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# *In vitro* antiparasitic activity and chemical composition of the essential oil from *Protium ovatum* leaves (Burceraceae)

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**ABSTRACT**

Leishmaniasis and trypanosomiasis are globally widespread parasitic diseases which have been responsible for high mortality rates. Since drugs available for their treatment are highly hepatotoxic, nephrotoxic and cardiotoxic, adherence to therapy has been affected. Thus, the search for new, more effective and safer drugs for the treatment of these diseases is necessary. Natural products have stood out as an alternative to searching for new bioactive molecules with therapeutic potential. In this study, the chemical composition and antiparasitic activity of the essential oil from *Protium ovatum* leaves against trypomastigote forms of *Trypanosoma cruzi* and the promastigote forms of *Leishmania amazonensis* were evaluated. The essential oil was promising against trypomastigote forms of *T. cruzi* ($IC_{50}$= 28.55 μg.mL$^{-1}$) and *L. amazonensis* promastigotes ($IC_{50}$ = 2.28 μg.mL$^{-1}$). Eighteen chemical constituents were identified by Gas Chromatography coupled to Mass Spectrometry (GC-MS) in the essential oil, whose major constituents were spathulenol (17.6 %), caryophyllene oxide (16.4 %), β-caryophyllene (14.0 %) and myrcene (8.4 %). In addition, the essential oil from *P. ovatum* leaves had moderate cytotoxicity against $LLCMK_2$ adherent epithelial cell at the concentration range under analysis ($CC_{50}$ = 150.9 μg.mL$^{-1}$). It should be highlighted that this is the first report of the chemical composition and anti-*Trypanosoma cruzi* and anti-*Leishmania amazonensis* activities of the essential oil from *Protium ovatum* leaves.

**Key words:** *Trypanosoma cruzi*, *Leishmania amazonensis,* essential oil, *Protium ovatum*, parasitic diseases.

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## INTRODUCTION

Leishmaniasis, which is a disease caused by a digenetic protozoan of the genus *Leishmania*, has affected about two million people per year. It has also been considered an endemic disease in 88 countries, where about 350 million people are at risk (Neto et al. 2016). Its treatment is based on chemotherapeutic agents, which are pentavalent antimonial compounds ($Sb^{+5}$), and on amphotericin B. However, these chemotherapeutic agents are considered toxic (Antinarelli et al. 2015).

American trypanosomiasis, also known as Chagas disease, which is caused by *Trypanosoma cruzi*, can be transmitted to humans through feces of infected triatomine insects, contaminated food, blood transfusion and organs donated by infected donors (Delmondes et al. 2014). Nifurtimox and benzonidazole are prominent drugs in the treatment of Chagas disease, due to their efficacy in its acute phase and their tolerance (Delmondes et al. 2014).

The species *Protium ovatum*, which has been popularly known as *almecega*, is a shrub whose height ranges from 0.4 to 4 m, blooms from April to September and yields fruits in September (Castelo et al. 2010). This species is native to Brazil and, although it occurs in both the *Cerrado* and the Amazon Forest biomes, it is more associated with savanna formations, such as the *Cerrado* (Lista de Espécies da Flora do Brasil 2016). The main characteristic of this genus is the production of resins, which are used in folk medicines, such as anti-inflammatory, immunostimulant, repellent, antinociceptive and antineoplastic ones (Dias et al. 2011).

Since natural products are important for the development and discovery of new drugs, the objective of this study was to describe the chemical composition and *in vitro* antiparasitic activity of the essential oil from *Protium ovatum* leaves, for the first time.

## MATERIALS AND METHODS

### PLANT MATERIAL

Leaves of *P. ovatum* were collected in the *Cerrado* region at the University of Rio Verde (UniRV), in Rio Verde, Goiás, Brazil (17º 47'53"S and 50º 55'41" W) in July 2015. The plant was identified by the botanist Erika Amaral and a sample was deposited at the Herbarium Jataiense Professor Germano Guarim Neto at exsiccate number HJ 742.

