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Anti-leishmanial activity and dereplication using GC-MS assisted by molecular networking of the essential oil from the leaves and inflorescence of *Melaleuca viminalis* (myrtaceae)

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Introduction: The urgent need for new antileishmanial agents is driven by the toxicity and emerging resistance associated with current therapies. Essential oils from *Melaleuca viminalis* (Myrtaceae) are rich in bioactive monoterpenes; however, integrated chemical annotation strategies combining advanced computational tools with biological evaluation remain limited. This study combined gas chromatography-mass spectrometry with molecular network-assisted dereplication to accelerate compound annotation and correlate chemical composition with antileishmanial activity against *Leishmania amazonensis*.

Methods: Essential oils were obtained by hydrodistillation from leaves (yield 0.54%) and inflorescences (yield 0.41%). Volatile constituents were analyzed by GC-MS and subjected to dereplication using the GNPS platform, allowing spectral matching and visualization of structural relationships. Antileishmanial activity was evaluated against the promastigote and intracellular amastigote forms of *L. amazonensis*, and cytotoxicity was evaluated in bone marrow-derived macrophages to determine selectivity indices.

Results and discussion: The molecular networking allowed the annotation of 86.92% and 90.71% of the constituents detected in the oils of the leaves and inflorescences, respectively, revealing a chemical profile dominated by monoterpenes. The main compound, 1,8-cineole (47.53%–50.53%), was grouped with other oxygenated monoterpenes. The leaf oil had effective concentrations of 27.78 µg/mL (promastigotes) and 6.80 µg/mL (amastigotes), while the inflorescence oil had 44.67 and 12.05 µg/mL. The cytotoxic concentrations were 36.55 and 73.72 µg/mL, producing selectivity indices of

5.37 and 6.12. Notably, 1,8-cineole exhibited an effective concentration of 5.58 μg/mL against amastigotes and a selectivity index ≥17.92, surpassing miltefosine.

KEYWORDS

essential oil, leaves, inflorescences, dereplication, molecular networking, antileishmanial activity

1 Introduction

Melaleuca viminalis (Sol. and Gaertn.) Byrnes (MV), a species belonging to the Myrtaceae family, is popularly known as "bottlebrush" due to the characteristic morphology of its flowers (Martins et al., 2020). Formerly known as *Callistemon viminalis* (Sol. ex Gaertn.) G. Don., this plant underwent a botanical reclassification based on genetic and morphological studies, which resulted in all species from the genus *Callistemon* being officially transferred to the genus *Melaleuca* (Craven, 2006; Royal Botanic GardensKew, 2025). Phytochemical studies conducted on MV extracts have demonstrated that the plant is rich in various classes of secondary metabolites, including triterpenoids, flavonoids, saponins, steroids, alkaloids, tannins, and amino acids (Abdelmalek et al., 2021; Celaj et al., 2021). Furthermore, polyphenolic compounds isolated from the aerial parts of this plant exhibit antioxidant and anticancer activities (Mahgoub et al., 2021). Although plant extracts have been widely studied, there is growing interest in the essential oil (EO) obtained from MV. Evidence indicates that the EO extracted from its leaves is predominantly composed of 1,8-cineole (eucalyptol), along with other monoterpenes such as α-pinene, α-terpineol, and *p*-cymene. However, the chemical composition of the oil varies across different geographic regions, which also reflects variations in its associated biological activities, including antioxidant, antibacterial, and fungicidal properties (Martins et al., 2020; Salem et al., 2021; Sharma et al., 2021). These findings highlight its potential to be further investigated as a therapeutic agent for other diseases.

Leishmaniasis is one of the most prevalent neglected diseases, affecting more than 12 million people worldwide. This disease is closely associated with poverty, poor sanitation, inadequate housing, poor nutrition, and other socioeconomic factors. The drugs currently available for the treatment of leishmaniasis, especially glucantime and amphotericin B, have been in during decades, with increasing reports of parasitic resistance and toxicity for humans (Chavy et al., 2019; World Health Organization, 2022). Despite advances in the study of bioactive substances of natural origin, challenges related to efficacy, toxicity, and the complex mechanisms of parasite-host interactions continue to hinder the development of cost- effective therapies.

