Chemical compositions and biological properties of the leaf essential oil of three *Melaleuca* species

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Abstract. The genus *Melaleuca* (Myrtaceae) is a valuable plant resource for research and applications in medicine, pharmacy, the pharmaceutical industry and folklore medicine. The present study aimed to investigate the chemical components and biological properties of the essential oils (EOs) of three *Melaleuca* species. Gas chromatography-mass spectrometry analyses revealed that the EOs extracted from the leaves of *M. cajuputi*, *M. quinquenervia* and *M. leucadendra* contained 19-21 compounds with high amounts of oxygenated monoterpenes and sesquiterpenes. Subsequent bioassays demonstrated that the EOs of *M. cajuputi*, *M. quinquenervia* and *M. leucadendra* exhibited antimicrobial activity against all tested microorganisms, including *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aegurinosa* and *Candida albicans*, with minimum inhibitory concentrations in the range 640-2,560 μg/ml. The same EOs exerted inhibitory effects against α-amylase, α-glucosidase, acetylcholinesterase and xanthine oxidase with IC_{50} values ranging from 331.9±20.64 to 1,453±93.79 μg/ml. The findings obtained in the present study provide additional insight into the enzyme inhibitory properties of the leaf EOs from *Melaleuca* species that have not been reported in previous research, at least to the best of our knowledge.

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Introduction

Vietnam is a Southeast Asian country characterized by a tropical monsoon climate with a rich vegetation system. Out of >12,000 plant species in Vietnam, 5,117 of these have medicinal value (1,2), representing a key source of active compounds for research or applications in medicine, pharmacy and the pharmaceutical industry. The objective of the present study was to investigate the chemical constituents and biological activities of the essential oils (EOs) of three *Melaleuca* species growing in Vietnam.

The genus *Melaleuca* (Myrtaceae) includes 280 species, of which four species have been found in Vietnam, including *Melaleuca alternifolia*, *Melaleuca cajuputi*, *Melaleuca leucadendra* and *Melaleuca quinquenervia* (1). According to traditional Vietnamese medicine, the species *M. cajuputi, M. leucadendra* and *M. quinquenervia* are widely used in the treatment of diseases, such as cold, flu, fever, malaria, indigestion, bone pain, diarrhea, inflammatory skin diseases, allergies and eczema (2). Previous studies on the chemical constituents of the plant EOs have demonstrated that the EOs of *M. cajuputi* are mainly composed of 1,8-cineol, α-pinene, γ-terpinene, *p*-cymene, α-terpineol, caryophyllene, α-humulene and α-gurjunene (3-6). The EOs of *M. leucadendra* L. have been found to be mainly composed of *p*-cymene, α-terpinene, γ-terpinene, 4-terpineol, caryophyllene, methyleugenol and *E*-nerolidol (7-10). The main components of the EOs of *M. quinquenervia* have been shown to be monoterpenes (1,8-cineole, α-pinene, α-terpineol, limonene) and sesquiterpene (viridiflorol, *E*-nerolidol) (11,12). Furthermore, pharmacological studies on the EOs from *M. cajuputi, M. quinquenervia* and *M. leucadendra* have demonstrated their antibacterial, antimycrobacterial, antivirus, antifungal, antiinsecticidal and antioxidant effects (4,6,9,10,13-18); however, other investigations have revealed that the chemical compositions of EOs of *M. cajuputi, M. quinquenervia* and *M. leucadendra* vary extensively in different geographic and ecological conditions, even from different regions of the same country, resulting in several chemotypes (7,9,10,12,18-20). In addition, studies on the biological activities of EOs from *M. cajuputi*, *M. quinquenervia* and *M. leucadendra* species grown in Vietnam are still limited and primarily focus on the EOs from *M. cajuputi* (4,6,14,18).

The present study evaluated the antimicrobial activity and, for the first time, to the best of our knowledge the enzyme inhibitory effects against α-amylase, α-glucosidase, acetylcholinesterase (AChE) and xanthine oxidase (XO) of EOs extracted from the leaves of three *Melaleuca* species widely distributed in Vietnam (Fig. 1).

Materials and methods

Plant materials. The leaves of *M. cajuputi* and *M. quinquenervia* were collected in Phong My, Phong Dien, Thua Thien Hue, Vietnam [(16˚30'56''N, 107˚18'08''E) and (16˚30'41''N, 107˚16'56''E)] in May, 2023, while the leaves of *M. leucadendra* were collected in An Minh Bac, U Minh Thuong, Kien Giang, Vietnam (9˚37'06''N, 105˚05'50''E) in June, 2023. These plant materials were identified by Dr Le Tuan Anh (Mientrung Institute for Scientific Research, Vietnam National Museum of Nature, Thua Thien Hue, Vietnam) and the voucher specimens (MISR2023-7, MISR2023-8 and MISR2023-11) were kept in the herbarium of the Mientrung Institute for Scientific Research, Vietnam National Museum of Nature.

Extraction of EOs. Fresh leaves of *M. cajuputi, M. quinquenervia* and *M. leucadendra* (200 g) were cut into small sections and then subjected to steam distillation using a glass apparatus to extract the EOs for 2 h at normal pressure. The EOs were then collected, dried with 0.5 g Na_2SO_4 (Merck, KGaA), stored in the dark and sealed in vials at 4˚C until further chemical analysis and biological activity testing.

Gas chromatography-mass spectrometry (GC-MS) analysis. The EOs extracted from the leaves of *M cajuputi, M. quinquenervia* and *M. leucadendra* were analyzed via GC-MS using a Shimadzu GCMS-QP2010 Plus system (Shimadzu Corporation). This system was equipped with a flamer ionization detector (FID) and an Equity-5 capillary column (30 m x 0.25 mm, 0.25 µm film thickness). The EOs were diluted to a concentration of 1% in n-hexane and 1.0 µl of the solutions were injected into the instrument for analysis. The GC was operated with helium as carrier gas at a flow rate of 1.5 ml/min. The GC oven temperature was initiated at 60˚C for 2 min, then increased to 240˚C at a rate of 4˚C/min and maintained for 10 min before further programming to 280˚C at a rate of 5˚C/min. The injector temperature was set at 260˚C. Mass spectrometry was performed at 70 eV in a mass range of 40-500 amu with a sampling rate of 0.5 scan/sec.

