Application of molecular networks in investigating metabolites from ethanolic extracts and fractions of *Piper multinodum* and activity evaluation against *Mycobacterium tuberculosis*

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Key words:
- GC-MS
- liquid-liquid extraction
- molecular annotation
- *Mycobacterium tuberculosis*
- Piperaceae

Abstract

The Piperaceae family comprises approximately 4,300 species with a pantropical distribution. *Piper multinodum* a species endemic to the *Cerrado* and Atlantic Forest, has shown promising bioactivity against *Mycobacterium tuberculosis* in preliminary studies of its volatile components. This study used Gas Chromatography-Mass Spectrometry (GC-MS) and molecular networking by the GNPS platform to elucidate the metabolomic profile of ethanolic extracts and solvent fractions from *P. multinodum* organs and assessed activity against *M. tuberculosis*. Static maceration yielded extracts, which were fractionated using *n*-hexane, dichloromethane, and ethyl acetate. GC-MS analysis resulted in the annotation of 45 metabolites arranged into eight molecular families, including phenylpropanoids, sesquiterpenes, steroids, long-chain aldehydes, and tocopherol derivatives. Notably, *α*-spinasterol was the major steroid in the *n*-hexane partition of stems, while eupomatenoid-5 isomers were predominant in the dichloromethane fractions. The antimycobacterial screening against *M. tuberculosis* H37Rv revealed minimum inhibitory concentration (MIC) values above 128 µg/mL, classifying the extracts as moderate candidates according to literature benchmarks (100-200 µg/mL). These findings contribute to the phytochemical characterization of *P. multinodum*, reinforcing its chemical diversity and potential as a source of antimycobacterial lead compounds for further pharmacological investigation.

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Palavras-chave:
- CG-EM
- extração líquido-líquido
- anotação molecular
- *Mycobacterium tuberculosis*
- Piperaceae

Resumo

A família Piperaceae compreende aproximadamente 4.300 espécies de distribuição pantropical. *Piper multinodum* espécie endêmica do Cerrado e da Mata Atlântica, demonstrou atividade bioativa promissora contra *Mycobacterium tuberculosis* em estudos preliminares de seus constituintes voláteis. Este estudo empregou Cromatografia em Fase Gasosa acoplada à Espectrometria de Massas (CG-EM) e análise por redes moleculares via a plataforma GNPS para elucidar o perfil metabolômico dos extratos etanólicos e frações obtidas dos orgãos de *P. Multinodum* e avaliou atividade contra *M. tuberculosis*. A extração foi realizada por maceração estática, seguida de partição líquido-líquido com hexano, diclorometano e acetato de etila. A análise por CG-EM permitiu a anotação de 49 metabólitos distribuídos em oito famílias moleculares, incluindo fenilpropanoides, sesquiterpenos, esteroides, aldeídos de cadeia longa e derivados de tocoferol. Destaque para o α-espinasterol, principal esteroide identificado na fração hexânica dos caules, enquanto isômeros do eupomatenoide-5 foram predominantes nas frações diclorometânicas. Os ensaios antimicobacterianos contra *M. tuberculosis* H37Rv revelaram valores de concentração inibitória mínima superiores a 128 µg/mL, classificando os extratos como candidatos moderados segundo parâmetros da literatura (100–200 µg/mL). Esses achados contribuem para a caracterização fitoquímica de *P. multinodum*, reforçando sua diversidade química e seu potencial como fonte de substâncias líderes para investigações farmacológicas futuras.

Introduction

Piper multinodum C. DC. belongs to the Piperaceae family and is a species found in the Southeast region of Brazil, specifically in biomes such as *Cerrado* and Atlantic Forest, with a restricted occurrence (Guimarães *et al*. 2025). The leaves have a smooth surface with secondary veins extending to the median portion and an upright spike-shaped inflorescence. This species belongs to taxonomic group IV, making it taxonomically related to *Piper aduncum* L. (Guimarães *et al*. 1994).

Piper multinodum plays an important role in the ecosystem resilience, as its complementary functions enhance the stability of ecological processes in response to environmental changes (Kubitzki & Gottlieb 1984). According to Gottlieb *et al*. (1996), special metabolites, when analyzed from the perspective of chemical language, tend to modulate and diversify in satellite species - those with restricted distribution, such as *P. multinodum*. This phenomenon is particularly evident in environments under high ecological pressure, such as the Serra dos Órgãos National Park (PARNASO), which is characterized by high-altitude conditions.

Despite its ecological importance, the chemical and pharmacological potential of *P. multinodum* remains largely unexplored. Until now, there were no recorded chemical, pharmacological, and/or toxicological studies on this species, except for an article published by our research group in 2021 (Ramos *et al*. 2021), which focused on the essential oils extracted from different plant organs. This study revealed that *P. multinodum* essential oils are rich in arylpropanoids, monoterpenes, and sesquiterpenes, demonstrating promising antimycobacterial activity. However, no studies have addressed the metabolite profile of ethanolic extracts and their potential biological effects.