Considering the rich biological diversity of MV, this study aimed to evaluate the antileishmanial and cytotoxic activities of the essential oil (EO) extracted from its leaves and inflorescences on extra and intracellular forms of *Leishmania amazonensis*. To obtain a detailed and comprehensive identification of the chemical composition of the EO, gas chromatography coupled with mass spectrometry (GC-MS) was employed, combined with a molecular networking approach using the Global Natural Products Social Molecular Networking (GNPS) platform. A search of the literature revealed a low number of studies using this tool, however, there has been growing interest in recent years, reinforcing its relevance in annotating compounds that have already been described in the literature more quickly.

2 Results

Hydrodistillation of leaves (MV-FOE) and inflorescences (MV-IOE) of MV yielded essential oil contents of 0.54% and 0.41%, respectively. The chemical composition of the essential oils (MV-FOE and MV-IOE) was analyzed by GC–MS, and compound annotation was performed by dereplication using the GNPS platform. This approach enabled the identification of 86.92% of the constituents in MV-FOE and 90.71% in MV-IOE (Table 1). GNPS compared the acquired spectra against extensive libraries (Fiehn, HMDB, MoNA, VocBinBase, NIST, 2017, and Wiley), comprising more than 19,000 reference spectra (Aksenov et al., 2021).

Molecular networking analysis allowed visualization of node sizes according to total ion intensity (Figure 1), in which 1,8-cineole appeared as the largest node, indicating that it is the major constituent of both oils. The chemical profiles of MV-FOE and MV-IOE revealed a predominance of monoterpenes, particularly 1,8-cineole (eucalyptol) at 47.53% and 50.53%, α-pinene at 14.57% and 21.04%, β-pinene at 1.98% and 0.65%, α-terpineol at 12.15% and 13.12%, and linalool at 1.32% and 1.64% (Table 1). The MV- IOE molecular networking displayed a single cluster predominantly composed of monoterpenes, but also containing two sesquiterpenes, spathulenol and α-cubebene.

Regarding biological activity against *L. amazonensis* (Table 2), MV-FOE and MV-IOE exhibited EC_{50} values of 27.78 μg/mL and 44.67 μg/mL against promastigotes, respectively, which, although indicative of bioactivity, were considerably less potent than the reference drug Miltefosine (EC_{50} = 8.26 μg/mL). Against intracellular amastigotes, MV-IOE exhibited an EC_{50} value of 12.05 μg/mL, approaching that of miltefosine (7.60 μg/mL), while MV-FOE and its main isolated constituent, 1,8-cineole, demonstrated EC_{50} values of 6.80 and 5.58 μg/mL, respectively. The isolated constituent, in addition to outperforming the standard drug, exhibited a favorable safety profile (CC_{50} > 100 μg/mL; SI ≥ 17.92).

3 Discussion

The yields obtained are consistent with those reported in the literature, where values range from 0.28% to 0.90% (Khubeiz and Mansour, 2016; Oyedeji et al., 2009; Silva et al., 2010). The lower yield observed for inflorescences compared to leaves was expected, as previously reported by Pires et al. (2013), who described that essential oils obtained from flowers or inflorescences generally

TABLE 1 Compounds identified by GNPS in the essential oil of *Melaleuca viminalis* leaves and inflorescences.

Compound	MV-FOE (peak area %)	Cosine score	MV-IOE (peak area %)	Cosine score
α-pinene	14.57	0.93	21.04	0.92
β-pinene	1.98	0.88	0.65	0.88
Myrcene	0.83	0.91	-	-
α-phellandrene	2.77	0.90	0.17	0.90
p-cymene	3.84	0.84	-	-
1,8-Cineole	47.53	0.79	50.53	0.81
γ-terpinene	1.24	0.84	-	-
Terpinolene	0.41	0.88	-	-
Linalol	1.32	0.96	1.64	0.98
Terpinen-4-ol	0.28	0.84	-	-
α-terpineol	12.15	0.95	13.12	0.92
Spathulenol	-	-	2.76	0.83
α-cubebene	-	-	0.80	0.84