Identification of the compounds. The chemical components of the EOs were identified by comparing their relative retention indices (RI) with those of a series of reference n-alkanes C7-C40 (Merck, KGaA). Additionally, the identification relied on computer matching against commercial libraries (WILEY7 Library and NIST11 Library), as well as MS and RI data of known compounds from the literature (21,22). The chemical structures of the compounds were drawn using ChemDraw Ultra 8.0 (CambridgeSoft Corporation).

Determination of biological activities of EOs: Antimicrobial activity. The antimicrobial activity of the EOs was evaluated against a panel of five reference microorganisms, including *Staphylococcus aureus* (ATCC 25923; *S. aureus*), *Enterococcus faecalis* (ATCC 29212; *E. faecalis*), *Escherichia coli* (ATCC 25922; *E. coli*), *Pseudomonas aeguri-nosa* (ATCC 27853; *P. aegurinosa*) and *Candida albicans* (ATCC 10231; *C. albicans*). The minimum inhibitory concentrations (MICs) of the EOs against these microorganisms were determined using the broth microdilution method as previously reported by Dat *et al* (23). Briefly, the bacterial inoculum (100 µl at a concentration of $1x10^6$ CFU/ml) was introduced into the wells of 96-well plates containing various concentrations of the EOs (100 µl) ranging from 1.0 to 2,560 µg/ml. The plate was incubated at 37˚C for 24 h, followed by the measurement of absorbance at 630 nm using an ELx800 absorbance microplate reader (BioTek Instruments, Inc.; Agilent Technologies). The MICs of the antibacterial EOs were defined at the lowest concentration where no bacterial growth was observed through absorbance records at 630 nm. Similarly, for yeast, the yeast inoculum (100 µl at a concentration of $2\text{-}5x10^5$ CFU/ml) was added to wells containing the EOs (100 µl) at various concentrations ranging from 1.0 to 2,560 µg/ml in 96-well plates, which were then incubated at 28˚C for 48 h. The MICs of the anti-yeast EOs were determined at the lowest concentration where no yeast growth was observed through absorbance records at 530 nm using an ELx800 absorbance microplate reader. The antibiotics, ciprofloxacin and fluconazole (MilliporeSigma) ranging from 0.5 to 8.0 µg/ml, served as the positive controls for bacteria and yeast, respectively. The experiments were performed in triplicate.

Determination of biological activities of EOs: Enzyme inhibition activity

i) Amylase inhibitory activity. The inhibitory effect of the EOs on α-amylase (MilliporeSigma) was assessed according to the method previously described by Nguyen *et al* (24). In brief, the starch azure solution supplemented with 0.01 M $CaCl_2$ in 0.05 M Tris-HCl buffer (pH 6.9) (MilliporeSigma) was boiled for 5 min and pre-incubated at 37˚C for 5 min. The reaction containing 50 µl of the EO, 50 µl of the substrate solution, and 25 µl of α-amylase solution (2 U/ml) was incubated in 96-well plates at 37˚C for 10 min. The reaction was terminated by the addition of 75 µl of 50% acetic acid, followed by the measurement of absorbance at 650 nm using an ELx800 absorbance microplate reader (BioTek Instruments, Inc.). The inhibitory activity was calculated as follows: Inhibition (%)=100 x $[1\text{-}(A_s\text{-}A_{bs})/(A_c\text{-}A_{cb})]$, where: A_s is the absorbance of the sample, A_{sb} is the absorbance of the sample blank, A_c is the absorbance of the control, and A_{cb} is the absorbance of the control blank. Acarbose (MilliporeSigma) was used as a positive control with tested concentrations ranging from 10 to 200 µl/ml. The IC_{50} value was calculated using GraphPad Prism v8.0 (Dotmatics). The experiments were performed in triplicate and data are expressed as the mean ± standard deviation.

ii) Glucosidase inhibitory activity. The inhibitory effect of the EOs on α-glucosidase (MilliporeSigma) was determined according to the method previously described by Nguyen *et al* (24). Briefly, the reaction containing 50 µl of the EO and 100 µl of α-glucosidase solution (0.5 U/ml) in 0.1 M potassium phosphate buffer (pH 6.8) (MilliporeSigma) was incubated in 96-well plates at 37˚C for 10 min. The reaction was initiated by the addition of 50 µl of 5 mM 4-Nitrophenyl

Melaleuca cajuputi *Melaleuca leucadendra* *Melaleuca quinquenervia*

Figure 1. *Melaleuca* species examined in the present study.

β-D-glucopyranoside (MilliporeSigma), followed by incubation at 37˚C for 30 min. The reaction was then terminated by the addition of 75 µl of 0.2 M Na_2CO_3 (MilliporeSigma) and absorbance was recorded at 405 nm using an ELx800 absorbance microplate reader. The inhibitory activity was calculated as follows: Inhibition (%)=100 x [1-(A_s-A_{bs})/(A_c-A_{cb})], where: A_s is the absorbance of the sample, A_{sb} is the absorbance of the sample blank, A_c is the absorbance of the control, and A_{cb} is the absorbance of the control blank. Acarbose (MilliporeSigma) was used as a positive control with tested concentrations ranging from 10 to 200 µl/ml. The IC_{50} value was calculated using GraphPad Prism v8.0 (Dotmatics). The experiments were performed in triplicate and data are expressed as the mean ± standard deviation.