### ESSENTIAL OIL EXTRACTION

The essential oil was obtained from fresh leaves of *P. ovatum* (100 g), which were reduced by a knife mill and had their essential oil extracted by the hydrodistillation method carried out by a Clevenger type apparatus at 100 °C for 4 h (Xavier et al. 2016). Thereafter, the hydrolate was submitted to liquid-liquid partition in a separatory funnel. Three washes of the hydrolate were performed with three 10 mL portions of dichloromethane. Total oil yield was expressed as percentage (g/100 g of fresh plant material). Essential oil samples were stored at −4 °C until further chemical and biological tests.

### GC-MS ANALYSIS OF ESSENTIAL OIL

Gas Chromatography coupled to Mass Spectrometry (GC-MS) analysis was done by a Shimadzu QP2010 with an AOC-20i auto-injector using a DB-5MS column (30 m x 0.25 mm, 0.25 mm in thickness). The carrier gas was He at pressure of 57.4 kPa and flow rate of 1.0 mL/min. The split ratio was 1/30, the injector temperature was 250 °C and the injected volume was 0.1 µL. Temperature ranged between 60 and 240 °C, having been increased 3 °C/min. MS were recorded on electron ionization (EI) mode, with ionization energy of 70 eV (scan time: 2 scan/s). The volatile chemical constituents were identified on the basis of their retention indices relative to a homologous series of *n*-alkanes ($C_{10}$–$C_{29}$) and by comparing mass spectra

with libraries (Wiley 7 and Nist 62) and references of previously published data (Adams 2007).

### LEISHMANICIDAL ACTIVITY AGAINST *Leishmania amazonensis* PROMASTIGOTE FORMS

In order to evaluate leishmanicidal activity, *L. amazonensis* promastigote forms (MHOM/BR/PH8) were maintained in RPMI 1640 (Gibco) culture medium supplemented with 10 % fetal bovine serum. Subsequently, about $1x10^6$ parasites were distributed on 96-well plates. The essential oil was previously dissolved in 100 % dimethylsulfoxide (DMSO, stock solution 10 $mg.mL^{-1}$ (Synth)) and added to the cultures at concentrations from 1.56 to 50 $\mu g.mL^{-1}$. Amphotericin B was previously dissolved in 100 % DMSO at concentration of 1 $mg.mL^{-1}$; afterwards, it was diluted in stock solution 500 $\mu g.mL^{-1}$ in the culture medium (Synth) and added to cultures at concentrations from 0.19 to 3.12 $\mu g.mL^{-1}$. Cultures were incubated at 25 °C in BOD ovens (Quimis) for 24 h and the leishmanicidal activity was determined by growth inhibition of promastigote forms by counting the total number of live promastigotes in the Neubauer chamber (Global Glass, Porto Alegre, BR), considering flagellar motility. RPMI 1640 medium (Gibco) containing 0.5 % DMSO (Synth) (highest concentration) was used as negative control and Amphotericin B (Eurofarma, São Paulo, BR) at 1 $\mu g.mL^{-1}$ concentration was used as positive control. Results were expressed as the mean of the lysis percentage relative to the negative control (0.1 % DMSO). Two experiments were performed in triplicate. Determination of 50 % inhibitory concentration values ($IC_{50}$) was carried out by non-linear regression curves of a GraphPad Prism version 5.0 Windows software (GraphPad software, USA). Maintenance of life cycle was approved by the Ethics Committee for Animal Care at the University of Franca, under protocol number 010/14.

### *In vitro* EVALUATION OF TRYPANOCIDAL AND CYTOTOXIC ACTIVITIES

The *in vitro* trypanocidal trial was performed with the *Trypanosoma cruzi* Y strain, constituted by thin trypomastigote forms. This strain has been maintained at the Franca University Vivarium through successive tests in Swiss mice by cardiac puncture on the day of the parasitemic peak (seventh day of infection). This procedure was approved by the National Council for Control of Animal Experimentation of the Ethics Committee at the University of Franca, under protocol number 010/14.