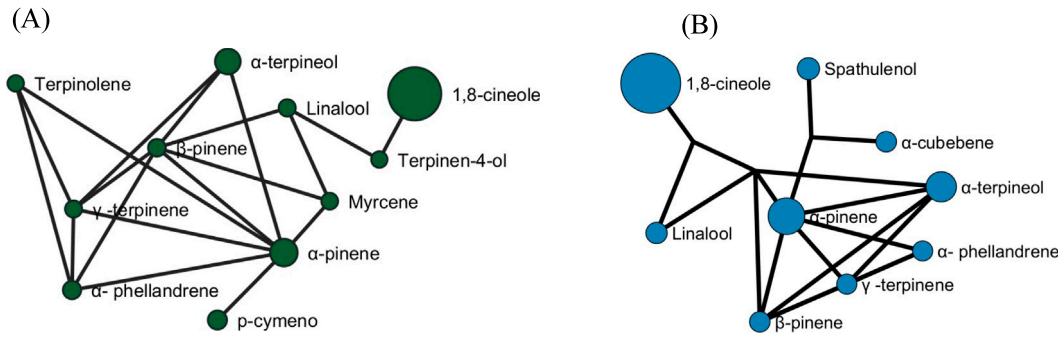


FIGURE 1
Molecular networking for the compounds identified in the essential oil of *Melaleuca viminalis* leaves **(A)** and inflorescences **(B)**.

present lower yields due to anatomical and functional differences between these plant organs.

The clustering of compounds in the molecular networking is attributed to their chemical and biosynthetic similarities. All monoterpenes annotated for MV-FOE from MV are derived from the methylerythritol phosphate (MEP) pathway, which converges to the formation of geranyl pyrophosphate (GPP). This precursor gives rise to the biosynthesis of cyclic and acyclic monoterpenes, such as α-pinene, β-pinene, myrcene, terpinolene, γ-terpinene, and α-phellandrene, which share similar structural features and characteristic fragmentation patterns (Dewick, 2009; Kumar and Bhattarai, 2024). The main fragments observed include *m/z* 136 $[M]^{\cdot+}$ (typical molecular ion of hydrocarbon monoterpenes), *m/z* 121 (associated with CH_3 loss), and *m/z*93 (resulting from cationic rearrangement) (Supplementary Figure S1).

The compounds 1,8-cineole, terpinen-4-ol, and linalool possess oxygenated structures and cyclic rearrangements that differ from those of hydrocarbon monoterpenes, which explains their fewer connections in the molecular networking. Their fragmentation patterns include *m/z* 154 $[M]^{\cdot+}$ (typical molecular ion of oxygenated monoterpenes), *m/z* 136 (associated with H_2O loss, observed for linalool and terpinen–4–ol), and *m/z*81 (characteristic of the cleavage of the oxygenated ring of 1,8-cineole). The observed connection between 1,8-cineole and terpinen-4-ol is supported by the biosynthetic pathway, in which terpinen-4-ol acts as a precursor of 1,8-cineole (Loza-Tavera, 1999; Croteau et al., 1994; Dewick, 2009).

The presence of sesquiterpenes in MV-IOE from MV has previously been reported by Sales et al. (2017), who described a similar compositional variation between flowers and leaves. These compounds appear more distant in the network but remain connected to α-pinene, reflecting similarities in their fragmentation profiles, although they are biosynthetically derived from farnesyl pyrophosphate (FPP) rather than directly from geranyl pyrophosphate (GPP). The formation of these sesquiterpenes involves carbocation-mediated cyclization, a mechanism analogous to that of bicyclic monoterpenes, generating common fragments such as *m/z* 93 and *m/z*

TABLE 2 Effective concentration 50% of compounds against intracellular amastigotes of *L. amazonensis* and promastigotes, cytotoxicity in macrophages.

