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Antimycobacterial Activity and Chemical Characterization of the Essential Oils from Reproductive Organs of *Piper lhotzkyanum* Kunth (Piperaceae)

Atividade Antimicobacteriana e Caracterização Química dos Óleos Essenciais dos Órgãos Reprodutivos de Piper lhotzkyanum Kunth (Piperaceae)

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Piper lhotzkyanum Kunth belongs to the Piperaceae family, is a medicinal plant also known as "Aperta-ruão" and "Beque-cheiroso". This species is used in folk medicine to treat rheumatism, sore throats, gastrointestinal problems, and bronchial conditions. The present study aimed to analyze the chemical composition and biological activity of the essential oils (EOs) from reproductive organs of *P. lhotzkyanum* against strains of *Mycobacterium tuberculosis* H37Rv (ATCC, 25618). The EOs were obtained by hydrodistillation and characterized by GC-MS and GC-FID. Chemical composition of the volatile mixture showed to be rich in monoterpenes for both inflorescences (IFl) and infructescences (IFr). These monoterpenes included α-phellandrene, β-phellandrene, α-pinene, and β-pinene for both samples. The antimycobacterial activity showed minimum inhibitory concentration (MIC) of 76.51 μg/mL and 128 μg/mL for IFr and IFl, respectively. By these results, the volatile fraction of IFr showed promising activity against *M. tuberculosis*. The chemical composition and antimicrobial properties of IFl and IFr EOs have been reported for the first time.

Keywords: Inflorescence; infructescence; Mycobacterium tuberculosis; monoterpenes, phellandrene.

1. Introduction

Piper genus (Piperaceae) has commercial, ecological, and medicinal importance.[1] Biological properties of essential oils (EOs) from plants of this genus have been reported. [2-8] The most popular species is *P. nigrum* L., known as black pepper, widely used as a condiment and as medicine, mainly in Asia.[9]

Piper lhotzkyanum Kunt is a perennial shrub from 1 to 2 m high and popularly known as "beque-cheiroso",[10-11] common in the Brazilian Amazon rainforest and the Brazilian Atlantic forest.[12] This species is used in folk medicine in infusion preparation for the treatment of rheumatism, sore throats, gastrointestinal problems, bronchial conditions, among others. The infructescences of *P. lhotzkyanum* have a strong flavor and produce a burning sensation and analgesia when chewed.[10,13-14]

Tuberculosis (TB) is a neglected disease caused by *Mycobacterium tuberculosis* and is one of the most significant motive of death since 19th century.[8] According to WHO 230,000 children died from one million infected with TB.[15] In this scenario, it is worth noting the increase in multidrug-resistant TB to drugs such as fluoroquinolones and injectables such as amikacin, kanamycin, capreomycin, further reducing the range of treatment options. There is an urgency regarding the discovery of new drugs and an alternative would be the combination of commercial drugs with natural compounds that could be tested to enhancer antibiotic activity.[15-19] It is reported that natural products, including EOs, and their isolated compounds have inhibitory activity against the growth of *M. tuberculosis*, while some have been selected as prototype molecules for the development of new anti-tuberculosis agents.[20-22]

Despite being well reported that EOs of the *Piper* genus have demonstrated biological activities,[2-8] such as antibacterial, antiproliferative,[23-26] antileishmanial,[16] insecticidal, fungicidal,[25] antioxidant, and cytotoxic, [26] to date there is nothing published demonstrating the EO composition of *P. lhotzkyanum* with biological properties, besides a study showing great ovicidal activity against *Anticarsia gemmatalis* (soybean caterpillar) (LC_{50} = 1.6%).[27]

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Also, the chemical composition of EOs from inflorescences (IFl) and infructescences (IFr) (reproductive organs) for *P. lhotzkyanum* has never been described. Therefore, the aim of this study was to perform an analysis of the EO chemical composition of reproductive organs of *P. lhotzkyanum* from altitude in addition to reporting its antimycobacterial activity.