The assay was performed with blood from infected albino mice by cardiac puncture at the parasitemic peak (seventh day of infection). The infected blood was diluted with physiological solution to achieve final blood concentration of $10^6$ trypomastigote $forms.mL^{-1}$. Samples of the essential oils were diluted in DMSO and aliquots of this stock solution were added to the infected blood on the microtiter plate (96 wells), totalling 200 µL. In the trypanocidal trial, samples were evaluated in triplicate at concentrations of 400, 200, 100, 50, 25, 12.5 and 6.25 $\mu g.mL^{-1}$. Regarding controls, the positive one was benzonidazole whereas the negative one was 0.5 % DMSO (atoxic concentration in this cell type). The microplate was incubated at 37 °C for 24 hours. Afterwards, the activity was quantitatively verified by counting the trypomastigote forms in agreement with the technique previously described in the literature (Rashed et al. 2016). Determination of the parasite lysis percentage was performed by comparison with the control group without treatment. Two experiments were performed in triplicate. Determination of 50 % inhibitory concentration values ($IC_{50}$) was carried out by non-linear regression curves of a GraphPad Prism version 5.0 Windows software (GraphPad software, USA).

$LLCMK_2$ adherent epithelial cells were grown in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg.mL$^{-1}$ streptomycin and 5 % inactivated fetal calf serum. They were kept at 37 °C in 5 % $CO_2$. A cell suspension was seeded at a concentration of 1 x $10^6$ cells.mL$^{-1}$ on a 96-well microplate with RPMI 1640 medium. Thereafter, adherent epithelial cells were treated with essential oil at different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 μg.mL$^{-1}$). Plates were incubated at 37 °C for 24 h and the biological activity was evaluated by the MTT colorimetric method [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by a microplate reader at 540 nm. RPMI 1640 medium was used as positive control whereas DMSO and RPMI 1640 media were used as negative ones. All experiments were performed in triplicate. The percentage of cell viability was determined by the following formula: % cell viability = 1 - [(Y-N)/(N-P)] x 100, where Y = absorbance of wells containing cells and essential oil at different concentrations; N = negative control; and P = positive control (Esperandim et al. 2013).

## RESULTS AND DISCUSSION

The essential oil from *P. ovatum* leaves yielded 0.3 %, which is higher than the percentage previously found by Castelo et al. (2010) in a study developed with the same species. The essential oil from *P. ovatum* leaves was obtained and analyzed by GC-MS to determine its composition. It was evaluated against *T. cruzi* and *L. amazonensis* to establish a possible antiparasitic effect. Twenty seven compounds were detected and 18 compounds were

**TABLE I**
**Substances identified in the essential oil from *Protium ovatum* leaves.**

| Substances | *Retention time* | $RI_{Lit}$ | $RI_{exp}$ | *% RA* |
|---|---|---|---|---|
| α-Pinene | 6.29 | 932 | 933 | 1.2 |
| Sabinene | 7.51 | 969 | 972 | 1.8 |
| β-Pinene | 7.63 | 974 | 976 | 2.1 |
| Myrcene | 8.07 | 988 | 991 | **8.4** |
| γ-3-Carene | 8.78 | 1008 | 1011 | 1.5 |
| *p*-Cimene | 9.30 | 1020 | 1024 | 0.3 |
| Limonene | 9.46 | 1024 | 1028 | 1.8 |
| Terpinen-4-ol | 15.62 | 1174 | 1178 | 0.4 |
| α-Copaene | 24.17 | 1374 | 1376 | 6.4 |
| Bourbonene | 24.52 | 1387 | 1386 | 0.3 |
| β-Caryophyllene | 24.03 | 1417 | 1420 | **14.0** |
| Spathulenol | 25.99 | 1577 | 1578 | **17.6** |
| α-Humulene | 27.39 | 1452 | 1453 | 1.3 |
| Allo-Aromadendrene | 27.68 | 1458 | 1462 | 0.3 |
| Dauca-5,8-diene | 28.50 | 1471 | 1471 | 0.6 |
| Cubebol | 29.83 | 1515 | 1515 | 0.2 |
| γ-Cadinene | 30.20 | 1522 | 1525 | 1.4 |
| Caryophyllene oxide | 32.31 | 1582 | 1583 | 16.4 |
| Monoterpenes | | | | 17.5 |
| Sesquiterpenes | | | | 58.5 |
| Total (%) | | | | 76.0 |

***$RI_{Lit}$*:** Retention index from the literature. ***$RI_{exp}$*:** Retention index relative to *n*-alkanes ($C_{10}$-$C_{29}$) on the DB-5MS column. ***%RA*:** Relative area (peak area relative to the total peak in the GC-MS chromatogram), average of three replicates.

