Sigma-Aldrich®), bovine hemin (Inlab®), sodium bicarbonate (Sigma-Aldrich®), gentamicin (40 mg.mL⁻¹) (Inlab®), and 5% human male sterile urine. Cultures were maintained in a BOD incubator at 23.6 °C, and were passaged every three to four days.

Amastigotes (Laboratório de Protozoologia/USP) were cultured with the murine monocyte cell line RAW 264.7 (Sigma-Aldrich®) in 96 well microplates. Cells were counted in a Neubauer chamber and plated at a density of 1 × 10⁴ cells/well. Promastigotes were added at a ratio 10:1 parasites to cell. Cells were cultured in Dulbecco's medium (Cultilab®) with 5% FCS, sodium bicarbonate, and 40 mg.mL⁻¹ gentamicin. Bottles were kept ajar and cultivated under glass with 5% CO₂ at 36.6 °C. After 24 hours, the amastigotes inside the monocyte cells were observed under inverted microscope.

5. Assays on *L. chagasi* promastigotes

Promastigotes were counted in a Neubauer chamber and used at a concentration of 1×10^5 promastigotes/well. On the first step, the oils of each plant were dissolved in ethanol, this solution was called the stock solution. On the second step, 100 μL of stock solution was diluted in milli-Q water add M199 medium for promastigotes or Dulbecco's medium for amastigotes. The oil solution was evaluated at 6.25, 12.5, 25, 50, and 100 $\mu\text{g.mL}^{-1}$, as previously described by Tempone et al. (2005). After a 24-h incubation with the relevant compound, a tetrazolium dye (MTT) colorimetric assay (bromide 3-4,5-dimethylthiazol-2-yl-2,5-dephenyltetrazolium) was performed on promastigotes to determine viability, and the IC50 of each treatment was calculated. The positive control for this assay was pentamidine, and the negative control was M199 medium. All oils were assayed in triplicate. The MTT assay was analyzed on a Multiskan MS (UNISCIENCE®) microplate reader at a wavelength of 570 nm.

6. Assays on *L. chagasi* amastigotes

Plant oils were diluted in milli-Q water to 6.25, 12.5, 25, 50 and 100 $\mu\text{g.mL}^{-1}$ and then added to microplates containing a confluent layer of cells and amastigotes. An *in situ* enzyme-linked immunosorbent assay (ELISA) to determine the IC50 was performed according to the method of Piazza et al. (1994). Prior to treatment, infected cells were stained, and parasites were observed by microscopy. Experiments were performed on cells exhibiting greater than 50% infection in a given microscope field. Briefly, after a 24 hours incubation with the indicated plant oil, a solution of 0.01% saponin (Sigma–Aldrich®) and 1% bovine serum albumin (BSA; Sigma–Aldrich®) in 1X phosphate-buffered saline (PBS) was added to each well, and plates were incubated for 30 minutes at 37 °C. Wells were subsequently blocked with 5% nonfat dry milk (Nestle®) in PBS for 30 minutes at 37 °C. Microplates were washed and dried three times and then anti-*L. chagasi* serum, diluted 1:500 in 1X PBS containing 3% milk, 0.05% Tween 20 (PBSLT) and 10% FCS, was added, and plates were incubated at 37 °C overnight. Horseradish peroxidase-conjugated anti-rabbit IgG (Sigma–Aldrich®) was diluted 1:10,000 in PBSLT and, after further washing, ortho-phenylenediamine chromogen (OPD) (Sigma–Aldrich®) was added. To stop the reaction, 4 N chloric acid (Novaquímica®) was added, and plates were read on a microplate reader using a 492 nm filter. The positive control for this assay was

40 $\mu\text{g.mL}^{-1}$ amphotericin B (Sigma–Aldrich®), and the negative control was Dulbecco medium alone. To generate anti-*L. chagasi* antibodies, rabbits were immunized with promastigotes, and serum was obtained 30 days after immunization.

7. Cytotoxicity assay

RAW 264.7 cells (Sigma–Aldrich®) were grown in 96 well microplates and treated with 100 $\mu\text{g.mL}^{-1}$ of plant oils. Subsequent testing was performed *in situ* by ELISA, as described above. The positive control in this step was 40 $\mu\text{g.mL}^{-1}$ amphotericin B (Sigma–Aldrich®), and the negative control was Dulbecco medium. Readings were performed using a 492 nm filter in a microplate reader.

