Trop J Nat Prod Res, November 2024; 8(11): 9231 - 9237

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Chemical Composition, Antibacterial, Antioxidant, and Toxicity Profiles of Cajuput Oils from Diverse Malaysian Districts

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ARTICLE INFO

Article history:
Received 29 August 2024
Revised 16 September 2024
Accepted 31 October2024
Published online 01 December 2024

ABSTRACT

Despite the known medicinal properties of cajuput oils, there is a lack of comprehensive research on their chemical composition and bioactivity across different Malaysian districts. Thus, this study examined the chemical composition, antibacterial, antioxidant, and toxicity profiles of cajuput oils from different districts in Terengganu and Kelantan, Malaysia. Gas chromatography-flame ionization detection (GC-FID) and mass spectrometry were used to identify the chemical constituents. Antibacterial effectiveness was measured via broth microdilution assays, while antioxidant properties were tested using the DPPH assay. The toxicity profiles were determined using the brine shrimp lethality assay (BSLA). Major compounds in Besut essential oil (BTEO) were 4-(methoxycarbonyl) phenol (17.27%), cyclopentasiloxane decamethyl (13.43%), and methyl-lathodoratin (13.03%). Pasir Mas essential oil (PMEO) was characterized by 3-phenylbutyrophenone (18.30%), 2-isopropyl-10-methylphenanthrene (15.44%), and octanoic acid hex-4-yn-3-yl ester (14.03%). Meanwhile, the major components of Pasir Puteh essential oil (PPEO) were octanoic acid hex-4-yn-3-yl ester (32.89%), N1-(44-dimethyl-13-thiazolan-2-yliden) cyclohexan-1-amine (15.10%), and methyl-lathodoratin (9.44%). BTEO exhibited substantial antibacterial effects, with minimum inhibitory concentration (MIC) values between 0.125 and 0.5 mg/mL. Furthermore, PPEO exhibited significant antioxidant activity, with an IC_{50} value of 6.06 mg/mL. BSLA demonstrated non-toxicity for PMEO (LC_{50} 1047.13 µg/mL) and PPEO (LC_{50} 13182.57 µg/mL), while BTEO exhibited moderate toxicity (LC_{50} 630.96 µg/mL). These findings suggest that cajuput oil has potential as a natural antioxidant and antibacterial agent.

***Keywords*:** Cajuput oils, Chemical composition, Antibacterial activity, Antioxidant properties, Toxicity.

Introduction

Natural products have consistently inspired the development of innovative therapeutic agents to combat microbial infections and reduce the spread of antimicrobial resistance.[1] Many pharmaceutical products have been discovered by exploring the pharmacological properties of plant bioactive compounds, including essential oils (EOs).[2,3] Plant EOs have garnered significant interest due to their therapeutic potential, including antifungal, anti-inflammatory, antibacterial, enzyme-inhibitory, and antioxidant activities. These natural products provide a promising alternative to synthetic drugs, which are often associated with numerous side effects.[4–6] The cajuput tree, *Melaleuca cajuputi* or Gelam in Malaysia, is an evergreen tree predominant in Southeast Asia and Australia. It is widely recognized for its medicinal and fragrant properties—with its EO, known as cajuput oil, mainly extracted through steam or hydro-distillation techniques.[7]

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Citation: Isah M, Wan-Nor-Amilah WAW, Sallehudin NA, Azmi MN, Sul'ain MD. Chemical Composition, Antibacterial, Antioxidant, and Toxicity Profiles of Cajuput Oils from Diverse Malaysian Districts. Trop J Nat Prod Res. 2024; 8(11): 9231 – 9237 https://doi.org/10.26538/tjnpr/v8i11.37

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Cajuput oils have traditionally been used to treat skin diseases, respiratory tract infections, joint pain, depression, and anxiety.[8,9] During World War II, these oils and plant extracts were employed by Aboriginals and soldiers as effective antimicrobial and insect repellents.[10] Previous studies have identified several bioactive compounds in cajuput oils, including 1,8-cineole, terpinene-4-ol, *β*-eudesmol, α-pinene, phenolics, α-eudesmol, *β*-pinene, polycyclic aromatic hydrocarbons, and limonene.[5,11,12] *M. cajuputi* EOs are currently the focus of numerous investigations due to their diverse chemical constituents and pharmacological properties, including antimicrobial,[5,13] anti-inflammatory,[14] anti-cancer,[15] and antioxidant activities.[10] Despite their medicinal benefits, it is crucial to evaluate their toxicity profile to ensure the safety of potential consumers. Investigating EOs sourced from various locations provides valuable insights into how environmental, genetic, and cultural factors influence their chemical composition and bioactivity.[16–18] With the rising interest in natural products, especially EOs, as alternative treatments for microbial infections and oxidative stress, this study comprehensively compares cajuput oils sourced from the Besut district in Terengganu and Pasir Mas and Pasir Puteh districts in Kelantan, Malaysia, examining their chemical compositions, antibacterial potential, antioxidant properties, and toxicity profiles. This study emphasizes the impact of regional variations on the bioactivity of oils and identifies distinct compounds found in different regions. The findings will help expand the understanding of cajuput oils' therapeutic potential.