iii) XO inhibitory activity. The inhibitory effect of the EOs on XO (MilliporeSigma) was determined according to the method described in the study by Dat *et al* (23). In summary, the reaction mixture containing 50 µl of the EO, 35 µl of 70 mM phosphate buffer (pH 7.5) and 30 µl of enzyme solution (0.01 U/ml) was pre-incubated at 25˚C for 15 min, followed by the addition of 60 µl of 150 mM xanthine (MilliporeSigma). Subsequently, the reaction was incubated at 25˚C for 30 min, followed by the addition of 25 µl of 1.0 N HCl (MilliporeSigma). The absorbance of the reaction was then measured at 290 nm using an ELx800 absorbance microplate reader. The inhibitory activity was calculated as follows: Inhibition (%)=100 x [1-(A_s-A_{bs})/(A_c-A_{cb})], where: A_s is the absorbance of the sample, A_{sb} is the absorbance of the sample blank, A_c is the absorbance of the control and A_{cb} is the absorbance of the control blank. Allopurinol (MilliporeSigma) was used as a positive control with tested concentrations ranging from 1.0 to 50 µl/ml. The IC_{50} was calculated using GraphPad Prism v8.0. The experiments were performed in triplicate and the data are expressed as the mean ± standard deviation.

iv) AChE inhibitory activity. The inhibitory activity of the EOs on AChE (MilliporeSigma) was determined according to the method previously described by Thai *et al* (25). The reaction containing 100 µl of 3 mM 5,5-dithiobis-2-nitrobenzoate (MilliporeSigma), 20 µl of the EO and 20 µl of AChE (0.2 U/ml; MilliporeSigma) was pre-incubated at 25˚C for 15 min, and then initiated by the addition of 20 µl of 15 mM acetylthiocholine iodide (MilliporeSigma). Following incubation at 25˚C for 20 min, the reaction was terminated by the addition of 20 µl of 4% SDS (MilliporeSigma). Subsequently, the absorbance of the reaction was measured at 415 nm using an ELx800 absorbance microplate reader. The inhibitory activity was calculated as follows: Inhibition (%)=100 x [1-(A_s-A_{bs})/(A_c-A_{cb})], where: A_s is the absorbance of the sample, A_{sb} is the absorbance of the sample blank, A_c is the absorbance of the control and A_{cb} is the absorbance of the control blank. Galantamine (MilliporeSigma) was used as a positive control with tested concentrations ranging from 1.0 to 20 µl/ml. The IC_{50} value was calculated using GraphPad Prism v8.0 software (Dotmatics). The experiments were performed in triplicate and the data are expressed as the mean ± standard deviation.

Results and Discussion

For each of the three steam distillations of the fresh leaves of *M. cajuputi, M. quinquenervia,* and *M. leucadendra* (200 g), the average yield of the EOs was 1.40 g (0.70%, wt/wt), 2.35 g (1.18%, wt/wt) and 0.62 g (0.31%, wt/wt), respectively. The GC chromatograms of the EOs of *M. cajuputi, M. leucadendra*, and *M. quinquenervia* are presented in the Figs. 2-4. The chemical constituents of the EOs are listed in Table I and the chemical structures of the main compounds are illustrated in Fig. 5.

The GC-MS analysis of the leaf EO of *M. cajuputi* and the comparisons of relative RIs of reference n-alkanes and the spectral databases of known compounds revealed the presence of 21 compounds, accounting for 92.50% of the total amount of the EO. Of these, monoterpene hydrocarbons accounted for 3.94%, oxygenated monoterpenes for 43.56%, sesquiterpene hydrocarbons for 5.68%, oxygenated sesquiterpenes for 39.00% and non-terpenoids for 0.32%. These results indicated that oxygenated monoterpenes and sesquiterpenes were the main components of the EO of *M. cajuputi*. Among these, 1,8-cineole and α-terpineol were the main compounds in the group of oxygenated monoterpenes, accounting for 30.87 and 8.31%, respectively. Moreover, guaiol (9.71%), γ-eudesmol (6.16%), β-eudesmol (9.23%) and α-cadinol (11.29%) were the main compounds in the oxygenated sesquiterpene component of the EO of *M. cajuputi*. Previous studies have indicated that