8. Statistical analysis

The drug concentrations that achieved a 50% inhibition in the growth of parasites (IC50) were calculated using a nonlinear regression curve, with a 95% confidence interval. A one-way analysis of variance was the statistical method used to evaluate the response. Pair wise comparisons of means were made using Tukeys procedure. A p-value <0.05 was considered significant. For normalization, 100% survival was computed as the OD of the control containing only promastigotes or amastigotes, and/or cells alone.

Results

The results of oil activity on promastigotes showed that *C. reticulata* oleoresin and *L. sidoides* essential oil were effective compared with positive control pentamidine ($p > 0.05$). *C. sativum* essential oil was not effective against promastigotes of *L. chagasi* and was different from positive control ($p < 0.05$). The assay on amastigotes demonstrated that the three oils were effective, and they were not significantly different from amphotericin B ($p > 0.05$) (Table 1).

The oleoresin from *C. reticulata* was not toxic to RAW 264.7 cells; the cells displayed 78.45% cell viability, which did not differ significantly from positive and negative controls ($p > 0.05$). In contrast, the other oils demonstrated some toxicity; 49.9% viability was observed in cells treated with *C. sativum*, and 57.8% viability was observed in cells treated with *L. sidoides*. These results were

Table 1. IC50 values of *Coriandrum sativum* and *Lippia sidoides* essential oils, and *Copaifera reticulata* oleoresin on promastigotes and amastigotes of *Leishmania chagasi*.

Plants	Promastigotes IC50 ($\mu\text{g.mL}^{-1}$)	CI95%	p values	Amastigotes IC50 ($\mu\text{g.mL}^{-1}$)	CI95%	p values
<i>Copaifera reticulata</i>	7.88 ^a	1.52-40.86	0.1458	0.52 ^a	0.05-5.39	0.0851
<i>Coriandrum sativum</i>	181.00 ^b	67.53-269.60	0.0019	1.51 ^a	0.06-37.64	0.0851
<i>Lippia sidoides</i>	19.76 ^a	11.00-38.98	0.1458	5.07 ^a	0.47-54.33	0.0851
Positive Control						
Pentamidine	2.149 ^a	0.07-58.14	0.1458	-	-	-
Amphotericin B	-	-	-	9.754 ^a	0.01-263.40	0.0851

Means with the same letter are not significantly different ($p > 0.05$).

significantly different from the positive and negative controls and to oleoresin *C. reticulata* ($p < 0.05$) (Figure 1).

Phytochemical analysis revealed the presence of terpenoids in the extracted oils. Further characterization determined that the principal constituents of *L. sidoides* essential oil, *C. sativum* essential oil and *C. reticulata* oleoresin were thymol (59.65%), β -linalool (73.21%) and β -caryophyllene (43.18%), respectively (Table 2).

