GELAM (MELALEUCA CAJUPUTI POWELL) : HONEY & ESSENTIAL OIL

ARTICLES FOR FACULTY MEMBERS

Title/Author	A comparative study of antibacterial and antivirulence activities of four selected honeys to Manuka honey / Al-Kafaween, M. A., & Al-Jamal, H. A. N.
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Title/Author	Antioxidant properties and characterization of Heterotrigona itama honey from various botanical origins according to their polyphenol compounds / Shamsudin, S., Selamat, J., Abdul Shomad, M., Ab Aziz, M. F., & Haque Akanda, M. J.
Source	*Journal of Food Quality* Volume 2022 (2022) 2893401 Pages 1-14 https://doi.org/10.1155/2022/2893401 (Database: Hindawi)

Title/Author	Evaluation of antibacterial activity of essential oils of Melaleuca cajuputi Powell / Wahab, N. Z. A., Ja'afar, N. S. A., & Ismail, S. B.
Source	*Journal of Pure and Applied Microbiology* Volume 16 Issue 1 (2022) Pages 549–556 https://doi.org/10.22207/JPAM.16.1.52 (Database: Journal of Pure and Applied Microbiology (JPAM)

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Source	*Sains Malaysiana* Volume 48 Issue 9 (2019) Pages 1919-1926 https://doi.org/10.17576/jsm-2019-4809-13 (Database: Jurnal Sains Malaysiana, UKM)

Title/Author	Green facile synthesis of cajuput (Melaleuca cajuputi Powell.) essential oil loaded chitosan film and evaluation of its effectiveness on shelf-life extension of white button mushroom / Chaudhari, A. K., Das, S., Singh, B. K., & Kishore Dubey, N.
Source	*Food Chemistry* Volume 401 (2023) 134114 Pages 1-11 https://doi.org/10.1016/J.FOODCHEM.2022.134114 (Database: ScienceDirect)

Title/Author	Physicochemical properties, chemical components, and antibacterial activity of Melaleuca cajuputi Powell essential oil leaves from Quang Tri Province, Vietnam / Quoc, L. P. T.
Source	*Bulletin of the Chemical Society of Ethiopia* Volume 35 Issue 3 (2021) Pages 677–683 https://doi.org/10.4314/bcse.v35i3.18 (Database: African Journals Online (AJOL)

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Title/Author	Screening of acetylcholinesterase inhibitory activity in essential oil from Myrtaceae / Petrachaianan, T., Chaiyasirisuwan, S., Athikomkulchai, S., & Sareedenchai, V.
Source	*Thai Journal of Pharmaceutical Sciences* Volume 43 Issue 1 (2019) Pages 63–68 https://www.thaiscience.info/Journals/Article/TJPS/10996329.pdf (Database: Thai Journal of Pharmaceutical Sciences (TJPS)

Title/Author	Unveiling the cellular and molecular mode of action of Melaleuca cajuputi Powell. essential oil against aflatoxigenic strains of Aspergillus flavus isolated from stored maize samples / Chaudhari, A. K., Singh, V. K., Das, S., Kujur, A., Deepika, & Dubey, N. K.
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ARTICLES FOR FACULTY MEMBERS

CULTURAL HERITAGE

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ORIGINAL ARTICLE

A comparative study of antibacterial and antivirulence activities of four selected honeys to Manuka honey

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ABSTRACT

Background and Objectives: Honey has excellent antibacterial properties against various microorganisms of several different species. To date, there is no comparative evaluation of the antibacterial activity of Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) with that of Manuka honey (MH). The purpose of this study was to conduct such study and to compare the antibacterial activity of JH, KMH, GH, and AH with that of MH against *Pseudomonas aeruginosa* and *Streptococcus pyogenes.*

Materials and Methods: Activity was assessed using broth microdilution, time kill viability, microtiter plate, scanning electron microscope (SEM) and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR).

Results: The susceptibility tests revealed promising antibacterial activities of all honeys against both bacteria. The MICs of JH, KMH, GH, and AH ranged from 20% to 25% compared to MH (12.5%) against both bacteria. The MBCs of JH, KMH, GH, and AH ranged from 20% to 50% compared to MH (20%) against both bacteria. Treatment of both bacteria with 2× MIC (Minimum inhibitory concentration) of MH, JH, KMH, GH, and AH for 9 hours resulted in reduction in colony-forming unit (CFU/ml). SEM images showed that the morphological changes, cell destruction, cell lysis and biofilm disruption in both bacteria after exposure to all honeys. RT-qPCR analysis revealed that the expression of all genes in both bacteria were downregulated following treatment with all honeys. Among the all-tested honeys, MH showed the highest total antibacterial and antivirulence activities.