Compound	EC_{50} in promastigotes (μg/mL)[a]	EC_{50} in amastigotes (μg/mL)[a]	CC_{50} (μg/mL)[b]	Selectivity index
	Mean ± standard deviation	Mean ± standard deviation	Mean ± standard deviation	
MV-FOE	27.78 ± 4.82	6.80 ± 2.86	36.55 ± 7.09	5.37
MV-IOE	44.67 ± 5.74	12.05 ± 2.86	73.72 ± 4.26	6.12
α-pinene	-	4.41 ± 0.20	31.67 ± 3.79	7.18
β-pinene	-	5.30 ± 0.43	34.35 ± 3.09	6.49
α-terpineol	-	6.56 ± 0.12	38.72 ± 2.49	5.91
Linalool	-	3.82 ± 0.41	>100	≥26.18
1,8-Cineole	-	5.58 ± 0.45	>100	≥17.92
Miltefosine[c]	8.26 ± 2.70	7.60 ± 0.90	43.12 ± 7.30	5.67

[a]Values represent effective concentration 50% in μg/mL.
[b]Cytotoxic concentration 50% in μg/mL.
[c]Miltefosine: standard drug.

121 through C–C bond cleavage, a process shared by both sesquiterpenes and certain monoterpenes (Zhang et al., 2018; Könen and Wüst, 2019).

Overall, the differences in chemical composition observed between the molecular networking (Figure 1) of MV-FOE and MV-IOE may reflect the distinct biological roles of these tissues, resulting in qualitative and quantitative variations in metabolite composition (Table 1). Such differences may contribute to the distinct biological activities observed between the essential oils. It is well established that flowers, as components of the inflorescence, are evolutionarily derived from modified leaves specialized for reproductive functions. This functional divergence can directly influence the biosynthesis and accumulation of secondary metabolites (Pichersky and Gershenzon, 2002; Wink, 2003; Dudareva et al., 2006). While leaves are primarily associated with defense mechanisms against herbivores and environmental stress, flowers are more closely related to reproductive success, including the attraction of pollinators and the protection of reproductive structures.

The obtained data demonstrate that the GNPS platform is an efficient tool for the rapid and reliable annotation of compounds in essential oils, enabling the structured visualization of clusters based on similarities in fragmentation patterns and biosynthetic pathways. Previous studies have highlighted its application in this context. Ibrahim et al. (2024) employed GNPS to support the characterization of essential oils from lemon, orange, and tangerine peels, demonstrating its effectiveness in annotating and organizing volatile constituents. Hegazi et al. (2022) used the platform to investigate discrepancies among the constituents of five *Melaleuca* species through molecular networking, allowing visualization and comparison of the annotated volatiles in the chemical composition of each oil. Furthermore, Pilon et al. (2022) demonstrated that molecular networking can be applied as a tool to integrate biological data, providing a clearer interpretation of the results.

The predominance of the oxygenated monoterpene 1,8-cineole (47.53%), also known as eucalyptol, corroborates previous reports in which its concentration ranged from 39.4% to 83.2%. Such variation is largely influenced by the geographic origin of the plant material, which directly affects both the qualitative and quantitative composition of the essential oil (Ahmad and Athar, 2017; Aweke and Yeshanew, 2016; Gohar et al., 2013; Ndomo et al., 2010; Prins et al., 2010; Silva et al., 2010; Vishwakarma et al., 2017). Previous studies on the essential oil of MV have demonstrated considerable compositional variability, both in the number of constituents and in their relative proportions, highlighting the influence of biological and environmental factors. Martins et al. (2020) reported a simpler profile for the essential oil from leaves, with approximately 12 identified compounds and a predominance of oxygenated monoterpenes, particularly 1,8-cineole as the major constituent. In contrast, Fall et al. (2017) described a more complex oil, with more than 30 components, including monoterpenes and sesquiterpenes distributed across different chemical classes and proportions. In the present study, marked differences were observed between MV-FOE and MV-IOE, both in terms of metabolite diversity and relative abundance as revealed by molecular networking. The MV-IOE profile, characterized by lower chemical diversity and a more restricted distribution of metabolites, shows greater similarity to the pattern described by Martins et al. (2020), whereas MV-FOE exhibits higher chemical complexity, approaching the more diverse profile reported by Fall et al. (2017). Although 1,8-cineole was also identified as one of the major constituents in both essential oils analyzed in this study, differences in the relative proportions of other compounds, particularly minor and intermediate constituents, suggest a relevant role in modulating the observed biological activity.