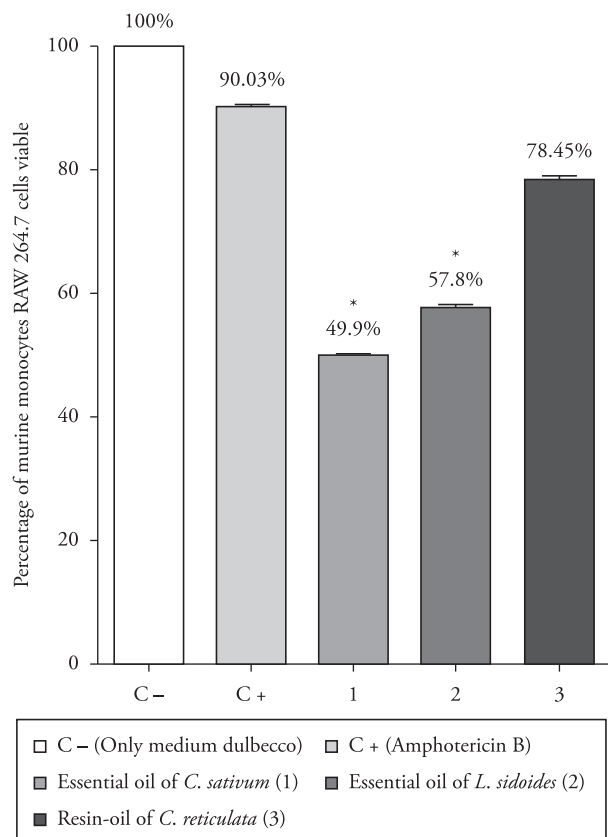


Figure 1. Effect of *C. sativum* and *L. sidoides* essentials oils, and *C. reticulata* oleoresin at a concentration of 100 $\mu\text{g.mL}^{-1}$, on the viability of the murine monocyte cell line, RAW 264.7. Amphotericin B (40 $\mu\text{g.mL}^{-1}$) was used as a reference, and Dulbecco medium served as the negative control. * $p < 0.05$. ¹The star symbol shows statistical difference between treatments 1 and 2 related to treatment 3 and the positive and negative controls ($p < 0.05$). The treatment 1 is not statistically different from treatment 2 ($p > 0.05$).

Table 2. Relative composition (%) of *Coriandrum sativum* and *Lippia sidoides* essential oils, and *Copaifera reticulata* oleoresin.

Plants	Constituents	Percentage (%)
<i>Copaifera reticulata</i>	β -Caryophyllene	43.18
	α -Bergamotene	8.76
	Copaene	8.69
<i>Coriandrum sativum</i>	β -linalool	73.21
	Camphor	4.25
	α -Pinene	4.20
<i>Lippia sidoides</i>	Thymol	59.65
	β -Caryophyllene	10.60
	Cymene	9.08

Discussion

A study among eight plant species of the genus *Copaifera* (*C. multijuga*, *C. officinalis*, *C. reticulata*, *C. lucens*, *C. langsdorffii*, *C. paupera*, *C. martii*, and *C. cearensis*), related that *C. reticulata* demonstrated the greatest efficacy against *Leishmania amazonensis*, with IC₅₀ values of 5 $\mu\text{g.mL}^{-1}$ on promastigotes and 20 $\mu\text{g.mL}^{-1}$ on amastigotes. This oil also exhibited a low level of toxicity toward the murine macrophage cell line J774G8 (SANTOS et al., 2008). The results showed that *C. reticulata* had IC₅₀ values against promastigotes of *L. chagasi* similar to IC₅₀ values on promastigotes of *L. amazonensis* found by Santos et al. (2008). However the oleoresin had proven to be even more effective against *L. chagasi* amastigotes (IC₅₀ 0.52 $\mu\text{g.mL}^{-1}$). The differing IC₅₀ values obtained with *C. reticulata* oleoresin on amastigotes of *L. amazonensis* and *L. chagasi* can be explained by differences in target. The potential targets of leishmanicidal drugs are glucose transport system, purines and other essential biomolecules and several important and specific enzyme systems of *Leishmania* (BARRETT et al., 1999; DOERIG et al., 2002; PADMANABHAN et al., 2005).

Oliveira et al. (2009) had previously investigated the activity of essential oils from *Cymbopogon citratus*, *L. sidoides* and *Ocimum gratissimum* against promastigotes of *L. chagasi*. In this study, the most effective compound was *C. citratus*, with an IC₅₀ of 45 $\mu\text{g.mL}^{-1}$, followed by the oil of *O. gratissimum*, with an IC₅₀ of 75 $\mu\text{g.mL}^{-1}$; the least effective essential oil was *L. sidoides*, with an IC₅₀ of 89 $\mu\text{g.mL}^{-1}$. Another study performed on promastigotes and amastigotes of *L. chagasi* reported that essential oils from plants of the genus *Lippia* were effective in inhibiting promastigote infection, with IC₅₀ values of 4.4 $\mu\text{g.mL}^{-1}$ for *L. origanoides*, 5.2 $\mu\text{g.mL}^{-1}$ for *L. citriodora*, 18.9 $\mu\text{g.mL}^{-1}$ for *L. alba*, and 51.8 $\mu\text{g.mL}^{-1}$ for *L. micromera*. No oil had demonstrated inhibition of intracellular amastigotes in the human monocyte line THP-1 (ESCOBAR et al., 2010). In contrast, in the present work, *L. sidoides* oil exhibited the greatest activity against both promastigotes and amastigotes of *L. chagasi*. The difference between the IC₅₀ values may be due to the composition of the oils, which is highly variable, depending on the soil where the plant was grown, the time of collection, environmental factors and other effects (MARTINS et al., 2006; NOGUEIRA et al., 2007).