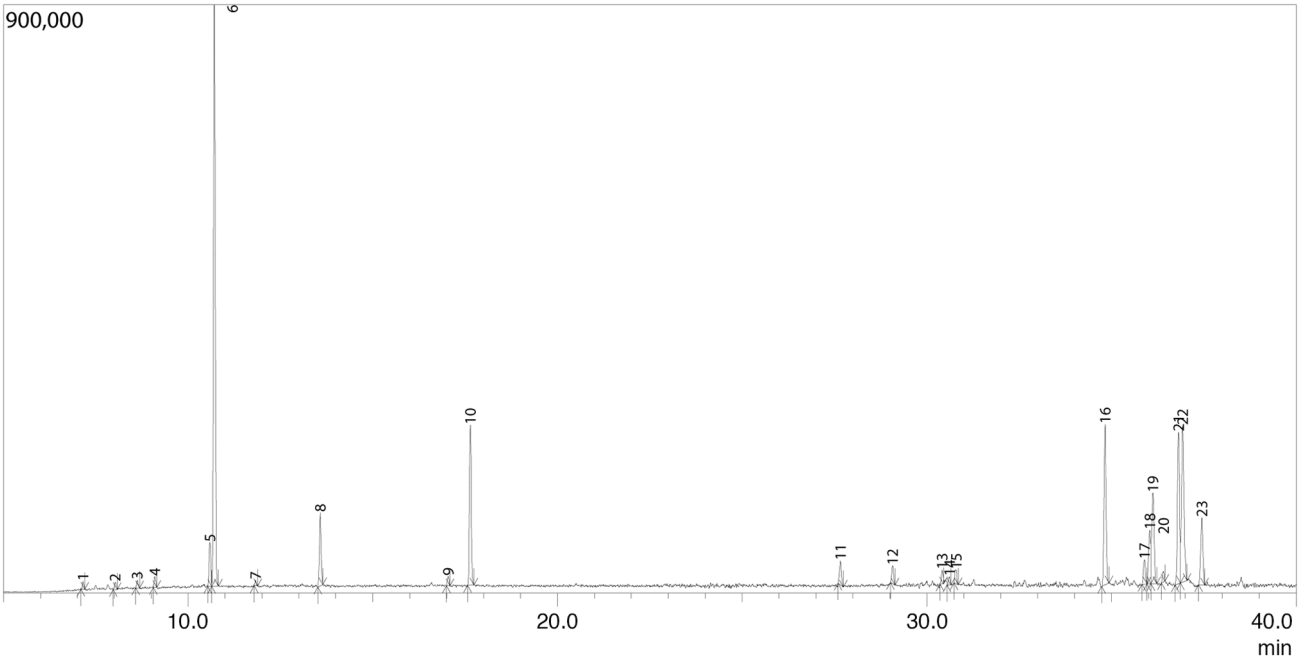

Figure 2. Gas chromatogram of the leaf EO of *M. cajuputi*.

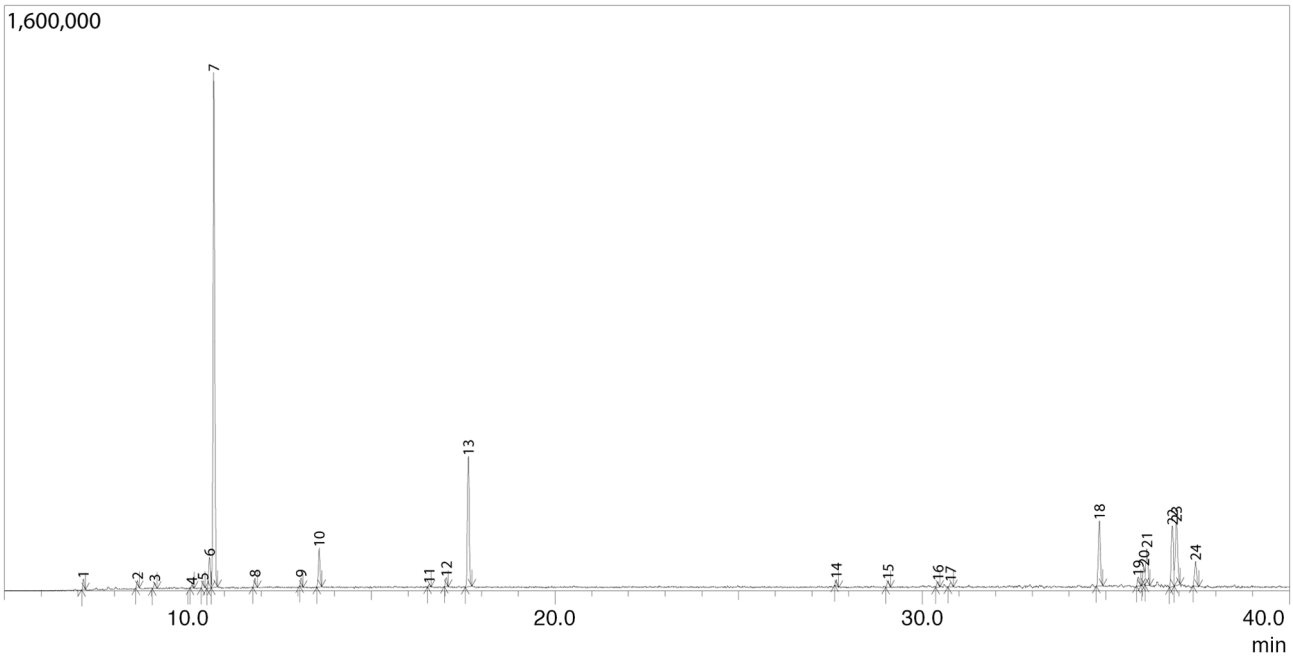

Figure 3. Gas chromatogram of the leaf EO of *M. quinquenervia*.

1,8-cineole is one of the main compounds in the leaf EO of *M. cajuputi*, with widely varying concentrations ranging of 27.78-59.90% (3-6,14).

In addition, the presence of 20 compounds was identified in the EO of *M. quinquenervia*. Among these, oxygenated monoterpenes (58.92%) accounted for the highest proportion, whereas other compound groups, including oxygenated sesquiterpenes, monoterpene hydrocarbons, sesquiterpene hydrocarbons and non-terpenoids accounted for 26.11, 6.48, 2.82 and 0.62%, respectively. The compounds 1,8-cineole (42.51%), α-terpineol (12.00%), guaiol (6.68%), β-eudesmol (6.53%) and α-cadinol (7.81%) were the main constituents of the EO extracted from *M. quinquenervia* species grown in Thua Thien Hue province, Vietnam. However, the main compounds in the EO of *M. quinquenervia* vary widely among different geographical regions. For example, a report on the chemical composition of the EO of *M. quinquenervia* in Australia and Papua New Guinea regions indicated that EOs extracted from *M. quinquenervia* species growing from Sydney, North along the East coast of Australia to Selection Flat, New South Wales and Maryborough, Queensland, typically contain linalool (14.0-30.0%) and *E*-nerolidol