Given the increasing global burden of tuberculosis, caused by *Mycobacterium tuberculosis*, the search for new antimycobacterial drugs is essential. Recent WHO data indicate approximately 10.6 million global cases and 1.3 million deaths in 2022 alone (WHO 2023). The disease remains the second leading cause of death from infectious diseases, and the rise of multidrug-resistant strains exacerbates the health crisis. In this context, natural products represent a promising avenue for the discovery of novel bioactive compounds (WHO 2023).

In this study, we used Gas Chromatography-Mass Spectrometry (GC-MS), integrated with the molecular networking approach, to investigate the chemical diversity of extracts and fractions obtained from the leaves, stems, and roots of *P. multinodum*. Additionally, we evaluated the biological activity of the ethanolic extract and its leaf fractions against *M. tuberculosis* H37Rv. Notably, this is the first report on the chemical characterization of this species' extracts using GC-MS.

Materials and Methods

Plant material

Adult specimens of *Piper multinodum* were collected in January 2019 at Serra dos Órgãos National Park (PARNASO) - Teresópolis, RJ, Brazil. Collection permit: SISBIO (issued by Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio) number 57296-4 - authentication code 47749568 (GPS coordinates: (22°27′00′′S, 42°59′20′′W, Elevation: 1,267 m). The collected plant material consisted of leaves (1,500 g, fresh weight), stems (1,100 g, fresh weight), and roots (500 g, fresh weight). The material was authenticated by Dr. Elsie Franklin Guimarães from the Research Institute of the Rio de Janeiro Botanical Garden (JBRJ) and Dr. George Azevedo Queiroz from the Rio de Janeiro State University where a voucher specimen was deposited under the number RB818479. The study was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number A001E32. The collected samples were transported under controlled conditions (4 °C) and processed immediately upon arrival at the laboratory to minimize degradation.

Preparation and fractionation of extract

The plant material was dried in an air-circulating oven at 40 °C (±2 °C) with controlled humidity (45–55%) until reaching a constant weight, resulting in 493.12 g of dried leaves, 1,001.22 g of dried stems, and 350.11 g of dried roots. The dried materials were pulverized using a knife mill (Willye model, Marconi, Brazil, equipped with a stainless steel sieve of 1 mm mesh size). The powder was stored in amber glass containers at room temperature (~25 °C) in a desiccator containing silica gel until extraction.

The extracts were obtained by static maceration for 7 days, at room temperature (25 °C ± 2 °C), with occasional manual agitation (twice a day), using 70% (*v*/*v*) ethanol/ultrapure water as solvent, in the ratio of 10:1 (solvent/sample, *v*/*w*). The solvent was replaced every day, and the extraction process was repeated three times to ensure maximum metabolite recovery.

The ethanolic extracts (PMEEL - leaves, PMEES - stems, and PMEER - roots) were concentrated under reduced pressure using a rotary evaporator (Fisatom 801, Brazil, coupled to a Büchi V-100 vacuum pump, Switzerland) with a heating bath at 40 °C ± 2 °C. The extracts yields obtained were 43.30 g (leaves), 13.44 g (stems), and 9.66 g (roots). These extracts were resuspended in methanol/ water (7:3, *v*/*v*) and subjected to liquid-liquid partitioning using a stepwise gradient solvent system (*n*-hexane, dichloromethane, and ethyl acetate, all of analytical grade, Sigma-Aldrich, Brazil). The aqueous residue was lyophilized (model L108, Liobras, Brazil). The solvents from the partitions were evaporated under reduced pressure using a rotary evaporator, generating the following fractions: Leaves: PMHPL (*n*-hexane; 2.86 g), PMDPL (dichloromethane; 3.03 g), PMEPL (ethyl acetate; 0.06 g); Stems: PMHPS (n-hexane; 2.41 g), PMDPS (dichloromethane; 1.23 g), PMEPS (ethyl acetate; 0.64 g); Roots: PMHPR (n-hexane; 1.62 g), PMDPR (dichloromethane; 2.16 g), PMEPR (ethyl acetate; 0.15 g). The fractions were analyzed by GC-MS, and the data were processed using the GNPS online platform.

Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

The crude extracts (10 mg/mL) were subjected to exploratory analysis by GC-MS on an HP Agilent GC model 6890 - MS 5973 system, with electron impact ionization (70 eV) in positive mode. A volume of 1.0 µL of solution for each sample, solubilized in dichloromethane (1,000 ppm), was injected, with an injector temperature of 280 °C and in splitless mode. The column used was HP-5MS (30 m x 0.32 mm i.d. x 0.25 µm ,film thickness), and the analysis was performed with a temperature program from 100 °C to 310 °C, with a 5 °C/min increment. The carrier gas used was helium (~99.99%) at a constant flow rate of 1 mL/min, and mass scanning was conducted between *m*/*z* 40 and 600 Da. The mass spectra were analyzed by consulting the WILEY No. 7 and NIST version 12 and 62 libraries, as well as by comparison with data from specialized literature.