Materials and Methods

Plant material collection and extraction

In November 2023, leaves of *M. cajuputi* were collected from the Besut (5° 49' 12.32" N latitude and 102° 32' 15.60" E longitude), Pasir Mas

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

(6° 02' 36.37" N latitude and 102° 08' 31.17" E longitude), and Pasir Puteh (5.836163° N latitude and 102.4077409° E longitude) districts in Malaysia. After the plant material authentication, an official voucher number (PIIUM 0304) was presented to the Kulliyyah of Pharmacy at the International Islamic University Malaysia (IIUM). The leaves were thoroughly rinsed with tap water to remove soil particles and scrubbed with sterile distilled water. The cleaned leaves were finely chopped to increase the surface area for better water contact. Subsequently, they were placed in a flask with a raw material-to-water ratio of 1:4. The mixture underwent a distillation process for 75 min at 105°C. The EO was volatilized with steam, passed through a condenser, and collected in a flask, forming a biphasic mixture with water. The upper layer of the EO was separated from the water using a funnel and purified with Na_2SO_4. The purified EO was preserved in an amber container for further experiments.[19]

Chemicals and instruments

In the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, analytical-grade methanol with 99% purity (R&M Chemical, UK) was used. DPPH with 95% purity (Thermo Scientific Chemicals, UK) was used as the reagent for assessing antioxidant activities. Analytical-grade sodium sulphate (Na_2SO_4) with 98.5% purity (MilliporeSigma, Germany) was used for EO purification. Mueller Hinton broth (MHB) and Mueller Hinton agar (MHA) (Hi-Media, India) were used as the culture media for the antibacterial assay. Reagent-grade dimethyl sulfoxide (DMSO) with 99.9% purity (Merck, Germany) was used as a control in antibacterial and toxicological assays. Artificial seawater prepared with distilled water and sea salt was used for the brine shrimp lethality assay. A PerkinElmer AutoSystem XL gas chromatograph (GC) with a flame ionization detector (FID) (Waltham, USA) was used to identify chemical ingredients in the EO. A DB-5 capillary column (Agilent Technologies, USA) was used with GC to separate the EO components, and 96-well microtiter plates (Thermo Fisher Scientific, Waltham, USA) were used for antibacterial and antioxidant experiments. Finally, an Epoch microplate spectrophotometer (BioTek, Santa Clara, CA, USA) was used to measure absorbance in the DPPH assay at 517 nm.

Chemical analysis of EOs using GC-FID/MS

The EO samples were analysed using a PerkinElmer AutoSystem XL gas chromatograph with an FID and a DB-5 capillary column. The oven temperature was set to change gradually from 70°C to 250°C, with increases of 3°C every minute. The temperature was then held steady for 2 min at the beginning and end of the procedure. Helium with a flow rate of 1.1 mL per minute served as the carrier gas. The temperature of the injector was maintained at 250°C and that of the FID was maintained at 280°C. A split ratio of 1:35 was used with an injection volume of 0.02 µL. For GC-MS, a Clarus 680 GC was used; a Clarus SQ 8C mass spectrometer outfitted with an Elite-5 MS fused-silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm) was linked to the gas chromatograph. The oven was heated to 240°C, with temperature increases of 3°C every minute, from 60°C to the final setting. At a pace of 5°C per minute, the temperature subsequently increased to 270°C. All other temperatures were maintained at 220°C, including that of the injector, which was set to 250°C. The split ratio was 1:50, with an injection volume of 0.03 µL. The ionization energy was 70 eV, and the mass scan ranged from 40 to 450 amu. Chemical constituents were identified using retention indices from coinjected n-alkanes and by comparing the MS library results (NIST 17 and Wiley Version 10) with literature fragmentation patterns. Each chemical's relative percentage was determined by calculating the GC peak area.[20,21]