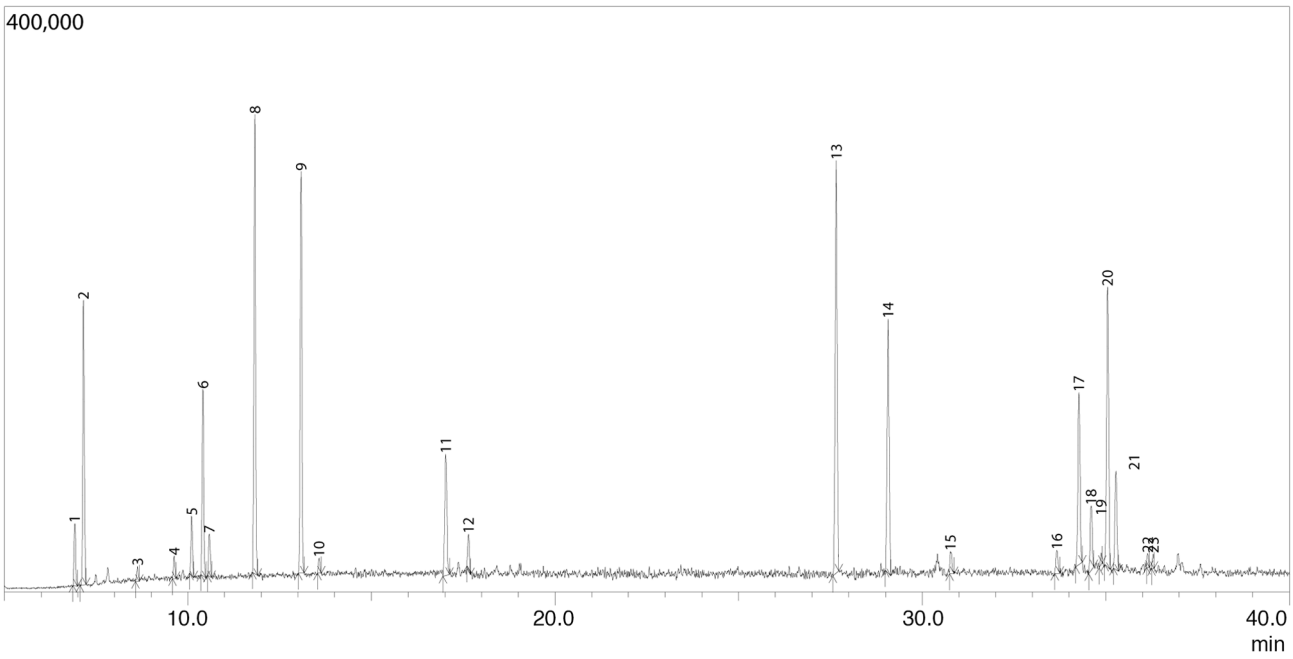

Figure 4. Gas chromatogram of the leaf EO of *M. leucadendra*.

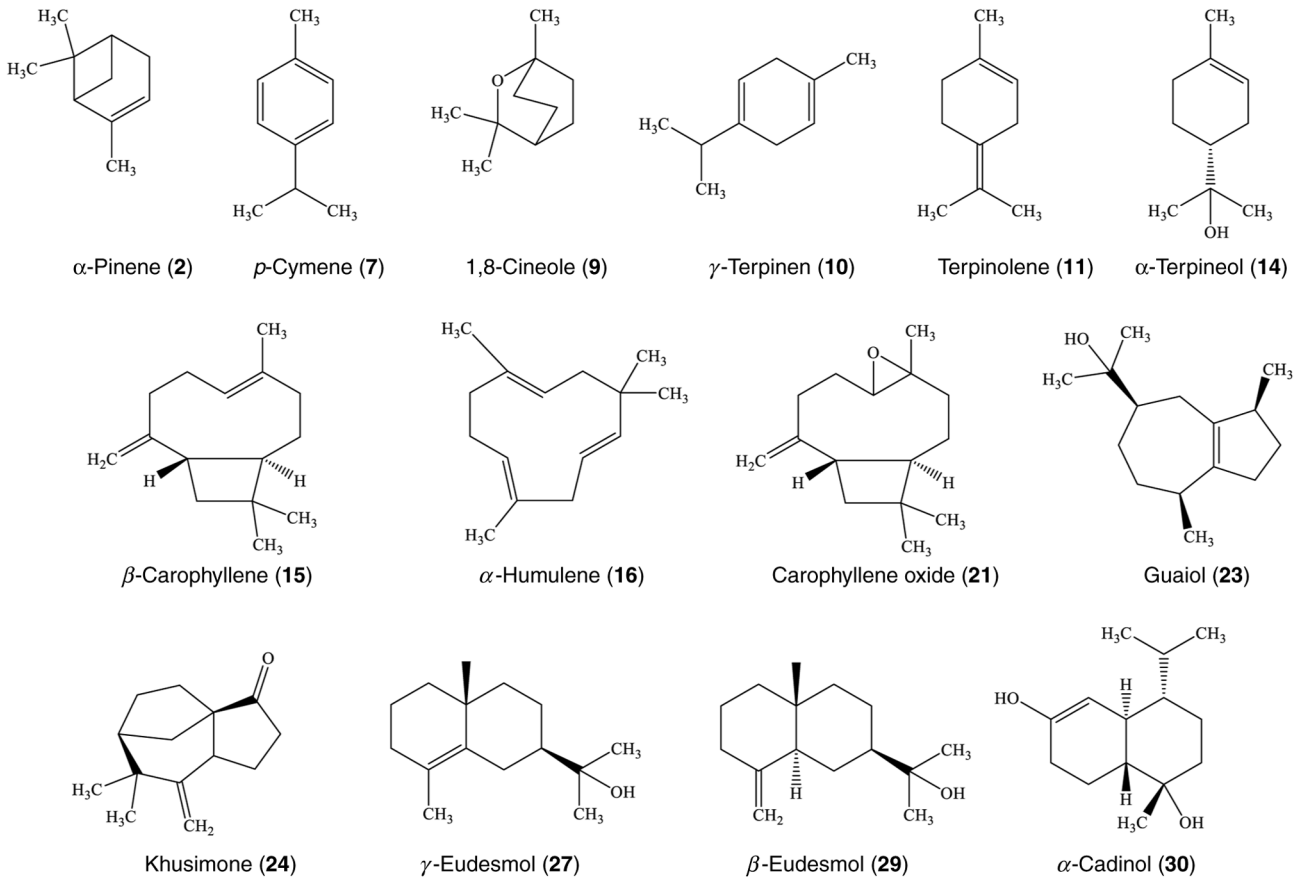

Figure 5. Chemical structure of the major compounds of the leaf EOs of *Melaleuca* species in Vietnam. The numbers in bold font in parentheses next to name of each compound correspond to their order of presentation in Table I.