Processing of CG/MS data by molecular network

The GC-MS data obtained were converted to .CDF format using the OpenChrom program (version 1.3.0, Lablicate GmbH, Germany). The processed data were then exported and analyzed using the Global Natural Product Social Molecular Network (GNPS) platform (<https://gnps.ucsd.edu>) (Wang *et al*. 2016), applying the following parameters: a precursor ion mass tolerance of 20.000 Da and a fragment ion tolerance of 0.5 Da. Edges were filtered to retain only those with a cosine score > 0.75 and more than six shared peaks. The molecular networks created on GNPS were imported and visualized using Cytoscape software (Version 3.8.0, Institute of Systems Biology, USA). The full molecular network dataset is publicly available at GNPS <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=3d90807926bd44c6900fda60b1eeccd1>.

Mycobacterium tuberculosis *H37Rv growth*

The virulent standard strain of *M. tuberculosis* H37Rv (ATCC 27294) was cultured in Middlebrook 7H9 broth (BD Difco, USA), supplemented with 10% albumin, dextrose, catalase (ADC; BD BBL), and 0.05% Tween 80. The cultures were maintained at 37 °C in a CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) under biosafety level 3 containment conditions until reaching the exponential growth phase.

Growth inhibition assay against Mycobacterium tuberculosis *H37Rv*

The antimycobacterial activity of the samples was evaluated using the colorimetric tetrazolium salt assay (MTT reduction assay) in a 96-well microplate format, following standardized protocols for drug susceptibility testing against *M. tuberculosis* H37Rv (ATCC 27294). For the assay, a bacterial suspension at a final concentration of 1×10^6 colony-forming units per well (CFU/well) was prepared in Middlebrook 7H9 broth (BD Difco, USA), supplemented with 10% albumin-dextrose-catalase (ADC, BD BBL) and 0.05% Tween 80, and then plated in the presence of the test samples or rifampicin (positive control). Test samples were evaluated at final concentrations of 32, 64, and 128 µg/mL, and rifampicin was tested at 0.008, 0.04, 0.2, and 1 µg/mL.

The microplates were sealed with parafilm and incubated at 37 °C with 5% CO_2 for 5 days under biosafety level 3 containment conditions. Following incubation, 100 µL of a freshly prepared 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in sterile phosphate-buffered saline, PBS; pH 7.4) was added to each well, and the plate was further incubated for 3 hours at 37 °C to allow enzymatic reduction of MTT into formazan by metabolically active cells. To stop the reaction and solubilize the formazan crystals, 100 µL of lysis buffer (20% w/v sodium dodecyl sulfate (SDS)/50% dimethylformamide (DMF) in distilled water, pH 4.7) was added to each well, and the microplate was incubated overnight at room temperature in the dark.

Optical density (O.D.) was measured at 570 nm using a microplate spectrophotometer (Epoch 2, BioTek, USA). Rifampicin-treated wells served as positive controls (C^+) for antimycobacterial activity, while untreated bacilli (growth control) were used as negative controls (C^-).

The percentage of *M. tuberculosis* growth inhibition was calculated using the equation:

$$100 - (O.D._{sample} - O.D._{blank}) \times 100 \,/\, (O.D._{C-} - O.D._{blank})$$

Statistical analysis

GraphPad Prism 5.0 was used for statistical analysis. The data were expressed as the mean±SEM and was applied one-way analysis of variance (ANOVA) followed by Tukey's post-test. The p-values $^*p < 0.05$ and $^{***}p < 0.001$ were considered significant compared to the negative control (Mtb H37Rv). MIC_{50} values were calculated by non-linear regression analysis of log[concentration]/inhibition curves applying a sigmoidal dose-response variable slope curve fitting using the different percentage obtained for each corresponding concentration in triplicate.

Results

Chemical composition analysis of extracts and fractions of leaves, stems and roots

The GC-MS data from ethanolic extracts and fractions of leaves, stems, and roots were analyzed using the online GNPS platform to construct the molecular network. The resulting molecular network displayed 324 nodes and 59 clusters formed by at least two ions with similar fragmentation spectra, with a cosine similarity score of 0.75.

The analysis of the data, with the assistance of different libraries, including suggestions from the GNPS database, led to the annotation of 45 substances. Among the annotated compound classes were steroids, saturated fatty acids, saturated fatty acid esters, long-chain aldehydes, phenylpropanoids, sesquiterpenes, a distinct molecular family of vitamin E, and a specific molecular family of eupomatenoid-5 isomers.