2. Material and Methods

2.1. Plant material and essential oil extraction

Inflorescences and infructescences of *Piper lhotzkyanum* Kunth were collected in a region of altitude in the Atlantic Forest at Serra dos Órgãos National Park, near the city of Teresópolis, Rio de Janeiro (Altitude: 1,144.69 m and GPS: 12°11′45′S; 38°58"05'W) in 2019. This study was registered in the Genetic Heritage Management Council (CGEN n. AE4E953) and in the Biodiversity Authorization and Information System (SISBIO n. 57296-1). The botanical identification was made by Dr. Elsie Franklin Guimarães and Msc. George Azevedo Queiroz at Rio de Janeiro Botanical Garden Research Institute (JBRJ). Herborized samples were deposited at the Herbarium RB (01426181). The fresh plant material (100 g, 700 mL of distilled water) was subjected to hydrodistillation for 2 h in a Clevenger-type apparatus for EOs extracting. The obtained samples were drying over anhydrous sodium sulfate (Na_2SO_4, Sigma-Aldrich, Brazil), kept in sealed amber vials, and stored at -20 °C for five days until gas chromatography (GC) analysis. The total yield of EO was registered as a percentage value, considering weight of EO (g)/ 100 g of fresh plant material.[28-30]

2.2. Essential oils analysis

The obtained EOs were diluted in dichloromethane (HPLC grade, Tedia, Brazil) until 1.000 ppm. All samples were injected 1 µL, splitless,[28-29] for chemical identification by gas chromatography coupled to mass spectrometry (GC-MS) and for quantification by GC coupled to Flame Ionization Detection (GC-FID).

GC-MS analysis was performed using a gas chromatograph 6890 GC coupled to an Agilent MS 5973N mass spectrometer (Hewlett-Packard, Brazil), operating at 70 eV of ionization energy, in positive mode, and mass range of *m/z* 40 – 600 atomic mass units (u). The GC conditions were an HP-5MS capillary column (30 m x 0.25 mm id x 0.25 µm film thickness), temperature programming from 60 °C to 240 °C with an increase of 3 °C/min, using helium (99.99%) as carrier gas at a constant flow rate of 1.0 mL/min. The injector and detector were set at 270 °C, the transfer line was set at 280 °C and samples were injected 1 µL splitless.

GC-FID analysis was achieved in an HP-Agilent 6890 gas chromatograph (Hewlett-Packard, Brazil) equipped with an HP-5MS capillary column (30 m x 0.25 mm id x 0.25 µm film thickness), temperature setting from 60 °C to 240 °C, with an increase of 3 °C/min, using hydrogen as carrier gas at a constant flow rate of 1.0 mL/min. The injector and detector temperatures were set at 270 °C. Samples were injected 1 µL splitless. Retention indices (RI) as well as the quantification of the peak area were achieved based on the results of the GC-FID. Relative percentage of individual components was calculated based on the peak areas of the GC without correction of the FID response factor. The compounds were identified based on the fragmentation pattern of the mass spectrum compared with literature records (National Institute of Standards and Technology – NIST, 2010; Wiley7n), as well as calculated RI referring to a homologous series of *n*-alkanes (C_8 -C_{28}, Sigma -Aldrich, Brazil).[31-32]

2.3. Antibacterial activity

The standard virulent strain of *Mycobacterium tuberculosis* H37Rv (ATCC, 25618) was grown in 7H9 (BACTO) culture medium, supplemented with 10% albumin, dextrose, catalase (ADC) (BC®), 0.05% of tween 80, and kept in an incubator (Scientific – Water-Jacketed incubator) at 37 °C and 5% CO_2, until the beginning of the growth phase. Samples were evaluated for their antimycobacterial activity using the tetrazole salt assay in a 96-well microplate at concentrations of 16, 32, 64 and 128 µg/mL. For this test, a suspension was prepared with *M. tuberculosis* H37Rv (300 µL of mycobacteria in 7.2 of 7H9 culture medium supplemented with 10% ADC, approximately 3 x10^7 Colony Forming Units – CFU/ mL) and kept in an incubator at 37 °C and 5% CO_2 until the beginning of the log phase (exponential growth phase). The CFU dosage for turbidity was standardized and monitored in a spectrophotometer (Hitachi – Model U-1100) at an optical density (O.D.) of 600 nm. Subsequently, in the logarithmic growth phase, 50 µL of this suspension were plated in a 96-well microplate (1x10^6 CFU/ well). The EO samples (50µL / well) were previously diluted in 7H9 supplemented with ADC in a concentration 2 times higher than the desired final concentration and added to the microplate where the mycobacteria already contained. The sealed plate was incubated at 37 °C and 5% CO_2 for 5 days. After this period, 10 µL per well of a 5 mg/ mL solution of tetrazole 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazole (MTT) in saline phosphate buffer (PBS) was added sterile. Three hours later, 100 µL of the lysis buffer was added (20% w/ v sodium dodecyl sulfate (SDS)/ 50% dimethylformamide (DMF) in distilled water – pH 4.7). The microplate reading was performed on a spectrophotometer at 570 nm (Hitachi – Model U-1100).[33] Treatment with rifampicin (0.032; 0.08; 0.2 and 1 µg/ mL) was used as a positive control of antimycobacterial activity in the wells containing only the bacilli. Negative control was set in wells containing bacilli and without treatment. To calculate the percentage of inhibition of mycobacterial growth, equation (1) was used.