Antibacterial assay

The bacterial strains used in this study were *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Klebsiella pneumoniae* ATCC 1706, and *Escherichia coli* ATCC 25922. These bacteria were collected from the Microbiology Laboratory Universiti Sains Malaysia Health Campus. The EOs were prepared at concentrations between 0.015 and 1 mg/mL. The diluted sample was transferred to 96-well plates. Each well contained 50 µL of the diluted EO, 40 µL of the MHB, and 10 µL of standardized inoculum (10^6 CFU/mL). A growth medium was used to check for sterility, and 2% DMSO was used as the control. The inoculated plates were incubated for 18 h at 37°C. The minimum inhibitory concentrations (MICs) were determined based on turbidimetric data, which showed the lowest concentration of bacterial growth inhibition. The minimum bactericidal concentrations (MBCs) were determined by transferring aliquots from wells with inhibited growth onto MHA and incubated for 18 h at 37°C. Bacterial death (99.9%) is considered MBC.[21]

DPPH radical scavenging activity

The antioxidant properties of the EOs were examined using the DPPH test.[22] A 100 mM DPPH solution in methanol was prepared by dissolving 3.9432 mg of DPPH in 100 mL of methanol. The EOs were prepared in concentrations of 3.125–50 mg/mL. The prepared solution containing the test sample, DPPH reagent, and methanol in 96-well microtiter plates was kept in the dark at 25°C for 30 min. Absorbance was measured at 517 nm using an Epoch microplate spectrophotometer (BioTek, Santa Clara, CA, USA). Methanol and ascorbic acid were used as the blank and positive controls, respectively. The following equation was used to ascertain the inhibition percentage in DPPH scavenging:

$$\text{DPPH Scavenging (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of the test}}{\text{Absorbance of control}} \times 100$$

Brine shrimp lethality test

The toxicity profile of the EOs was investigated in accordance with the process outlined by Isah et al.[23] One litre of distilled water was mixed with 38 g of sea salt to prepare artificial seawater, followed by filtration to obtain a homogenous solution. Brine shrimp (*Artemia salina*) eggs, sourced from pet shops, were dispersed in synthetic seawater with a pH of 7. The eggs hatched into active nauplii over 48 h under continuous illumination and oxygenation. Ten nauplii were transferred to plates containing 5 mL of seawater using a pipette. The brine solution was supplemented with EO at varying concentrations (62.5–1000 µg/mL). The results were documented following a 24-h incubation period. Absolute DMSO was used as the positive control, while 2% DMSO was used as the negative control. The following equation was used to determine the percentage mortality rate after three experiments were conducted:

$$\text{Percentage mortality} = \frac{\text{Total nauplii} - \text{survived nauplii}}{\text{Total nauplii}} \times 100$$

Statistical Analysis

The IC_{50} values for the cajuput oils were calculated using GraphPad Prism 8.0.1 through nonlinear regression, while the LC_{50} values were calculated using probit regression analysis in Microsoft Office Excel 2016. ANOVA and post hoc were used to determine statistical significance. All experiments were conducted in triplicate.

Results and Discussion

Chemical constituents of EOs

The phytochemical analysis revealed that BTEO predominantly contained phenolic compounds [4-(methoxycarbonyl) phenol (17.27%), cyclic siloxane (cyclopentasiloxane, decamethyl) (13.23%), flavonoids (methyl-lathodoratin) (13.04%), and 3-phenylbutyrophenone (7.57%)] and other polycyclic aromatic hydrocarbons, as presented in Table 1. The major constituents identified in PMEO were 3-phenylbutyrophenone (18.30%), polycyclic aromatic hydrocarbons (2-isopropyl-10-methylphenanthrene (15.44%)), fatty acids (octanoic acid, hex-4-yn-3-yl ester (14.03%)), naphthalene, 1-phenyl (8.12%), and 10-methylanthracene-9-carboxaldehyde (6.14%) (Table 2). The main bioactive compounds in PPEO included fatty acids (octanoic acid, hex-4-yn-3-yl ester (32.89%), thiazole derivatives (N1-(4,4-dimethyl-1,3-thiazolan-2-yliden) cyclohexan-1-amine (15.10%), flavonoids (methyl-lathodoratin (9.44%)), and polycyclic aromatic hydrocarbons (2-isopropyl-10-methylphenanthrene (9.13%)), as presented in Table 3. The GC-FID/MS analysis results indicated that only PMEO and PPEO contained fatty acids. However, phenolic acid (24-dihydroxybenzoic acid) was exclusive to BTEO.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Analysis of *M. cajuputi* BTEO using GC-FID/MS