Figure 1 presents the molecular network generated from GNPS, highlighting the 7 annotated molecular families, with one of them divided into subgroups according to their chemical classes. The annotated substances are listed in Table 1.

Activity against Mycobacterium tuberculosis *H37Rv*

The evaluation of the antimycobacterial activity of *P. multinodum* extracts and fractions resulted in minimum inhibitory concentration (MIC) values above 128 µg/mL, as shown in Table 2 and Figure 2.

Discussion

Chemical analysis of extracts and partitions of leaves, stems and roots

The molecular network analysis enabled the annotation of various compound classes in the extracts of *P. multinodum* leaves, stems, and roots. In a molecular family composed of 6 nodes, the following steroids were annotated: campesterol (36, MW 400.69), stigmasterol (37, MW 412.70), alpha-

spinasterol (38, MW 412.70), stigmasta-7,25-dien-3β-ol (39, MW 412.70), sitosterol (40, MW 414.72), and sitostenone (41, MW 412.10). These steroids were mainly detected in the ethanolic extracts and hexane partitions of stems and roots, except for stigmasta-7,25-dien-3β-ol (39), which was exclusively found in the roots of *P. multinodum*. Compounds 37, 38, and 39 were characterized by the presence of the base ion (*m*/*z* 55), and the fragmentation pattern of this family displayed similar fragments: *m*/*z* 145, *m*/*z* 81, *m*/*z* 55, and *m*/*z* 43. Compounds 36, 37, and 40 had previously been described in *P. solmsianum* C.DC. by Moreira *et al*. (2000).

The GNPS analysis generated a molecular family consisting of 80 nodes, with the GNPS database annotating 8 saturated fatty acid esters, 11 compounds corresponding to long-chain aldehydes, and 6 other compounds belonging to the fatty acids and derivatives class. The saturated fatty acid esters were represented by ethyl pentadecanoate (11, MW 270.46), ethyl hexadecanoate (16, MW 284.48), ethyl octadecanoate (23, MW 312.54), ethyl nonadecanoate (25, MW 326.57), ethyl arachidate (27, MW 340.59), ethyl docosanoate (31, MW 368.65), ethyl tetracosanoate (33, MW 396.70), and ethyl dotriacontanoate (43, MW 508.92). These compounds were detected in the extracts and hexane partitions of stems and roots, as well as in the hexane partition of leaves. In the MS fragmentation profile of these esters, the same fragmentation pattern was observed, in addition to the base ion (*m*/*z* 88) for all. To date, there are no reports of these compounds in the *Piper* genus.

The compounds annotated as long-chain aldehydes, decanal (10, MW 156.27), undecanal (12, MW 170.30), 14-methylpentadecan-1-ol (18, MW 242.45), tridecanal (19, MW 198.35), tetradecanal (24, MW 212.38), pentadecanal (26, MW 226.40), hexadecanal (29, MW 240.43), heptadecanal (32, MW 254.46), tricosanal (42, MW 338.62), tetracosanal (44, MW 352.65), and pentacosanal (45, MW 366.67) were predominantly found in the hexane partition of leaves. The MS spectra of these compounds were characterized by the presence of fragment ions *m*/*z* 82, *m*/*z* 69, *m*/*z* 57, *m*/*z* 55, *m*/*z* 43, and *m*/*z* 41. Compound 26 had been previously described as one of the main components identified in the essential oil of *Piper arborescens* Roxb. (Daniel *et al*. 2019).

The fatty acids and derivatives were identified as 9,12-hexadecadienoic acid (13, MW 252.40),