(74.0-95.0%) as major components. Moreover, oils from *M. quinquenervia* species in areas ranging from Sydney to Papua New Guinea and New Caledonia often contain main constituents, such as α-terpineol (0.5-14.0%), β-caryophyllene (0.5-28.0%), viridiflorol (13.0-66.0%) and 1,8-cineole (10.0-75.0%) (12). The EO extracted from *M. quinquenervia*

Table I. Chemical composition of the leaf EOs of *M. cajuputi, M. quinquenervia* and *M. leucadendra*.

Compound no.	Compounds	RI[a]	RI[b]	*M. cajuputi* (%)	*M. quinquenervia* (%)	*M. leucadendra* (%)
1	α-Thujene	925	924	-	-	1.76
2	α-Pipene	932	932	0.44	0.84	**7.69**
3	Benzaldehyde	957	960	0.32	-	-
4	β-Pinene	975	974	0.42	0.79	0.44
5	Myrcene	989	990	0.58	0.58	-
6	α-Phellandrene	1,004	1,002	-	-	0.73
7	p-Cymene	1,023	1,024	-	0.62	**5.38**
8	(-)-Limonene	1,027	1,029	2.16	2.66	1.43
9	1,8-Cineole	1,030	1,031	**30.87**	**42.51**	-
10	γ-Terpinene	1,057	1,059	0.35	0.79	**12.94**
11	Terpinolene	1,087	1,088	-	0.83	**11.77**
12	Linalool	1,099	1,096	3.86	3.38	0.55
13	Terpinen-4-ol	1,177	1,177	0.52	1.02	4.65
14	α-Terpineol	1,190	1,188	**8.31**	**12.00**	1.25
15	β-Caryophyllene	1,420	1,419	1.61	0.95	**14.11**
16	α-Humulene	1,454	1,454	1.12	0.75	**8.54**
17	β-Selinene	1,487	1,490	1.17	0.50	-
18	δ-Selinene	1,492	1,492	0.52	-	-
19	α-Selinene	1,496	1,498	1.26	0.63	0.65
20	Palustrol	1,569	1,568	-	-	1.06
21	Caryophyllene oxide	1,584	1,583	-	-	**7.22**
22	Viridiflorol	1,592	1,592	-	-	2.48
23	Guaiol	1,598	1,600	**9.71**	**6.86**	-
24	Khusimone	1,604	1604	-	-	**9.87**
25	Isolongifolanone	1,610	1613	-	-	3.42
26	1-epi-cubenol	1,627	1,628	1.59	1.01	-
27	γ-Eudesmol	1,633	1,632	**6.16**	3.90	-
28	Alloaromadendrene epoxide	1,641	1,641	1.02	-	-
29	β-Eudesmol	1,652	1,650	**9.23**	**6.53**	-
30	α-Cadinol	1,655	1,654	**11.29**	**7.81**	-
Total identified				**92.50**	**95.95**	**95.93**
- Non-terpenoids				0.32	0.62	5.38
- Monoterpene hydrocarbons				3.94	6.48	36.76
- Oxygenated monoterpenes				43.56	58.92	6.45
- Sesquiterpene hydrocarbons				5.68	2.82	23.30
- Oxygenated sesquiterpenes				39.00	26.11	24.04
Total unidentified				**7.50**	**4.05**	**4.07**

RI[a], retention indices calculated from retention time in relation to those of a series of C7-C40 n-alkanes on an Equity-5 capillary column; Rib, literature retention indices (17,18,23). EO, essential oil; *M. cajuputi*, *Melaleuca cajuputi*; *M. quinquenervia*, *Melaleuca quinquenervia*; *M. leucadendra*, *Melaleuca leucadendra*. Values in bold indicate main compounds in essential oils.

species harvested in Taiwan contains main chemical components, including α-terpineol (13.73%), viridiflorol (14.55%), α-pinene (15.93%) and 1,8-cineole (21.60%) (11). On the other hand, the EO of *M. quinquenervia* collected in Costa Rica exhibits different compositions, including α-terpineol (6.5%), α-pinene (17.9%), viridiflorol (21.7%) and 1,8-cineole (31.5%) (26).

The results of the chemical composition analysis of the leaf EO of *M. leucadendra* identified 19 compounds, accounting for 95.93% of the EO. Compounds, such as α-pinene (7.69%), p-cymene (5.38%), γ-terpinene (12.94%), terpinolene (11.77%), β-caryophyllene (14.11%), α-humulene (8.54%), caryophyllene oxide (7.22%) and khusimone (9.87%) were the main compounds found in the EO of *M. leucadendra*. Previous

Table II. Antimicrobial activity of the EOs (MIC, μg/ml).