The essential oil and its main pure compounds were tested on the promastigote and amastigote stages of Leishmania. Promastigotes are the extracellular form of the parasite found in the sandfly vector, whereas amastigotes are found intracellularly in vertebrate hosts. Although amastigotes are significant in inducing pathological changes in the vertebrate host (Carvalho et al., 2012; Passero et al., 2010), promastigotes are among the most commonly

assayed forms in biomonitored studies. This is because promastigotes can be easily manipulated *in vitro*, grow rapidly in culture media, enabling to perform assays that are less expensive and time-consuming than experiments involving intracellular amastigotes (Passero et al., 2009). Furthermore, some compounds active against extracellular forms may also act on amastigotes (De Jesus et al., 2021), likely due to physiological similarities between the two parasite stages. Thus, it is a common practice to analyze the effects of compounds on both promastigote and amastigote forms. Regarding the biological assay, the EC_{50} values obtained against the promastigote form, when compared with other Myrtaceae species such as Myrciaria plinioides (IC_{50} = 14.16 μg/mL) (Kauffmann et al., 2019) and Eugenia uniflora (IC_{50} = 6.96 μg/mL) (Rodrigues et al., 2013), indicate that the essential oils of MV are less active. These findings suggest that not all essential oils within this botanical family exhibit equivalent potency. This observation reinforces the notion that chemical composition, as well as synergistic or antagonistic interactions among constituents, may strongly influence the overall biological activity of complex mixtures such as essential oils.

In contrast, against the intracellular amastigote form the clinically most relevant stage of the parasite the results highlighted the therapeutic potential of isolated monoterpenes, even when the crude oils did not reproduce comparable potency. Notably, 1,8-cineole exhibited EC_{50} values superior to those of the reference drug Miltefosine. The reduced efficacy of the whole essential oils compared to their active constituents may be attributed to the presence of less active or even antagonistic compounds within the mixtures, a phenomenon that has also been reported in other studies involving essential oils (Hamdeni et al., 2026; Mohammed et al., 2026).

The leishmanicidal activity of monoterpenes has been increasingly recognized. For instance, 1,8-cineole, α-pinene, and p-cymene demonstrated activity against amastigotes of *Leishmania amazonensis*, with IC_{50} values of 48.4, 37, and 46 μg/mL, respectively (Santana et al., 2020). Although the activity of 1,8-cineole observed in the present study was superior to previously reported values, variability among results may be attributed to differences in experimental conditions, parasite strains, or the purity of the tested compounds. Furthermore, α-pinene, which is also abundant in the essential oils of MV, has been reported as active against *L. amazonensis* when isolated from *Syzygium cumini*, with IC_{50} values of 19.7 μg/mL (promastigotes) and 16.1 μg/mL (amastigotes) (Da Franca Rodrigues et al., 2015). These findings suggest that monoterpenes commonly occurring in the Myrtaceae family possess a conserved leishmanicidal potential, reinforcing their role as bioactive markers within this botanical group.

Although 1,8-cineole is present in similar proportions in both essential oils, this compound alone does not appear to be primarily responsible for the observed differences in biological activity. Thus, although isolated compounds such as α-pinene have reported activity against amastigotes, the activity of essential oils should be interpreted as the result of the combined effects of their constituents, involving synergistic and antagonistic interactions that cannot be predicted based on individual compounds. Variations in the relative abundance of other constituents, including minor compounds, may exert a significant influence on the overall activity, either through direct effects or by modulating the activity of major components. In this context, the higher EC_{50} observed for MV-IOE may be associated with quantitative differences in chemical composition or the presence of metabolites with potential antagonistic effects, which may reduce the overall efficacy of the oil.