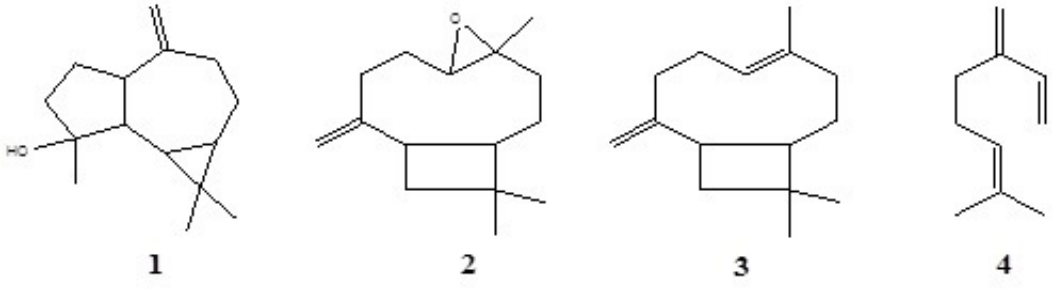


**Figure 1 -** Chemical structures of the four major constituents identified in the essential oil from *P. ovatum* leaves: **(1)** spathulenol, **(2)** caryophyllene oxide, **(3)** β-caryophyllene and **(4)** myrcene.

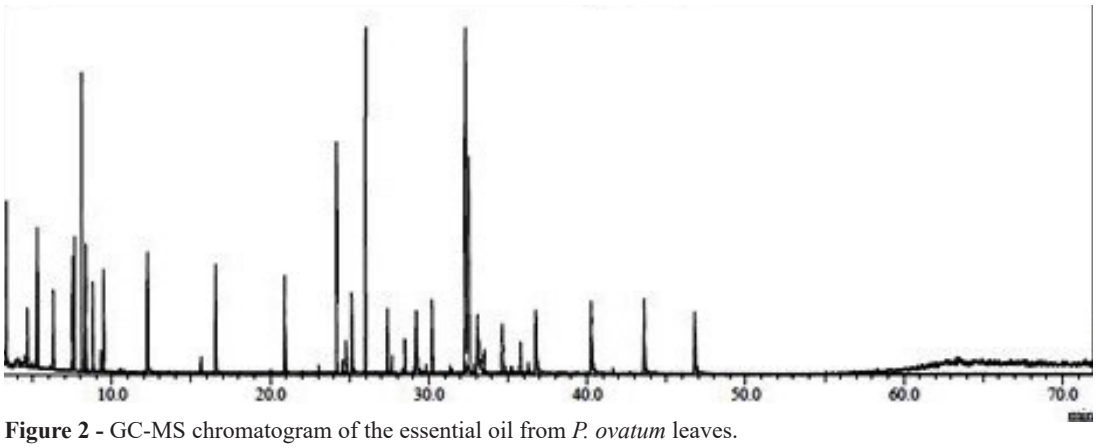


**Figure 2 -** GC-MS chromatogram of the essential oil from *P. ovatum* leaves.

identified (Table I, Figure 2). Major constituents were spathulenol (17.6 %, **1**), caryophyllene oxide (16.4 %, **2**), β-caryophyllene (14.0 %, **3**) and myrcene (8.4 %, **4**) (Figure 1). Spathulenol, caryophyllene oxide, β-caryophyllene and myrcene totalled 56.4 % of the chemical composition of the essential oil from *P. ovatum*. Identified compounds, retention time, retention index and relative percentage (%) are shown in Table I.

The chemical composition and biological properties of many essential oils have drawn the interest of researchers who have studied them widely in several areas (Estevam et al. 2016). In the literature, previous studies of essential oils from other species of the genus *Protium* showed similar chemical composition to the one found by this study. Sesquiterpene caryophyllene oxide (16.4 %) was identified as the major component in the essential oil from *P. bahianum* leaves and from leaves and branches of the *P. hebetatum* species (Moraes et al. 2013). Both compounds limonene (1.8 %) and *p*-cimene (0.3 %) were also found in the essential oil extracted from *P. strumosum*, *P. altsonii* and *P. hebetatum* resins (Zoghbi et al. 2005, Pinto et al. 2010). Compounds α-pinene (1.2 %) and myrcene (8.4 %) were identified at high concentrations in the essential oil from resins extracted from the *P. heptaphyllum* species (Siani et al. 2011). Relative concentration of spathulenol, by comparison with the concentration found in *P. ovatum* leaves (17.6 %), was lower in essential oils from *P. hebetatum* leaves and branches (Pinto et al. 2010), *P. pilosum* leaves and *P. strumosum* resins (Zoghbi et al. 2005). In the analysis of the chemical composition of the essential oil from *P. heptaphyllum* leaves, sesquiterpene spathulenol was identified as one of the major constituents (Carvalho et al. 2013).