The effect of *C. sativum* essential oil against *Leishmania* parasites had never been reported, actually only one study was published with three fractions of *C. sativum* against *Leishmania infantum* (RONDON et al., 2011). In this study, the results against promastigotes and amastigotes were contradictory because all fractions had effect on promastigotes but the ethyl acetate fraction did not act on amastigotes. Among the results on promastigotes, the chloroform fractions demonstrated the higher IC₅₀ value and only this fraction showed terpenoids in qualitative chemical analysis. Other studies reported the effect of *C. sativum* essential oil against microorganisms like *Candida* spp. (SILVA et al., 2011a), Gram-positive and Gram-negative bacteria (SILVA et al., 2011b).

The main constituents of *C. sativum*, *L. sidoides* and *C. reticulata* oils were the monoterpenes β -linalool and thymol, and the sesquiterpene β -caryophyllene, respectively. Similar findings were obtained by Ghannadi and Sadeh (1999) for *C. sativum*,

Camurça-Vasconcelos et al. (2007) for *L. sidoides* and Santos et al. (2008) for *C. reticulata*.

The mechanism of action of linalool found in the fruit of *C. sativum* is to stimulate the production of reactive oxygen species, such as nitric oxide (NO), to inhibit the activity of mitochondrial respiratory chain and to decrease levels of ATP and glutathione in the cells (USTA et al., 2009). Rosa et al. (2003) investigated the effects of linalool from essential oil of *Croton cajucara* against *Leishmania amazonensis* and on macrophage-parasite interactions. This compound had effect on both parasites forms and produced twice the amount of NO as the non treated macrophages. It reinforces the model that linalool acts indirectly to favor NO production by macrophages to eradicate the infection with *Leishmania* spp. *C. sativum* essential oil is rich in linalool. Such oil was more effective on amastigotes.

Thymol, a terpenoid, causes structural and functional damage to the cell membrane and acts directly on the synthesis and activity of ATPase and on the concentration of potassium and phosphate ions in the parasite. These activities have been characterized in bacteria, and this is the likely mechanism of action against *Leishmania* (LAMBERT et al., 2001; OSORIO et al., 2006). Synthetic thymol compounds exhibited an IC₅₀ of 194.3 µg.mL⁻¹ against promastigotes of *Leishmania panamensis* (ROBLEDO et al., 2005). In another study, a thymol-rich *L. sidoides* essential oil showed an IC₅₀ of 89 µg.mL⁻¹ against *L. chagasi* (OLIVEIRA et al., 2009). The essential oil of *L. citriodora*, which is also rich in thymol, demonstrated an IC₅₀ of 4.4 µg.mL⁻¹ on *L. chagasi* promastigotes (ESCOBAR et al., 2010). In this report, the essential oil of *L. sidoides* proved to be rich in thymol and yielded an IC₅₀ of 19.76 µg.mL⁻¹ on promastigotes of the same species. Synthetic thymol exhibited higher IC₅₀ values than the essential oils, which are composed of natural thymol and other substances. Thus, the more effective action of the oils may be due to the synergistic effect of other compounds, such as caryophyllene and cymene, or perhaps thymol is not as potent against *L. panamensis* compared to *L. chagasi*.

While the mechanism of action of β-caryophyllene is not well understood, it is effective against *L. amazonensis* (SANTOS et al., 2008) and exhibits anti-inflammatory (SHIMIZU et al., 1990), antimicrobial and antioxidant properties (SAHIN et al., 2004).

Investigations using natural products have shown great potential in finding new agents to fight tropical diseases, and the results here demonstrate that *C. reticulata* oleoresin is highly effective against promastigotes and amastigotes of *L. chagasi*, while exhibiting no toxicity toward the RAW 264.7 cell line. While further research is needed to prove its efficacy *in vivo*, *C. reticulata* oleoresin may be a promising new treatment for canine and human visceral leishmaniasis.

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