$$100 - (O.D_{\cdot Sample} - O.D_{\cdot C+}) \times (O.D_{\cdot C} - O.D_{C+} \quad \textbf{(1)}$$

2.4. Statistical analysis

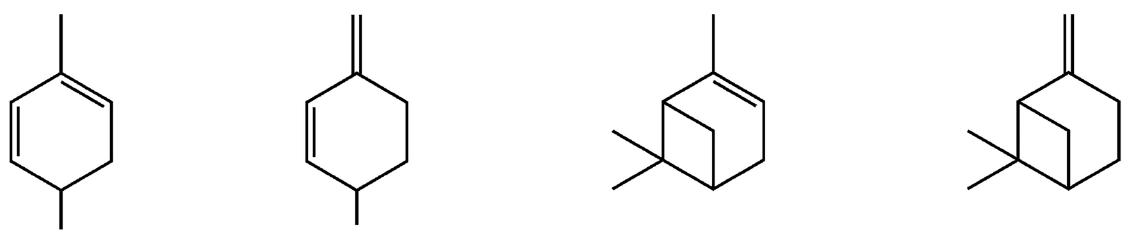
The statistical analysis to show differences in the antimicrobial activity of the analyzed EOs was performed by the ANOVA test, using Statistica® software. The value of $p < 0.05$ was considered statistically significant.

3. Results

Strongly yellowish essential oils were obtained from fresh IFr and IFl of *P. lhotzkyanum*. The yield of EOs was at 2.4% for IFl and 2.5% for IFr (w/ w). It was possible to identify a total of 39 and 20 compounds that correspond to 93.9% and 97.1% of total chemical composition, respectively, which are shown in Table 1. A total of 29 compounds for IFl and 12 for IFr was identified in less than 1.0% of relative percentage, and corresponding to 11.3% and 3.2%, respectively.

The relative percentage of monoterpenes in the EOs of the reproductive organs was quite pronounced (IFl – 86.16%; IFr – 92.34%). The major constituents were identified as non-oxygenated monoterpenes α-phellandrene (IFr – 56.4%; IFl – 48.52%), β-phellandrene (IFr – 14.5%; IFl – 8.49%), α-pinene (IFr – 6.8%; IFl – 11.25) and β-pinene (IFr – 6.9%; IFl – 8.35) (Figure 1). The mass spectra of the main components from the reproductive organs of *P. lhotzkyanum* (70 eV, positive mode, *m/z* 40 – 600 u) is shown on the supplementary material (Figures S1-4).

The antimicrobial activity of the EOs is shown in Figure 2. The sample IFr showed greater activity than IFl (MIC of 76 and 128 µg/mL, respectively).



α -phellandrene β-phellandrene α-pinene β-pinene

Figure 1. Chemical structures of the main identified constituents in the essential oils from inflorescences and infructescences of *Piper lhotzkyanum* Kunth