Results of the evaluation of the leishmanicidal activity of the essential oil from *P. ovatum* leaves against the promastigote forms of *L. amazonensis* are shown in Table II.

$IC_{50}$ value = 2.28 μg.mL$^{-1}$ is considered to be very promising by comparison with that of Amphotericin B ($IC_{50}$ = 0.60 μg.mL$^{-1}$) (Table II). The literature describes that α-pinene has recognised leishmanicidal activity against *L. brasiliensis*, a fact that may influence the antiparasitic activity of *P. ovatum* oil, even though this terpene is at concentration below 10 % (Table I) (Sobral et al. 2014). In addition, the literature has shown that β-caryophyllene (14.0 %) is also active against parasites of the genus *Leishmania* (Meneguetti et al. 2015). It is a relevant fact since this terpene is found at a concentration above 10 % in essential oil from *P. ovatum* leaves, a justification for the promising leishmanicidal potential of the species. Another important factor that can lead to good performance of antiparasitic activity is either the synergism of the major chemical constituents found in the essential oil or the presence of other constituents that can also be active at even lower concentrations (Melo et al. 2011).

It is relevant to mention the efficacy of the essential oils that have promising biological activities when the mixture of all their constituents, minor and major ones, is evaluated (Raut and Karuppayil 2014). It has been suggested, therefore, that minor constituents, due to the synergistic effect, are also crucial for the biological activities of essential oils (Silva et al. 2015a).

Essential oils of *P. ovatum* leaves also showed expressive trypanocidal activity when tested against trypomastigote forms of *T. cruzi*. An increase in the lysis of the parasites was observed as the concentration of the essential oil increased, with $IC_{50}$ values of 28.55 μg.mL$^{-1}$ and benzonidazole ($IC_{50}$ = 9.8 μg.mL$^{-1}$) as positive control (Table III).

**TABLE II**
**Leishmanicidal activity of essential oil from *P. ovatum* leaves.**

| EO | % of lysis ± S.D./Concentrations (μg.mL$^{-1}$) | | | | | | $IC_{50}$ (μg.mL$^{-1}$) |
|---|---|---|---|---|---|---|---|
| | **50** | **25** | **12.5** | **6.25** | **3.12** | **1.56** | |
| ***Protium ovatum*** | 90.54 ± 2.25 | 88.32 ± 3.40 | 79.55 ± 1.36 | 76.59 ± 2.26 | 58.17 ± 4.06 | 38.63 ± 4.18 | 2.28 |
| | **3.12** | **1.56** | **0.78** | **0.39** | **0.19** | | |
| **Amph. B** | 98.14 ± 0.44 | 89.08 ± 0.89 | 75.73 ± 1.17 | 13.06 ± 0.94 | 4.87 ± 1.31 | - | 0.60 |

**EO:** essential oil; **Amph. B:** Amphotericin B.

**TABLE III**
**Trypanocidal activity of essential oil from *P. ovatum* leaves against *T. cruzi* trypomastigote forms.**

| EO | % of lysis ± S.D./Concentration (μg.mL$^{-1}$) | | | | | | | $IC_{50}$ (μg.mL$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | **6.25** | **12.5** | **25** | **50** | **100** | **200** | **400** | |
| ***Protium ovatum*** | 23.76 ± 2.94 | 32.58 ± 1.12 | 41.40 ± 1.37 | 58.44 ± 3.73 | 76.38 ± 10.55 | 99.25 ± 0.93 | 100 ± 0 | 28.55 |