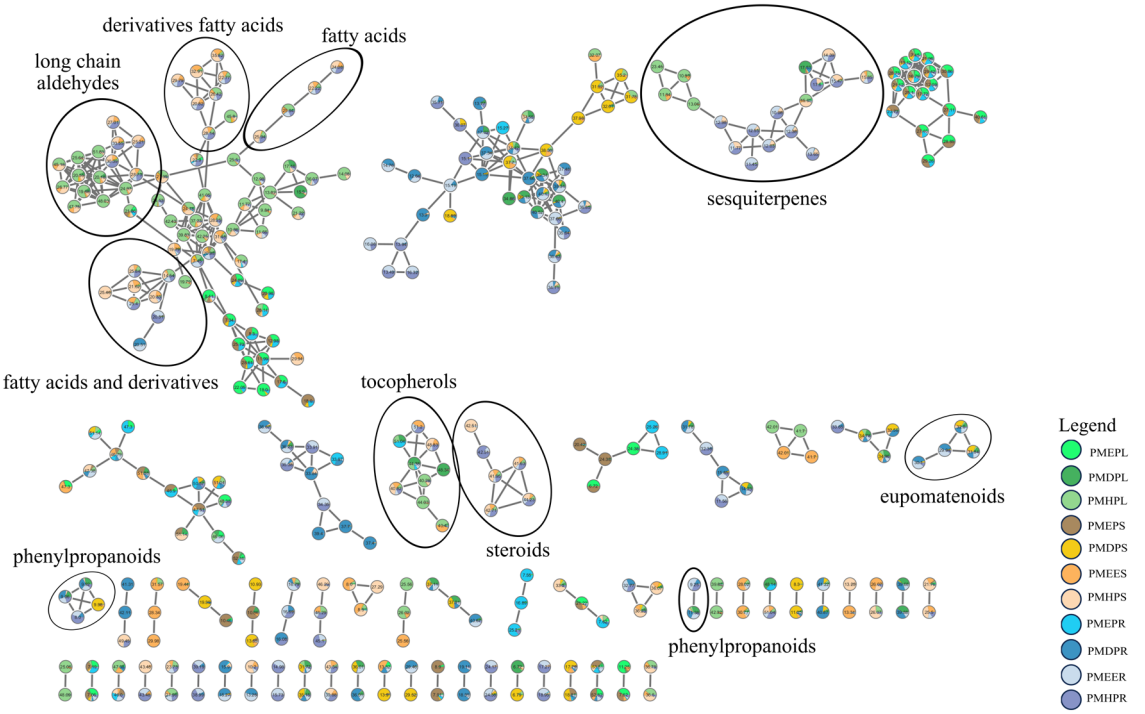

Figure 1 – Molecular network of extracts and partitions of leaves (PMHPL = hexane; PMDPL = dichloromethane; PMEPL = ethyl acetate); stems (PMEES = crude extract; PMHPS = hexane; PMDPS = dichloromethane; PMEPS = ethyl acetate), and roots (PMEER = crude extract; PMHPR = hexane; PMDPR = dichloromethane; PMEPR = ethyl acetate) of *Piper multinodum*. The seven annotated molecular families are highlighted. Node colors correspond to plant parts (leaves, stems, roots), pie charts indicate the relative abundance of each compound in the respective samples.

Table 1 – GC-MS analysis of the chemical composition of extracts and fractions from leaves, stems, and roots of *Piper multinodum* C.DC.