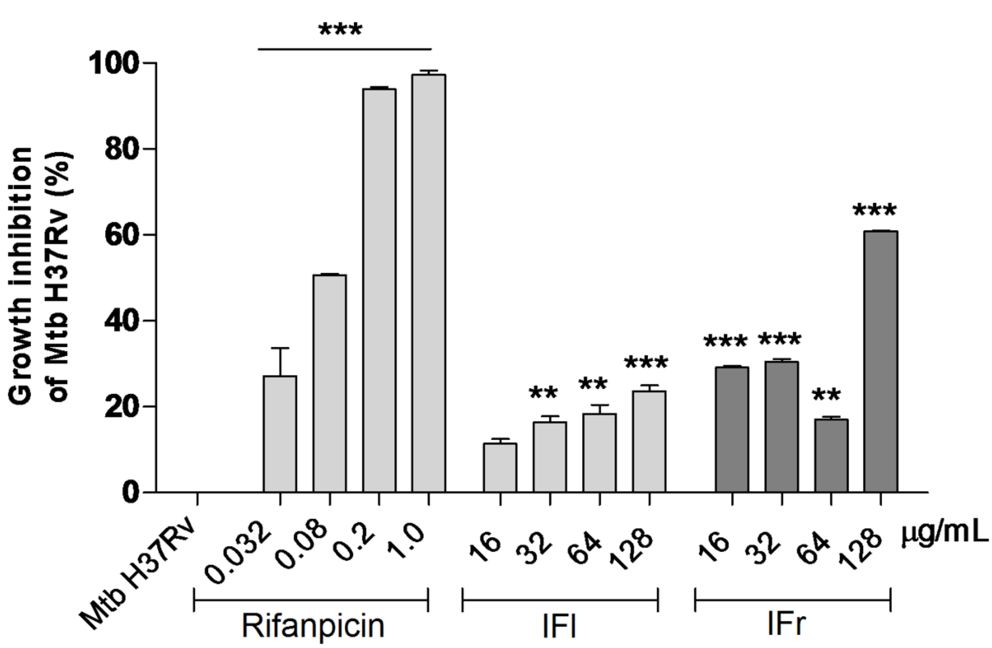


Figure 2. Growth inhibition of *Mycobacterium tuberculosis* H37Rv after treatment with *Piper lhotzkyanum* Kunth essential oils
Legend. Results of MTT assay after 5 days of incubation in the presence of samples at concentrations of 16, 32, 64 and 128 µg/mL. Positive control = *M. tuberculosis* H37Rv treated with rifampicin (reference drug); negative control = *M. tuberculosis* H37Rv without treatment. Statistical analysis: Oneway ANOVA followed by the Tukey test *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared to the negative control (Mtb H37Rv 1x10^6 CFU / mL). Triplicate results represented as mean ± standard error. IFl - Essential oil from inflorescences; IFr - Essential oil from infructescences.

Table 1. Chemical composition of the essential oil from reproductive organs of *Piper lhotzkyanum* Kunth.

Compounds[a]	**RI cal**[b]	**RI lit**[c]	**Relative percentage (%)**	
			IFl	**IFr**
α-thujene	923	924	0.1	tr
α-pinene	931	932	11.2	6.8
camphene	944	946	0.3	-
β-pinene	970	974	8.3	6.9
6-methyl-5-hepten-2-one	988	989	tr	tr
myrcene	995	988	1.1	0.7
α–phellandrene	1009	1002	48.5	56.4
δ-3-carene	1010	1008	3.5	3.7
α–terpinene	1012	1014	1.3	-
p-cymene	1022	1020	tr	tr
o-cymene	1023	1022	-	tr
limonene	1025	1024	tr	1.9
β-phellandrene	1028	1025	8.5	14.5
cis-β-ocimene	1036	1032	2.6	0.6
γ-terpinene	1057	1054	0.2	0.3
terpinolene	1085	1086	0.4	0.5
linalool	1103	1095	1.7	-
unidentified (monoterpene)	-	-	-	1.8
trans-sabinene hydrate	1104	1098	tr	-
menth-2-en-1-ol	1116	1118	tr	-
terpinen 4-ol	1178	1174	tr	-
α-terpineol	1189	1186	0.2	-
cis-piperitol	1205	1195	tr	-
α-copaene	1381	1374	0.2	-
β-elemene	1407	1389	0.0	-
α-gurjunene	1411	1409	0.1	-
cis-caryophyllene	1425	1417	2.4	2.0
γ-elemene	1433	1434	0.5	-
aromadendrene	1440	1439	0.1	-
α-humulene	1456	1452	0.3	-
β-selinene	1480	1489	0.5	0.5
α-selinene	1494	1498	0.6	0.1
β-curcumene	1496	1514	-	0.3
γ-cadinene	1509	1513	0.1	-
δ-cadinene	1518	1522	0.5	0.1
zonarene	1524	1528	0.1	-
selina-3,7(11)-diene	1547	1545	tr	-
germacrene B	1553	1559	0.1	-
caryophyllene oxide	1581	1582	tr	-
guaiol	1596	1600	tr	-
α-epi-muurolol	1645	1640	0.1	-
Non-Oxygenated Monoterpenes			86.16	92.34
Oxygenated Monoterpenes			1.99	1.77
Non-Oxygenated Sesquiterpenes			5.50	3.03
Oxygenated Sesquiterpenes			0.18	0.00
Total			93.85	98.91