No.	Name of compounds	RT (mins)	RI	RI ref	M. F.	Area %
1	3-Phenylbutyrophenone	9.859	1435	1837	C16H16O	7.67
2	Cyclopentasiloxane, decamethyl-	10.915	1473	1215	C10H30O5Si5	13.43
3	4-(methoxycarbonyl) phenol	11.830	1506	1504	C11H16O3Si	17.27
4	Phenanthrene, 4,5-dimethyl-	18.363	1744	1963	C16H14	0.82
5	10-Methylanthracene-9-carboxaldehyde	19.863	1805	383	C16H12O	2.96
6	Methyl lathodoratin	20.852	1845	-	C12H12O4	13.04
7	2-Isopropyl-10-methyl phenanthrene	21.604	1875	-	C18H18	5.45
8	2,4-Dihydroxybenzoic acid	24.897	2017	1800	C7H6O4	2.52
9	Cyclononasiloxane, octadecamethyl-	25.383	2039	1855	C18H54O9Si9	5.03
10	Cycloheptasiloxane	26.237	2078	1538	O7Si7	2.44

RT stands for 'retention time', RI refers to the 'retention index', RI Ref refers to the 'retention index references', and M.F. stands for molecular formula.

Table 2: Analysis of *M. cajuputi* PMEO using GC-FID/MS

No.	Name of compounds	RT (min)	RI	RI ref	M. F.	Area %
1	Cyclohexasiloxane, dodecamethyl-	9.225	1412	1350	$C_{18}H_{54}O_9Si_9$	2.68
2	1,2-Benzenediol, o-(3-cyclopentyl propionyl)-	9.583	1425	-	$C_{20}H_{22}O_4$	2.77
3	Naphthalene, 1-phenyl-	9.873	1435	1832	$C_{16}H_{12}$	8.12
4	Octanoic acid, hex-4-yn-3-yl ester	10.926	1473	1533	$C_{14}H_{24}O_2$	14.03
5	3-Phenylbutyrophenone	11.845	1506	1837	$C_{16}H_{16}O$	18.30
6	Methyl lathodoratin	19.891	1806	-	$C_{12}H_{12}O_4$	3.41
7	2-Isopropyl-10-methyl phenanthrene	20.872	1846	-	$C_{18}H_{18}$	15.44
8	10-Methylanthracene-9-carboxaldehyde	21.610	1876	383	$C_{16}H_{12}O$	6.14
9	Cyclononasiloxane, octadecamethyl-	25.348	2037	1858	$C_{18}H_{54}O_9Si_9$	5.75

RT stands for 'retention time', RI refers to the 'retention index', RI Ref refers to the 'retention index references', and M.F. stands for molecular formula.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

All EO samples contained the flavonoid methyl-lathodoratin, with BTEO showing a higher concentration than PMEO and PPEO. Moreover, polycyclic aromatic hydrocarbons were present in all samples. These variations in chemical composition are attributable to differences in geographical regions.[24] Previous studies have identified similar compounds,[25,26] although some studies have reported different major constituents such as guaiol, α-eudesmol, *β*-selinenol, terpineol, and cineol.[27] The discrepancies in the phytochemical constituents could be due to the EO extraction methods, ecological conditions, and the timing of sample collection.[24,28] The phenolic compounds, flavonoids, aromatic hydrocarbons, and terpenoids in these EOs possess a wide range of bioactivities.[3,29] As compared to synthetic drugs, plant-based antimicrobials present significant therapeutic potential with fewer side effects.[30] Therefore, it is imperative to focus on natural products, such as EOs, as alternative therapeutic sources to combat microbial infections and oxidative stress.