**EO:** essential oil; **S.D.:** standard deviation; positive control: benzonidazole ($IC_{50}$ = 9.8 μg.mL$^{-1}$).

**TABLE IV**
**Cytotoxic activity of essential oil from *Protium ovatum* leaves.**

| | % of lysis ± S.D./Concentration (μg.mL$^{-1}$) | | | | | | | $CC_{50}$ (μg.mL$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | **6.25** | **12.5** | **25** | **50** | **100** | **200** | **400** | |
| **EO** | 100 ± 0 | 85.2 ± 1.8 | 80.3 ± 1.5 | 72.9 ± 1.7 | 42.8 ± 1.1 | 35.6 ± 1.4 | 30.7 ± 1.7 | 150.9 |

**EO:** essential oil from *Protium ovatum* leaves. **S.D.:** standard deviation.

The trypanocidal activity of the essential oil from *P. ovatum* leaves is described by this paper for the first time. The literature describes that samples with $IC_{50} < 10$ μg.mL$^{-1}$ had highly active trypanocidal activity, whereas others were active ($IC_{50} > 10 < 50$ μg.mL$^{-1}$), moderately active ($IC_{50} > 50 < 100$ μg.mL$^{-1}$) and inactive ($IC_{50} > 100$ μg.mL$^{-1}$) (Estevam et al. 2016).

The promising trypanocidal activity exhibited by the essential oil from *P. ovatum* leaves can be justified by the following terpenes: β-caryophyllene (14.0 %), *p*-cymene (0.3 %), limonene (1.8 %), spathulenol (17.6 %) and caryophyllene oxide (16.4 %), as previously identified in the essential oil from different species of *Lippia* spp, with recognised antiparasitic activity (Escobar et al. 2010). The compound α-pinene (1.2 %), which is also found in the essential oil from *Schinus terebinthifolius*, has recognised trypanocidal activity (Sartorelli et al. 2012). Other notable compounds identified in the essential oil from *P. ovatum* leaves have already had their antiparasitic activity reported by the literature are: myrcene (8.4 %), sabinene (1.8 %), terpinen-4-ol (0.4 %) and β-pinene (2.1 %) (Silva et al. 2015b). To sum up, bioactive compounds that were identified by this study in the essential oil from *P. ovatum* leaves may justify the promising anti-*Trypanosoma cruzi* and anti-*Leishmania amazonensis* activities that it found.

Regarding cytotoxic activity, cultures of $LLCMK_2$ adherent epithelial cells were treated with essential oil at concentrations of 6.25, 12.5, 25.0, 50.0, 100, 200 and 400 μg.mL$^{-1}$ for 24 h. Results showed that the essential oil from leaves have moderate toxicity at the concentration evaluated with $CC_{50}$ 150.9 μg.mL$^{-1}$ (Table IV), by comparison with benzonidazole positive control, with $CC_{50}$ 147.3 μg.mL$^{-1}$.

Essential oil from *P. ovatum* fresh leaves was classified as moderately cytotoxic ($CC_{50} > 100 < 1000$ μg.mL$^{-1}$), by comparison with data found in the literature (Alves et al. 2012). The moderate cytotoxicity of this oil is an indicator of the fact that this plant can be well tolerated by the biological system. However, further detailed studies are required to evaluate the toxicity of these bioactive oils with other models *in vivo*. There is evidence that essential oils have low density and rapid diffusion across cell membranes because of their lipid solubility. As a result, they could damage the parasite cell membrane structure, which would lead to cellular lysis (Anthony et al. 2005). In addition, there could be synergistic and/or additive effects of the constituents of essential oils (Melo et al. 2011).

## CONCLUSIONS

To sum up, results of this study showed that the essential oil from *Protium ovatum* leaves found in the Brazilian *Cerrado*, which is located in the country's central-west region, has promising antiparasitic potential with moderate cytotoxicity towards $LLCMK_2$ adherent epithelial cells. The chemical composition of the essential oil from *P. ovatum* leaves had a mixture of mono and sesquiterpenes and its major constituents were spathulenol, caryophyllene oxide, β-caryophyllene and myrcene. Since diseases caused by the parasites under investigation affect millions of people worldwide, results of this study are relevant because the chemical composition of the essential oil from *P. ovatum* leaves was described for the first time. It is noteworthy that, in the face of medical advances, plants are still considered promising sources and bioactive compounds found in essential oils from plants of the genus *Protium* may serve as prototypes for the development of new antiparasitic drugs.

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