[a]All compounds were identified by MS and RI in accordance with experimental. [a]Compounds are listed in order of elution. [b]Retention indices calculated from retention times in relation to those of the *n*-alkanes series on a HP-5MS analytical column (see experimental). [c]Retention indices from the literature. IFl = Inflorescences, IFr = Infructescences. tr = compound < 0.05%.

4. Discussion

In this article we analyzed for the first time the chemical composition and the antimycobacterial potential of reproductive organs of the species *P. lhotzkyanum*. This work represents the first biological activity described for the inflorescences and infructescences of this species. Studies referring to the leaf EO of *P. lhotzkyanum* was published before by Krinski (2018),[27] and showed ovicidal activity.

The EO yields for the reproductive organs (~ 2.5%, w/w) were considered high, a fact that is not common for species of *Piper*, with some exceptions, for example, *Piper cubeba* L. (4.4%, w/w). However more studies is necessary to evaluate this yield during different phenological periods.[34-39] This fact is crucial in natural products, mainly, to be exploited commercially or in the production of pharmacological assets. Besides, this high EO yield may have an importance role in the ecological issues related to *P. lhotzkyanum*, such as pollinators attraction or herbivore repellency. Considering herbivore repellency, the identified compounds in the volatile mixture may present important biological properties such as antimicrobial.

This study reported a predominance of non-oxygenated monoterpenes in the reproductive organs which differs from some results found in the literature for other *Piper* species,[39-40]. The high relative percentage of α- and β-phellandrenes, that are used in the food and perfume industries are very interesting.[41-42] Compound α-phellandrene, a cyclic monoterpene, is also found in the EOs from plants such as *Schinus terebinthifolius* Raddi (15.7%), *Solanum erianthum* D.Don (17.5%), *Thymus kotschyanus* Boiss and Hohen (10.8%), *Cupressus atlantica* Gaussen (5.5%), *Anethum graveolens* L. (32.0%), *Myrica gale* L. (8.0%) and *Piper mullesua* D. Don (22.8%). Literature records showed antinociceptive, anti-inflammatory,[43] antimicrobial,[44] anticancer,[45] and hyperalgesic activities[46] related to this monoterpene.

The evaluation of antimycobacterial activity for both EOs against strains of *M. tuberculosis* showed promising activity for IFl and IFr. MIC values < 100 µg/mL have been found to be ideal candidates against *M. tuberculosis*, while values of 100 – 200 µg/mL are considered moderate candidates.[8,47] Therefore, EO from IFr (MIC = 76 mg/ mL) could be a promising candidate to proceed in a further study since synergism of EOs with standard drugs used to treat TB may be an option in the treatment of this condition.[17] Some studies relate the antimicrobial activity of EOs, mainly, to their monoterpenoid constituents. Mechanism of action proposed that monoterpenes act on the disruption and dysregulation of the bacterial membrane function.[48-51] Interestingly to note that the richest EO in monoterpenes from infructescences showed greater activity, strengthening the hypothesis that they may interact with the phospholipid membranes of *M. tuberculosis*.

5. Conclusions

The chemical composition of the essential oil from reproductive organs of *Piper lhotzkyanum* is described here for the first time. The studied essential oils and their volatile components can provide an important source of new antimycobacterial agents. In addition, the high essential oils´ yield and the great relative percentage of phellandrene may represent an important source of this monoterpene for the industry. The essential oil from infructescences was the most active against *M. tuberculosis*. These findings contribute with new data on the chemical constitution and antimycobacterial potential of the essential oils from reproductive organs of *P. lhotzkyanum* collected at high altitude site in the Atlantic Forest.

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