Compound	Molecular formula	Molecular mass	t_R (min)	Shared peaks	Fragment ions
safrole (1)	$C_{10}H_{10}O_2$	169.12	8.00	8	162; 135; 131; 104; 103; 77; 63; 51
isosafrole (2)	$C_{10}H_{10}O_2$	162.19	9.06	6	162; 131; 121; 103; 91; 63; 51
eugenol (3)	$C_{10}H_{12}O_2$	164.20	9.25	8	164; 149; 131; 103; 91; 77; 51; 55; 43
caryophyllene (4)	$C_{15}H_{24}$	204.36	10.08	7	189; 147; 133; 121; 107; 93; 81; 67; 55; 41
acetyleugenol (5)	$C_{12}H_{14}O_3$	206.24	11.38	9	164; 149; 133; 131; 121; 103; 91; 77; 55
aromadendrene (6)	$C_{15}H_{24}$	204.36	11.77	9	204; 161; 147; 133; 119; 105; 91; 79; 41
α-selinene (7)	$C_{15}H_{24}$	204.36	12.38	7	204; 147; 133; 121; 107; 93; 81; 67; 41
δ-cadinene (8)	$C_{15}H2_4$	204.36	12.98	10	204; 161; 134; 119; 105; 91; 81; 55; 41
elemol (9)	$C_{15}H_{26}O$	222.37	13.65	9	204; 189; 161; 135; 121; 107; 93; 59; 43
decanal (10)	$C_{10}H_{20}O$	156.27	19.68	6	123; 109; 95; 82; 81; 69; 68; 57; 43; 41
ethyl pentadecanoate (11)	$C_{17}H_{34}O_2$	270.46	20.82	11	270; 157; 101; 88; 73; 57; 55; 43; 41
undecanal (12)	$C_{11}H_{22}O$	170.30	21.35	6	111; 97; 85; 84; 71; 69; 57; 55; 43; 41
9,12-hexadecadienoic acid (13)	$C_{16}H_{28}O_2$	252.40	21.62	6	149; 135; 121; 109; 95; 81; 67; 55; 41
oleic acid (14)	$C_{18}H_{34}O_2$	282.47	21.76	6	236; 111; 97; 83; 73; 69; 55; 43; 41
palmitic acid (15)	$C_{16}H_{32}O_2$	256.43	22.22	14	256; 213; 129; 73; 60; 57; 55; 43; 41
ethyl hexadecanoate (16)	$C_{18}H_{36}O_2$	284.48	22.77	13	284; 157; 101; 88; 73; 57; 55; 43; 41
heptadecanoic acid (17)	$C_{17}H_{34}O_2$	270.46	24.08	12	214; 185; 129; 73; 60; 57; 55; 43; 41
14-methylpentadecan-1-ol (18)	$C_{16}H_{34}O$	242.45	24.53	7	125; 111; 97; 83; 69; 57; 55; 43; 41
tridecanal (19)	$C_{13}H_{26}O$	198.35	25.21	6	137; 123; 109; 96; 82; 67; 57; 55; 43; 41
linoleic acid (20)	$C1_8H_{32}O_2$	280.45	25.46	8	281; 109; 95; 81; 68; 55; 41
ethyl linoleate (21)	$C_{20}H_{36}O_2$	308.51	25.84	12	263; 109; 95; 81; 67; 55; 41
stearic acid (22)	$C_{18}H_{36}O_2$	284.48	25.94	8	185; 129; 95; 81; 73; 71; 60; 57; 43; 41
ethyl octadecanoate (23)	$C_{20}H_{40}O_2$	312.54	26.42	11	255; 157; 101; 88; 73; 57; 55; 43;41
tetradecanal (24)	$C_{14}H_{28}O$	212.38	27.01	6	109; 96; 95; 83; 82; 67; 57; 55; 43
ethyl nonadecanoate (25)	$C_{21}H_{42}O_2$	326.57	28.14	6	157; 101; 88; 69; 57;55
pentadecanal (26)	$C_{15}H_{30}O$	226.40	28.73	7	123; 96; 82; 71; 57; 55; 43;41
ethyl arachidate (27)	$C_{22}H_{44}O_2$	340.59	29.79	7	157; 143; 101; 89; 88; 69; 57; 43; 41
eupomatenoid-5 isomer (28)	$C_{19}H_{18}O_3$	294.35	29.94	6	294; 279; 135; 129; 115; 91; 77; 51
hexadecanal (29)	$C_{16}H_{32}O_2$	240.43	30.39	7	138; 123; 111; 96; 82; 69; 57; 43; 41
eupomatenoid-5 (30)	$C_{19}H_{18}O_3$	294.35	31.84	6	294; 279; 135; 128; 115; 91; 77
ethyl docosanoate (31)	$C_{24}H_{48}O_2$	368.65	32.91	10	213; 157; 101; 88; 73; 55; 43; 41
heptadecanal (32)	$C_{17}H_{34}O$	254.46	33.55	7	137; 124; 111; 96; 83; 82; 69; 57; 43
ethyl tetracosanoate (33)	$C_{26}H_{52}O_2$	396.70	35.82	18	396; 157; 101; 89; 88; 57; 55; 43;41
tocopherol (34)	$C_{29}H_{50}O_2$	430.72	40.28	12	430; 205; 165; 121
α-tocopheryl acetate (35)	$C_{31}H_{52}O_3$	472.75	40.82	13	472; 430; 205; 165; 55; 43

Compound	Molecular formula	Molecular mass	t_R (min)	Shared peaks	Fragment ions
campesterol (36)	$C_{28}H_{48}O$	400.69	41.63	16	400; 382; 315; 289; 213; 145; 107; 55; 43
stigmasterol (37)	$C_{29}H_{48}O$	412.70	41.95	20	412; 351; 300; 271; 255; 159; 133; 83;55
α-epinasterol (38)	$C_{29}H_{48}O$	412.70	42.51	14	412; 271; 213; 159; 145; 97, 81, 67,55
stigmasta-7,25-dien-3β-ol (39)	$C_{29}H_{48}O$	412.70	42.51	9	255; 213; 173; 159; 145;119; 105; 81; 55
sitosterol (40)	$C_{29}H_{50}O$	414.72	42.71	20	414; 329; 303; 213; 145; 107; 81; 55; 43
sitostenone (41)	$C_{29}H_{48}O$	412.10	44.23	11	327; 299; 271; 229; 147; 124; 95; 55; 43
tricosanal (42)	$C_{23}H_{46}O$	338.62	45.18	6	123; 109; 95; 82; 69; 57; 43
ethyl dotriacontanoate (43)	$C_{34}H_{68}O_2$	509.92	45.8	6	157; 101; 97; 88; 83; 69; 57
tetracosanal (44)	$C_{24}H_{48}O$	352.65	48.03	6	123; 109; 95; 82; 69; 57; 43; 41
pentacosanal (45)	$C_{25}H_{50}O$	366.67	51.83	6	123; 109; 95; 83; 69; 57; 55; 43; 41

hexadecanoic acid (15, MW 256.43), heptadecanoic acid (17, MW 270.46), linoleic acid (20, MW 280.45), ethyl linoleate (21, MW 308.51), and octadecanoic acid (22, MW 284.48). These compounds were found in the ethanolic extracts of stems and roots and the hexane partitions of all plant organs. The MS spectra of the annotated fatty acid derivatives showed that compounds 13 and 21 shared the same base ion (*m*/*z* 67), while compounds 15, 17, and 21 presented *m*/*z* 73 as the base ion, in addition to a characteristic fragmentation pattern involving CH_2 cleavages (-14 u.m.a). Compound 20 had been previously reported in *P. aduncum* by Luna *et al*. (2024). It is worth noting that fatty acid derivatives are common in species of the *Piper* genus (Péres *et al*. 2006; Chibuzo *et al*. 2022).