In addition to antiparasitic activity, the cytotoxicity of the essential oils was also evaluated based on CC_{50} values in macrophages. MV-IOE exhibited a higher CC_{50} value (73.72 ± 4.26 μg/mL) compared to MV-FOE (36.55 ± 7.09 μg/mL), indicating lower cytotoxicity. When analyzed in conjunction with EC_{50} values, these results suggest that, although MV-IOE shows lower antiparasitic potency, it presents a more favorable safety profile. This relationship is reflected in the selectivity index, which simultaneously considers efficacy and cytotoxicity. Therefore, the combined evaluation of EC_{50} and CC_{50} is essential for a more robust interpretation of the biological potential of essential oils, avoiding conclusions based solely on antiparasitic activity.

Overall, the results indicate that although the essential oils of MV are less potent than some previously studied Myrtaceae oils, their major constituents particularly 1,8-cineole exhibit strong and selective activity. This highlights the importance of prioritizing the investigation of isolated compounds or enriched fractions rather than whole essential oils in the search for novel therapeutic agents. Future studies should focus on exploring the pharmacological potential of these monoterpenes, assessing possible synergistic combinations, and elucidating their mechanisms of action to support the rational development of more effective antileishmanial therapies, in addition to conducting *in vivo* tests.

Therefore, the predominance of monoterpenes in the chemical composition of the essential oils of MV can be directly associated with their antiparasitic activity against *Leishmania amazonensis*. Potential synergistic interactions among these compounds may further contribute to the overall antileishmanial effect. Moreover, the integration of dereplication tools such as GNPS with GC–MS analyses enable the rapid and reliable identification of bioactive metabolites. This strategy is particularly valuable for advancing the discovery of novel therapeutic options for leishmaniasis, especially in light of the increasing resistance to conventional treatments.

4 Conclusion

The application of dereplication techniques using GC-MS combined with GNPS proved to be an efficient approach for compound annotation, allowing researchers to identify the chemical composition of essential oils in a faster and more streamlined manner, particularly considering the large number of compounds typically present in these matrices. The results indicate that the differences in biological activity between MV-FOE and MV-IOE are directly related to qualitative and quantitative variations in the chemical composition of the essential oils. MV-IOE exhibited a lower diversity of components and differences in the relative proportions of metabolites compared to MV-FOE, which may contribute to its reduced antiparasitic activity. Although major compounds such as 1,8-cineole are present in similar proportions in both oils, the biological activity appears to be modulated by the overall metabolite profile, including minor constituents. In this context, possible interactions among components, including synergistic or antagonistic effects, may contribute to the observed

activity profiles, although these effects are dependent on the specific chemical composition of each essential oil. This highlights need for further studies to better understand these interactions. In this regard, essential oils extracted from the leaves and inflorescences of *Melaleuca viminalis* emerge as promising candidates as new drug prototypes for the development of alternative therapies to the treatment of leishmaniasis, underscoring the importance of Brazilian biodiversity as a source of novel therapeutic agents. However, additional studies, including *in vivo* and clinical assays, are essential to evaluate the efficacy, safety, and feasibility of these compounds for the treatment of leishmaniasis.

5 Materials and methods

5.1 Plant material

The leaves were collected in June 2022, and inflorescences in August 2022, both during the winter season at a private farm in Gaspar Lopes, Alfenas, MG, in the Atlantic Forest region (21°25′27.5″S, 45°56′51.2″W). A voucher specimen was deposited in the herbarium of the Federal University of Alfenas, Brazil, under the voucher number 968, a voucher specimen has been identified by Dr. Marcelo Polo. Research on this species was registered on the platform of the Genetic Heritage and Associated Traditional Knowledge System (SisGen) under the code A20 C5 C9.

5.2 Essential oil extraction

Essential oil from the leaves (MV-FOE) and inflorescences (MV-IOE) of MV was obtained via hydrodistillation using a modified Clevenger apparatus for 2 h. The obtained essential oils were then stored in a refrigerated environment, between 5 °C and 10 °C, for subsequent analysis (Author Anonymous, 2010). The mass used for the extraction of MV-FOE was 50.4 g of dried leaves, which resulted in a mass of 0.2724 g, For MV- IOE, a mass of 127.7 g was used, and at the end of the process, we obtained a mass of 0.5231 g.