EOs	*S. aureus*	*E. faecalis*	*E. coli*	*P. aegurinosa*	*C. albicans*
M. cajuputi	1,280	1,280	640	640	2,560
M. quinquenervia	640	2,560	1,280	1,280	2,560
M. leucadendra	640	1,280	1,280	2,560	1,280
Ciprofloxacin	2	4	2	2	-
Fluconazole	-	-	-	-	4

EO, essential oil; *M. cajuputi*, *Melaleuca cajuputi*; *M. quinquenervia*, *Melaleuca quinquenervia*; *M. leucadendra*, *Melaleuca leucadendra*; *S. aureus*, *Staphylococcus aureus*; *E. faecalis*, *Enterococcus faecalis*; *E. coli, Escherichia coli*; *P. aegurinosa*, *Pseudomonas aegurinosa*; *C. albicans*, *Candida albicans*.

investigations on the chemical composition of the EO have revealed marked differences among *M. leucadendra*. In particular, the EO extracted from *M. leucadendra* species harvested in Fujian, China, has been reported to be rich in compounds, such as α-pinene (4.96%), α-terpinene (7.82%), p-cymene (5.74%), γ-terpinene (18.4%), α-terpineol (4.92%) and 4-terpineol (36.85%) (8). Furthermore, compounds such as limonene (4.8%), 1,8-cineole (61.0%), α-terpineol (15.6%) and viridiflorol (7.9%) dominate in the composition of the EO extracted from *M. leucadendra* species harvested in Havana, Cuba (9). Moreover, the EO extracted from *M. leucadendra* leaves in India has been shown to contain (*E*)-nerolidol (90.85%) as the absolute predominant compound among the 28 identified compounds present in the oil (7). Additionally, the EO of *M. leucadendra* in Senegal has been shown to contain methyleugenol (98.4-99.5%) as the main component (10). Furthermore, a report on the composition of the EO of *M. leucadendra* from Danang, Vietnam, indicated that α-humulene (4.4%), *β*-selinene (3.7%), α-selinene (3.7%), guaiol (10.9%) and α-eudesmol (17.6%) are the major compounds in this oil (18). The findings of the present study, as well as those of previous reports (7-10,18), indicate that the chemical composition of the leaf EO of *M. leucadendra* varies significantly due to geographical differences.

Although numerous studies have indicated that the EO of *Melaleuca* species includes several chemotypes, the environmental factors responsible for essential oil chemotype distribution of these species remain unclear. Therefore, further studies on environmental factors influencing the EO chemotype of *Melaleuca* species are required. Several investigations of EO chemotypes from other plants have revealed that the environment factors responsible for EO chemotype distribution include soil properties and nutrients (pH, Ca^{2+}, K^{+}, organic matter, aridity and texture), bioclimatic regions (temperature, precipitation, altitude and seasonal variation), cultivating conditions, maturation of harvested plants, plant storage, plant preparation and methods of extraction (27-31).

Biological activities of essential oils

Antimicrobial activity. The antimicrobial activity of the EOs presented in Table II demonstrated that the EOs of the *Melaleuca* species examined in the present study exhibited antimicrobial activity against all tested microorganisms (*S. aureus, E. faecalis, E. coli, P. aegurinosa, C. albicans*) with MICs in the range of 640-2560 μg/ml. The leaf EO of *M. cajuputi* exhibited the highest antimicrobial activity against *E. coli* and *P. aegurinosa* with MICs of 640 μg/ml, whereas the leaf EOs of *M. quinquenervia* and *M. leucadendra* exhibited the highest antimicrobial activity againts *S. aureus* with MIC of 640 μg/ml. The difference in the antimicrobial activity of the EOs derived from the *Melaleuca* species may be attributed to the varying chemical composition and content of bioactive compounds present in the EOs.

Previous investigations have revealed that the EOs of *Melaleuca* species exhibit antimicrobial activity against a broad spectrum of pathogenic microbes. The EO of *M. cajuputi* has been shown to inhibit the growth of a range of Gram-positive bacteria, including *Bacillus cereus, Bacillus subtilis, Corynebacterium diphtheriae, Corynebacterium minutissimum, Enterococcus faecium, Listeria monocytogenes, Micrococcus luteus, S. aureus, Staphylococcus capitis, Staphylococcus epidermidis, E. faecalis* and *Klebsiella* spp. at concentrations ranging of 0.4-0.6% (32,33), Gram-negative bacteria such as *Alcaligenes faecalis, Enterobacter cloacae, E. coli* and *Proteus vulgaris*, as well as the fungi such as *C. albicans, Gardnerella. vaginalis, Candida glabrata*, *Aspergillus niger*, *Penicillium notatum* at concentrations ranging of 0.4-0.6% (15,34,35). The EO of *M. quinquenervia* has been reported to have effective antimicrobial activity against bacteria, including *E. coli, S. aureus, P. aeruginosa, Staphylococcus epidermidis, Propionibacterium acnes, Streptococcus peroris, Klebsiella pneumonia, Acinetobacter baumannii* and *Proteus vulgaris* with MICs of 0.5-16 mg/ml and fungi *C. albicans*, *Candida tropicalis*, *Aspergillus niger* with MICs of 0.2-4 mg/ml (17). The EO from *M. leucadendra* leaf has been shown to exhibit antibacterial activity against *S. aureus* and *E. coli*, *Salmonella thiphymurium*, *Proteus mirabilis*, *Klebsiella pneumonie*, *E. coli*, *Enterobacter aerogenes*, *Providencia rettgeri*, *Shigella fexnerii*, *P. aeruginosa*, *E. faecalis*, *Staphylococcus saprophyticus* and *S. aureus* with MICs of 7.8-62.5 mg/ml (10,36).

In vitro and *in silico* investigations have demonstrated that terpenes and terpenoids are the main active compounds in antimicrobial EOs (17,37). Furthermore, other biomolecules, such as phenols, alcohols and aldehydes found in the EOs, induce antimicrobial activity with varying specificity and effectiveness. These variations are often attributed to the functional groups within the EO and the hydrogen bonding dynamics

Table III. α-Amylase, α-glucosidase, acetylcholinesterase, and xanthine oxidase inhibitory activities of the EOs (IC_{50}, μg/ml).