Table 3: Analysis of *M. cajuputi* PPEO using GC-FID/MS

No.	Name of compounds	RT (min)	RI	RI ref	M. F.	Area %
1	Octanoic acid, hex-4-yn-3-yl ester	9.930	1437	-	$C_{14}H_{24}O_2$	32.89
2	6-Phenylisoquinoline	11.793	1505	2030	$C_{15}H_{11}N$	1.40
3	3-Phenylbutyrophenone	13.187	1554	1837	$C_{16}H_{16}O$	4.04
4	N1-(4,4-Dimethyl-1,3-thiazolan-2-yliden) cyclohexan-1-amine	14.593	1605	-	$C_{11}H_20N_2S$	15.10
5	3,6-Dimethylphenanthrene	18.013	1733	1983	$C_{16}H_{14}$	6.37
6	10-Methylanthracene-9-carbaldehyde	19.832	1804	383	$C_{16}H_{12}O$	4.01
7	Eicosamethyl-cyclodecasiloxane	22.513	1913	2023	$C_{20}H_{60}O_{10}Si1_0$	2.96
8	Methyl lathodoratin	24.697	2008	-	$C_{12}H_{12}O_4$	9.44
9	2-Isopropyl-10-methyl phenanthrene	25.285	2034	-	$C_{18}H_{18}$	9.13
10	Oxymorphone	27.328	2129	2550	$C_{17}H_{19}NO_4$	3.12

RT stands for 'retention time', RI refers to the 'retention index', RI Ref refers to the 'retention index references', and M.F. stands for molecular formula.

Antibacterial activity

M. cajuputi EOs showed remarkable antibacterial activity, even at relatively low MIC values. BTEO (MIC value 0.125–0.5 mg/m) was the most potent EO, followed by PMEO and PPEO, with identical MIC values (0.5–1 mg/mL). However, no activity was determined against *E. coli* at the doses used in this study. In contrast, *S. aureus* showed the highest susceptibility to EOs, while *S. epidermidis* and *K. pneumoniae* showed lower susceptibility (Table 4). The antibacterial assays showed that none of the tested EOs inhibited *E. coli* growth. Additionally, *K. pneumoniae* was resistant to PMEO. Gram-negative bacteria mostly exhibit lower susceptibility to EOs due to their complex cell wall structures.[21] BTEO exhibited potent antibacterial activity compared to PMEO and PPEO, likely attributable to its high concentration of phenolics and flavonoids, including methyl-lathodoratin and dihydroxybenzoic acid.[31,32] In previous research, cajuput oil exhibited antimicrobial activity against *S. aureus* and *E. coli* (MICs between 800 and 6400 µg/mL).[33] Similarly, Wahab et al.[13] demonstrated that *M. cajuputi* EO exhibited antibacterial activity against *E. coli, K. pneumoniae, S. aureus, S. pyogenes,* and methicillin-resistant *S. aureus,* with MIC values of 0.714%. The observed resistance of *E. coli* in this study is attributable to the differences in the bacterial strains used across studies. Our findings are particularly significant, especially regarding *S. aureus and S. epidermidis*, which are known to cause severe skin infections in humans.[34]

Antioxidant activity

Figure 1 presents the results from the DPPH assay, showing IC_{50} values for BTEO, PMEO, and PPEO. According to the figure, PPEO exhibited the highest antioxidant potency (IC_{50} 6.60 mg/mL), followed by PMEO (IC_{50} 23.51 mg/mL) and BTEO (IC_{50} 33.30 mg/mL). The positive control (ascorbic acid) was the most effective antioxidant among the groups tested, as it had the lowest IC_{50} value (0.02 mg/mL), and was substantially more effective than all EOs (p-values = 0.001). The antioxidant activities observed, particularly in PPEO, are attributable to the methyl-lathodoratin (flavonoid) and ketone compounds (3-phenylbutyrophenone), known for their substantial antioxidant properties.[35] These findings are consistent with those of Rini et al.,[36] who documented the antioxidant properties of *M. Leucadendron* EO with an IC_{50} value of 4.24 mg/mL, similar to the IC_{50} value of PPEO. Yasin et al.[10] showed that tea tree oil significantly scavenges free radicals, as evidenced by its DPPH value of 86.85 ± 2.43 mg/mL. These values from previous research are relatively higher than the IC_{50} values obtained in this study, highlighting the novelty and significance of the present study. The distinct chemical compositions of each sample might have caused variations in the antioxidant properties of these EOs.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

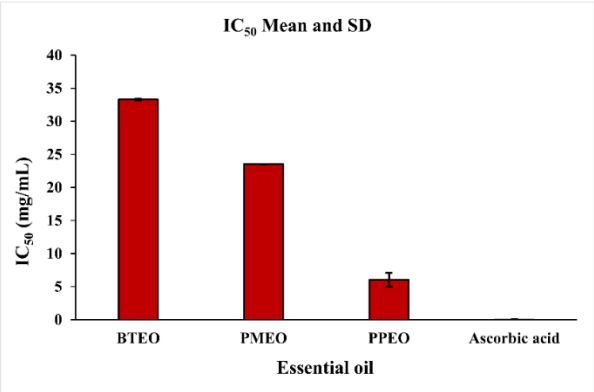

Figure 1: Antioxidant properties of three samples of *M. cajuputi* EO and a control (ascorbic acid). Values are presented as mean (n = 3) ± standard deviation (SD). BTEO: Besut essential oil; PMEO: Pasir Mas essential oil; PPEO: Pasir Puteh essential oil. (p-values = 0.001).