5.3 Analysis by GC-MS

The essential oils were analyzed by GC-MS, using a Shimadzu GC-17A chromatograph interfaced with a mass spectrometer MS-QP-5050A. Helium was used as the carrier gas. The operating conditions of the GC/MS were an ionization voltage of 70 eV and an ion source temperature of 230 °C (DB-5 Agilent®J&W GC Columns, Santa Clara, CA, United States – 30 m × 0.25 mm×0.25 μm). The annotation of volatile components was based on the fragmentation pattern observed in the mass spectra, compared with authentic samples available in reference spectral libraries (Adams, 2007).

5.4 Dereplication assisted by GNPS–molecular networking

The annotation of compounds was performed through dereplication of the data obtained by GC-MS using the GNPS platform - "GC-MS EI Data Analysis". For comparative purposes, Adams (2007) was used to corroborate the annotations made by the dereplication platform. A molecular networking was created using the Library Search/Molecular Networking GC workflow on GNPS (//gnps.ucsd.edu). The data were filtered with a fragment ion mass tolerance of 0.5 Da. The MS spectra were filtered by selecting only the top 6 fragment ions in the ± 50 Da window throughout the spectrum. The precursor ion mass tolerance was set at 20,000 Da, the maximum analog search mass difference at 100 Da, and the maximum precursor alignment difference at 500 Da. A molecular networking was then created with filters set to obtain a cosine score greater than 0.7 and more than 6 matching peaks, with a maximum connected component size of 100. The molecular networking was visualized using Cytoscape software 3.10.3 (Wang et al., 2016; Aksenov et al., 2021).

5.5 Parasites and mammalian cell maintenance

RPMI 1640 medium (Thermo Scientific, United States) was supplemented with 10% heat- inactivated fetal bovine serum (FBS), 1% pyruvate, 1% non-essential amino acids, 10 μg/mL gentamicin, 1,000 U/mL penicillin, and 0.1% 2-mercaptoethanol (R10). Schneider's medium (Sigma-Aldrich, United States) was supplemented with 10% FBS, 10 μg/mL gentamicin, and 1,000 U/mL penicillin (S10). Miltefosine (purity >99%) was obtained from Sigma-Aldrich (United States).

5.6 Bone marrow-derived macrophages (BMM)

BALB/c mice aged 7 weeks were euthanized according to the protocol established by the Animal Ethics Committee (CEUA) n° 1648/2022, and the femur and tibia were aseptically collected. The epiphyses of these bones were cut, and the bone marrow content was extracted by injecting Hank's Balanced Salt Solution (HBSS) into the medullary canal. The bone marrow was subjected to two washing steps at 2,000 RPM, 5 min, 4 °C, with HBSS. Then, 0.17 M NH4Cl (pH 7.4) was added to the cell suspension and incubated at 4 °C for 2 min. After the specified time, HBSS was added, stopping the erythrocyte lysis reaction; the cells were centrifuged at 2,000 RPM, 5 min, 4 °C, and the supernatant was discarded.

The cells were seeded in sterile tissue culture-treated Petri dishes in R10 medium with 15% of L929 fibroblast conditioned medium (LCCM) as a source of colony-stimulating factor-1 (Lima et al., 2023), and incubated at 35 °C with 5% CO_2. After 24 h of incubation, the non-adherent cells were collected and added to sterile Petri dishes with R10% and 15% of LCCM. On days 2, 3, 5, and 7 of the experiment, the medium was supplemented with 15% LCCM. On the eighth day, the adherent cells were collected using a cell scraper, counted using a Neubauer chamber, and added to sterile 96-well plates or round coverslips in 24-well plates.

5.7 Activity of EO on promastigote forms of *L. amazonensis*

To evaluate the leishmanicidal potential of MVFOE and MVIOE, promastigote forms of *L. amazonensis* (MHOM/BR/73/M2269) were cultivated in S10 medium and incubated at