Oleic acid (14, MW 282.47) was identified by the GNPS library in a separate cluster of two nodes, predominantly detected in the hexane partition of stems. The main fragment recorded in the MS spectrum was the base ion *m*/*z* 55. Compound 14 had been previously described in *P. colubrinum* Link by Sruthi *et al*. (2024).

The sesquiterpene molecular family consisted of 18 nodes, with 5 compounds annotated as caryophyllene (4, MW 204.36), aromadendrene (6, MW 204.36), alpha-selinene (7, MW 204.36), delta-cadinene (8, MW 204.36), and elemol (9, MW 222.37). These compounds were predominantly found in the ethanolic extract of roots and the hexane partitions of stems and roots. The MS fragmentation profile of these sesquiterpenes exhibited several similar fragments: *m*/*z* 133, *m*/*z* 121, *m*/*z* 107, *m*/*z* 93, *m*/*z* 81, *m*/*z* 55, and *m*/*z* 41, suggesting the formation of the molecular family. It is important to highlight that sesquiterpenes are commonly found in the essential oils of *Piper* species (Da Silva *et al*. 2017; Yousuf *et al*. 2022).

The phenylpropanoids were identified as safrole (1, MW 162.19) and isosafrole (2, MW 162.19) in a group containing four nodes. Eugenol (3, MW 164.20) and acetyl eugenol (5, MW 206.24) were assigned to another molecular family with three nodes. These phenylpropanoids were detected in the ethanolic extract of roots, as well as in the hexane and dichloromethane partitions of leaves and roots, except for safrole (1), which was exclusively found in roots. The MS fragmentation spectra of these compounds exhibited the same fragmentation pattern, with base ions at *m*/*z* 162 for compounds 1 and 2 and at *m*/*z* 164 for compounds 3 and 5. Phenylpropanoids are commonly found in Piperaceae species (Parmar *et al*. 1997).

GNPS analysis also revealed a specific molecular family of neolignan eupomatenoid-5 isomers, comprising four nodes. Eupomatenoid-5 isomer (28, MW 294.35) and eupomatenoid-5 (30, MW 294.35) were detected in the hexane and dichloromethane partitions of all plant organs. The primary fragment recorded in the MS spectra of this family was the base ion *m*/*z* 294. Eupomatenoid-5 had been previously described and isolated in *Piper rivinoides* Kunth (Felisberto *et al*. 2021; Ramos *et al*. 2024).

In another molecular family consisting of 9 nodes, 2 compounds were annotated: tocopherol (34, MW 430.72), predominantly found in the hexane partition of leaves, and α-tocopheryl acetate (35, MW 475.75), detected in the ethanolic extracts of stems and roots and the hexane partitions of all plant organs. The fragmentation profile of these compounds showed similarities in the fragments *m*/*z* 430, *m*/*z* 205, and *m*/*z* 165. Compound 34 had been previously reported in *Piper* L. (Rintu *et al*. 2015); however, this is the first report of α-tocopheryl acetate (35) in this genus.

Table 2 – Inhibitory effect of the extracts and partitions of *Piper multinodum* C.DC. against *Mycobacterium tuberculosis* H37RV. (PMEEL = ethanolic extract from leaves; PMHPL = hexane partition from leave ethanolic extract; PMDPL = dichloromethane partition from leaves ethanolic extract; PMEPL = ethyl acetate partition from leaves ethanolic extract).

Sample	H37Rv MIC_{50} (μg/mL)
PMEEL	890.4 ± 2.17
PMHPL	538.0 ± 2.08
PMDPL	400.7 ± 1.26
PMEPL	468.5 ± 3.85

Activity against Mycobacterium tuberculosis *H37Rv*

The results found for *P. multinodum* are relevant, with MIC values recorded above 128 μg/mL. It has been described that MIC values between 100 and 200 μg/mL classify an extract, partition, or isolated compound as a moderate candidate against *M. tuberculosis* (Bernuci *et al*. 2016).