EOs	Inhibitory activity			
	α-Amylase	α-Glucosidase	Acetylcholinesterase	Xanthine oxidase
M. cajuputi	862.6±65.74	1212±73.49	765.7±26.14	331.9±20.64
M. quinquenervia	786.3±58.42	1453±93.79	815.5±20.72	380.1±17.85
M. leucadendra	714.3±38.09	1066±45.01	535.5±19.84	433.8±18.02
Acarbose	60.84±2.23	111.5±2.96	-	-
Galantamine	-	-	5.95±0.14	-
Allopurinol	-	-	-	7.44±0.37

EO, essential oil; *M. cajuputi*, *Melaleuca cajuputi*; *M. quinquenervia*, *Melaleuca quinquenervia*; *M. leucadendra*, *Melaleuca leucadendra*.

during their interactions (38). Terpenes are recognized for their antimicrobial properties, mainly attributed to their ability to disrupt cell membranes, inhibit cell growth, and interfere with protein and DNA synthesis (39). Specific terpenes, such as carvone, carvacrol, eugenol, thymol and geraniol have demonstrated antibacterial effects (40), whereas compounds such as menthol, azadirachtin, ascaridol, toosendanin, methyl eugenol and volkensin have exhibited anti-fungal effects (41,42). These compounds have exhibited antimicrobial effects by compromising cellular membrane integrity (43). Monoterpenoids also exhibit antibacterial effects by disrupting microbial multiplication and development, as well as by interfering with their metabolic and physiological functions (44). Monoterpene terpineol isomers, such as terpinen-4-ol, α-terpineol and δ-terpineol have demonstrated effective inhibition against Gram-negative bacteria, particularly *Shigella flexneri*, by inducing permeability changes in bacterial membranes, leading to the release of nucleic acids and proteins, alongside a decrease in membrane potential (45). Eugenol exhibits potent bactericidal activity against *Salmonella enterica* serovar Typhimurium and *S. aureus* (46).

Previous investigations have also demonstrated the mechanisms of action of the main antimicrobial compounds in the EOs of *Melaleuca*. For instance, limonene and 1,8-cineole enhance the permeability of the bacterial membrane, whereas 1,8-cineole and viridiforol inhibit enzyme peptidoglycan glycosyltransferase (36). Limonene affects cell membrane permeability of in Gram-positive bacteria by attacking cell integrity and cell wall structure (47). Other compounds, such as lupene, guaiol and 1,8-cineole exhibit antifungal effects by inhibiting the target enzymes cellobiohydrolase, laccase and lignin peroxidase (48).

Enzyme inhibition activity. The enzyme inhibitory activities of the EOs presented in Table III revealed that the EOs of *Melaleuca* species exerted inhibitory effects on the enzymes α-amylase, α-glucosidase, AChE and XO. The leaf EO of *M. cajuputi* exhibited inhibitory activities against the enzymes α-amylase, α-glucosidase, AChE and XO with IC_{50} values of 862.6±65.74, 1212±73.49, 765.7±26.14 and 331.9±20.64 μg/ml, respectively; the leaf EO of *M. quinquenervia* exhibited enzyme inhibitory effects with IC_{50} of 786.3±58.42, 1453±93.79, 815.5±20.72, and 380.1±17.85 μg/ml, respectively; and the leaf EO of *M. leucadendra* exhibited enzyme inhibitory effects IC_{50} of 714.3±38.09, 1066±45.01, 535.5±19.84 and 433.8±18.02 μg/ml, respectively. The variations in the composition and content of bioactive compounds presented in the EOs of *Melaleuca* species may contribute to the differences in their enzyme inhibitory properties. Therefore, further research is needed to identify key compounds in the EOs that inhibit the enzymes.

Although the antimicrobial activity of the EOs of *Melaleuca* species has been reported in recent investigations, only a limited number studies on the enzyme inhibitory activity of *Melaleuca* species against α-amylase, α-glucosidase, AChE, XO have been identified to date. As regards the AChE and BChE enzymes, the EOs of *M. cajuputi* leaf and *M. citrina* leaf exhibited AChE inhibitory effects with 21.18±0.54 and 71.77±2.11% inhibition, respectively at a concentration of 100 μg/ml (49). The methanol extract from *M. cajuputi* leaf also exhibited potent AChE inhibitory effects with IC_{50} of 282 μg/ml (50). The EO from *M. alternifolia* leaf showed strong AChE and BChE inhibitory effects with IC_{50} of 153.7±1.25 and 85.6±0.7 μg/ml, respectively (51). In the case of the XO enzyme, the methanol and methanol-water extracts from *M. leucadendra* have been reported to have a XO inhibitory activity with IC_{50} values of 76.7 and 78.9 μg/ml, respectively (52). Involving α-amylase and α-glucosidase enzymes, the EOs from *M. alternifolia* leaf and *M. viridiflora* leaf demonstrated α-amylase inhibitory activity with 14 and 28% inhibition at a concentration of 0.67 mg/ml (53). To the best of our knowledge, the findings of the present study provide additional insight into the enzyme inhibitory properties of the leaf EO from *Melaleuca* species that have not been reported in previous research. However, a limitation of the present study is that the potential activity of compounds in the EOs was not investigated. Therefore, further studies are required to determine the most active compounds in the EO, such as molecular docking, *in silico* methods for drug design and discovery, from which the most potential.

The present study determined the chemical composition of the leaf EOs of *M. cajuputi*, *M. quinquenervia* and *M. leucadendra* with the dominance of oxygenated monoterpenes and sesquiterpenes. The present study also highlighted the chemical composition variation of EOs of *Melaleuca* species over geographical regions, in agreement with previous observations (7-12,14,26). The subsequent bioassays revealed that the leaf EOs of *M. cajuputi*, *M. quinquenervia* and

M. leucadendra exhibited antimicrobial, as well as enzyme inhibitory activities against α-amylase, α-glucosidase, AChE and XO. The findings presented herein provide additional insight into the enzyme inhibitory properties of the leaf EO from *Melaleuca* species not reported in previous research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

THDT and CVCL were involved in the conceptualization and methodology of the study, as well as in the formal analysis, writing of the original draft, and in the writing, review and editing of the manuscript. PHT and TTTV were involved in the methodology of the study, and in data investigation and formal analysis. TDTP, VMN and TNMN were involved in the formal analysis. PHT and TTTV confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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