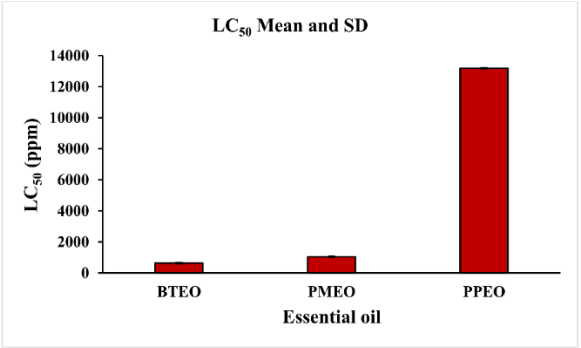

Figure 2: Brine shrimp lethality test of different EOs from *M. cajuputi* leaf. Values are presented as mean (n = 3) ± standard deviation (SD). BTEO: Besut essential oil, PMEO: Pasir Mas essential oil, PPEO: Pasir Puteh essential oil. (p-values = 0.001).

Table 4: Antibacterial results of *M. cajuputi* EOs from different districts in Malaysia

	S. aureus		*S. epidermidis*		*K. pneumoniae*		*E. coli*	
EO (mg/mL)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
BTEO	0.125	0.25	0.50	0.50	0.50	1.00	ND	ND
PMEO	0.50	1.00	1.00	1.00	ND	ND	ND	ND
PPEO	0.50	0.50	0.50	0.50	1.00	1.00	ND	ND

BTEO: Besut essential oil, PMEO: Pasir Mas essential oil, PPEO: Pasir Puteh essential oil, ND: not determined.

Brine shrimp lethality

Figure 2 presents the LC_{50} values of the tested EOs from the *M. cajuputi* leaf. The toxicity analysis showed that BTEO had mild toxicity (LC_{50} value of 630.96 µg/mL), while PMEO (LC_{50} value of 1047.13 µg/mL) and PPEO (LC_{50} value of 13182.57 µg/mL) were non-toxic. All EOs exhibited notable differences in toxicity, with p-values of 0.001 for each pairwise comparison. Clarkson's toxicity classification index categorizes substances based on their LC_{50} values. Samples with LC_{50} values >1000 µg/mL are classified as non-toxic, while those within 1000–500 µg/mL are considered less toxic. The samples with LC_{50} values ranging between 500 and 100 µg/mL are classified as moderately toxic and those with <100 µg/mL are considered highly toxic.[23] Our findings align with the toxicity effect of EO from *M. cajuputi,* which has an LC_{50} value of 2131 µg/mL. [28] The mild toxicity observed in BTEO might be due to its high phenolic and flavonoid contents.[37] Additionally, the high contents of these compounds in EOs have been reported to increase their cytotoxicity.[38] The chemical composition of the extracted EOs might have been influenced by the geographical origin of the *M. cajuputi* plant—which, in turn, impacts their biological activities.[35,39]

Conclusion

The chemical compositions and antibacterial, antioxidant, and toxicological profiles of cajuput oils sourced from the Besut and Kelantan districts in Malaysia were investigated in this study. The findings demonstrated the therapeutic potential of these EOs in combating bacterial infections and oxidative stress. BTEO exhibited remarkable antibacterial properties, whereas PPEO demonstrated better antioxidant properties. Exploring EOs sourced from diverse regions enhances our understanding and opens new avenues for pharmaceutical advancements. Overall, cajuput oils sourced from Malaysia demonstrate potential as a natural antibacterial and antioxidant agent suitable for pharmaceuticals, personal care, and sustainable agricultural products. Their unique regional characteristics present opportunities for tailored formulations across several industries.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims related to the content of this article will be borne by them.

Acknowledgements

We sincerely thank the School of Health Sciences, Universiti Sains Malaysia, for providing the necessary facilities to conduct this research. We also appreciate Siti Kurunisa Binti Mohd Hanafiah for her invaluable technical assistance. Her support was instrumental in completing this study.

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

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