Several biological studies have documented the antimycobacterial efficacy of essential oils derived from different *Piper* species, showing moderate to good activity against *M. tuberculosis* (Fernandez *et al*. 2019; Boren *et al*. 2020). Specifically, essential oils from infructescences and inflorescences of *Piper lhotzkyanum* Kunth exhibited minimum inhibitory concentrations (MICs) of 76 μg/mL and 128 μg/mL, respectively (Costa-Oliveira *et al*. 2021). Similarly, leaf essential oils from *P. cernuum* Vell., *P. diospyrifolium* Kunth, and *P. rivinoides* Kunth demonstrated MIC values of 125 μg/mL, while *P. ovatum* Vahl reported an MIC of 250 μg/mL (Bernuci *et al*. 2016). Further research indicated that root and infructescence essential oils from *P. multinodum* exhibited MICs of 78.51 μg/mL and 85.91 μg/mL, respectively (Ramos *et al*. 2021). The antimicrobial potential extends beyond essential oils to extracts, fractions, and isolated compounds. The methanolic extract of *P. guineense* Schumach. and Thonn. seeds exhibited an MIC of 256 μg/mL (Tekwu *et al*. 2012), while the ethyl acetate fraction from the methanolic extract of *P. sarmentosum* Robx. leaves showed an MIC of 3.12 μg/mL (Hussain *et al*. 2009).

Eupomatenoid-5 (30), identified in the dichloromethane partition (PMDPL), which exhibited the best antimycobacterial activity (MIC 400.7), was one of the three neolignans isolated from the leaf

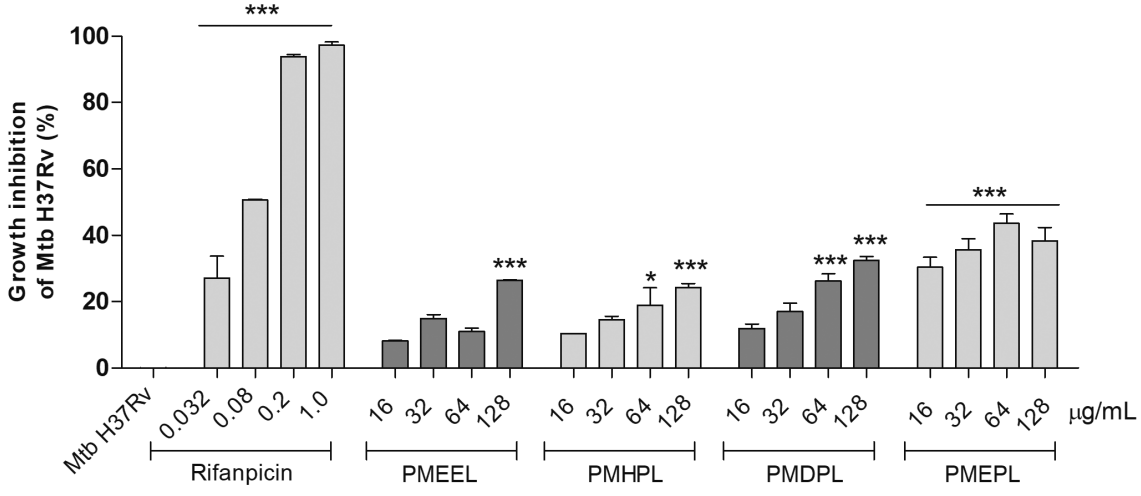

Figure 2 – Inhibition of *Mycobacterium tuberculosis* H37Rv growth after treatment with extracts and partitions of *Piper multinodum* MTT assay performed after 5 days of incubation with samples at concentrations of 32, 64, and 128 μg/mL. Positive control: *M. tuberculosis* H37Rv (1 × 106 CFU/mL) treated with rifampicin (reference drug), and negative control *M. tuberculosis* H37Rv untreated (1 × 106 CFU/mL). Statistical analysis: one-way ANOVA followed by the Tukey test. ***$p < 0.001$ and $p < 0.05$ compared to negative control. Triplicate results are represented as mean ± standard error. RIF = Rifampicin; PMEEL = Ethanolic extract from leaves; PMHPL = Hexane partition from leaves ethanolic extract; PMDPL = Dichloromethane partition from leaves ethanolic extract; PMEPL = Ethyl acetate partition from leaves ethanolic extract.

extract of *P. regnellii* (Miq.) C.DC. by Scodro *et al*. (2013). Eupomatenoid-5 was the most active against *M. tuberculosis* H37Rv, with a MIC of 1.9 μg/mL, thus suggesting its potential as a candidate for future anti-TB pharmacotherapy. Eugenol (3) was detected in the ethanolic extract of roots, as well as in the hexane and dichloromethane partitions of leaves and roots. Eugenol is known to be one of the substances commonly found in plants' essential oils. It has been investigated for its anti-*Mycobacterium tuberculosis* activity, showing a MIC of 2.5 (Raj *et al*. 2022).

These studies highlight the antimycobacterial potential of *Piper* species. However, further *in vivo* studies and deeper investigations into their mechanisms of action are necessary. The active compounds identified in these species appear to be directly related to the observed activity, making them promising candidates for the development of new anti-tuberculosis drugs.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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