25 °C until they reached the stationary phase of growth. They were then harvested, centrifuged (1,200 × g, 10 min, 4 °C), and counted using a Neubauer chamber. Parasite concentration was adjusted to 2×10^6 parasites/well in a sterile 96-well plate. MV-FOE and MV-IOE samples were solubilized with dimethyl sulfoxide (DMSO, never exceeding 1%) and added to the parasites at concentrations ranging from 0.08 to 100 µg/mL, along with the parasites. Miltefosine was used as a control drug (0.08 to 100 µg/mL). The control group consisted of S10 medium with 1% DMSO and parasites (2×10^6 parasites/well). The negative control consisted only of S10 medium with 1% DMSO. The parasites were incubated at 25 °C for 24 h, then centrifuged (1200 × g, 10 min, 4 °C), washed three times with 200 µL sterile saline buffered with 0.01M phosphate pH 7.2 (PBS), and the parasite viability was analyzed using the PrestoBlue® reagent (Thermo Fisher Scientific, United States) diluted in S10 medium (1:10). After being added to the cells, PrestoBlue® is reduced by mitochondrial enzymes of viable parasites, becoming reddish and highly fluorescent. Thus, wells with high fluorescence are associated with a high percentage of viable cells, whereas samples with blue coloration indicate low cell viability. After 45 min of incubation, the plates were read using a microplate reader with a fluorescence probe set at 416 nm excitation and 574 nm emission (GloMax®- Multi + Detection System, Promega©, United States). The 50% effective concentration (EC_{50}) was estimated using GraphPad Prism 8.0 software.

5.8 Cytotoxicity assay

BMMs were adjusted to a concentration of 10^6 macrophages/well, added to a 96-well culture plate with R10 medium, and incubated for 24 h in a humidified incubator at 37 °C and 5% CO_2. After this period, MV-FOE, MV-IOE, or miltefosine (0.7 to 100 µg/mL) were added to the macrophages, and the cells were incubated for 24 h in a humidified incubator at 35 °C and 5% CO_2. Control BMMs were incubated only with R10. The cytotoxicity of the compounds was analyzed after 24 h of incubation with PrestoBlue reagent as detailed in the *Activity of EO on promastigote forms of L. amazonensis*. The 50% cytotoxic concentration (CC_{50}) was estimated using GraphPad Prism 8.0 software. The selectivity index (SI) of MV-FOE and MV-IOE was determined by the ratio between CC_{50} and EC_{50} (Lima et al. 2023).

5.9 Activity of EO on intracellular forms of *L. amazonensis*

BMMs were cultured in round coverslips (10^5 macrophage/coverslip) in 24-well plates for 24 h, followed by infection with *L. amazonensis* promastigote forms at a ratio of 10 parasites per 1 macrophage in a humidified incubator with 5% CO_2 at 35 °C. Twenty-four hours later, each well was washed three times with warm PBS to withdraw the free parasites. Then, MV-FOE, MV-IOE, or miltefosine (1.25–10 µg/mL) was added to the infected cells during 24 h. Infected controls were cultured only with R10. After the incubation period, wells were washed three times with warm PBS, coverslips were dried at room temperature, fixed with methanol, and stained by Giemsa (Sigma-Aldrich, St Louis, MO, United States). At least 100 cells/coverslip were quantified and the infection indices were estimated according to the expression:

$$\text{II} = \%\text{Infected macrophages} \times \text{Internalized amastigotes/Infected Macrophages}$$

6 Statistical analysis

In all biological assays, at least three independent experiments were carried out with four replicates in each experimental situation. The data were expressed as the mean ± standard deviation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

All animal procedures were conducted in accordance with the ethical principles for animal experimentation and approved by the Committee on Ethics in the Use of Animals and the National Council for the Control of Animal Experimentation of the Medical School of Sao Paulo University (No 1963/2023). All experiments were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Author contributions

AS: Writing – review and editing, Investigation, Writing – original draft, Formal Analysis, Methodology. MS: Methodology, Data curation, Writing – original draft. EC: Data curation, Methodology, Writing – original draft. GA: Methodology, Formal Analysis, Writing – review and editing, Resources. LP: Resources, Methodology, Writing – review and editing, Conceptualization. ML: Methodology, Writing – original draft, Formal Analysis. PS: Project administration, Supervision, Conceptualization, Writing – review and editing, Methodology, Investigation, Writing – original draft, Funding acquisition, Resources.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fntpr.2026.1826685/full#supplementary-material

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