Conclusion: Our results indicate that all honeys activity included inhibition of both bacteria due to a decrease in expression of essential genes associated with both bacteria, suggesting that all honeys could potentially be used as an alternative therapeutic agent against certain microorganisms particularly against *P. aeruginosa* and *S. pyogenes.*

Keywords: Honey; *Pseudomonas aeruginosa*; *Streptococcus pyogenes*; Gene expression profiling; Real-time polymerase chain reaction

INTRODUCTION

The biofilm trait of high antimicrobial resistance to antibiotics and disinfectants is a multifactorial and is attributed to slow antibiotic penetration, reduced microbial growth rates, persisters and unique physiology (1). Bacterial biofilms are normally pathogenic and can cause nosocomial infections. The National Institutes of Health (NIH) reported that among all microbial and chronic infections, 65% and 80%, re-

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spectively, are associated with biofilm development (2). Bacterial infections are becoming more difficult to treat due to higher numbers of patients with multiple underlying conditions and the rise in pathogens, which are resistant to modern antimicrobial treatments (3). This is difficult with a rarity of new antibiotics in development and has urged renewed interest in several novel antimicrobial therapeutics. Part of the challenge in treating bacterial infections is biofilm formation. When bacteria exist as a biofilm, they are significantly less sensitive to antibiotics; this is a result of metabolic changes to cells within the biofilm and structural features influencing drug permeability (4). The development and range of antibiotic resistance are an alarming threat to the effective treatment and inhibition of bacterial infections in humans and animals (4). Solving this problem requires searching for natural antimicrobial alternatives (5). Presently, more researchers are turning their attention to conventional medicines as a possible source of antimicrobial agents (6). Honey is one of the oldest traditional remedies that has been extremely reputed and extensively utilised for the treatment of various human infections for over 2000 years ago (7). Nowadays, different kinds of honey have been used in several nations as an alternative to pharmaceutical products for treating infected, burn wounds and contaminated. The antimicrobial properties of honey may be attributed to many factors, including high osmolarity, acidity, in addition to the presence of hydrogen peroxide (H_2O_2) and non-peroxide components, such as methylglyoxal (8). Honey's composition is reliant on the environmental and geographical areas from which the original nectar was collected (8). This is attributed to natural variations in floral sources and climatic conditions at different locations (8). Therefore, several researchers have investigated the therapeutic effects of kinds of honey obtained from different geographical areas worldwide (9, 10). In addition, some honey varieties have been implicated in the differential expression of a number of genes essential for bacterial survival and virulence, including those involved in stress tolerance, virulence factor production, as well as multicellular behaviors, such as biofilm formation, and quorum sensing (11, 12). The present study aimed to investigate the effects of five kinds of honey on *P. aeruginosa* and *S. pyogenes* with a view to better understanding its potential to impact virulence and to compare the antibacterial activity of Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) with that of Manuka honey (UMF +10 (MH).

MATERIALS AND METHODS

Honey samples. Manuka honey (UMF +10 (MH), Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) were purchased from commercial supplier. The samples were packed and sealed in amber glass bottles and stored at 4ºC in the dark until processed (13).

Microorganisms and culture conditions. A reference strains of *P. aeruginosa* ATCC 15692 and *S. pyogenes* ATCC 49399 were obtained from the American Type Culture Collection (ATCC). *P. aeruginosa* and *S. pyogenes* were stored at -80ºC in nutrient broth (NB) medium (Oxoid, UK) with 20% (v/v) glycerol. Prior to each assay, *P. aeruginosa* and *S. pyogenes* strains were sub-cultured from the frozen stock preparations onto nutrient agar (NA) plates (Oxoid, UK). The plates were incubated at 37ºC for 24 hours. Pure liquid cultures (pre-inocula) of *P. aeruginosa* and *S. pyogenes* were maintained in NB (13, 14).

Agar well diffusion assay. The inoculum density of *P. aeruginosa* and *S. pyogenes* was adjusted to be 0.5 McFarland. A sterile cotton swab was dipped into the bacterial suspension and was rotated onto the tube with firm pressure to remove excess fluid. The swab was streaked over the entire surface of Muller Hinton agar plate (Oxoid, UK) for three times and each time the plate was rotated approximately 90ºC to ensure even distribution. A sterile 9 mm cork borer was used to create six wells of agar plate. The wells of agar plate were added with 150 µL of 100%, 75%, 50%, and 25% (w/v) concentrations of MH, JH, KMH, GH and AH. Distilled water was used as a negative control. The plates were incubated at 37ºC for 24 hours. Digital venire calliper was used to measure the zones of inhibition (13, 14).

Minimum inhibitory concentration (MIC). The concentrations of MH, JH, KMH, GH and AH; 50%, 25%, 12.5%, 6.25%, 3.125% and 1.562% (w/v) were freshly prepared with NB broth. The minimum inhibitory concentration (MIC) value was determined using broth microdilution method. Briefly, the cell density for both bacteria was adjusted to be 1×10^8

CFU/mL. A 100 µL was transferred into microtiter plate with 100 µL of each concentration of MH, JH, KMH, GH and AH. Broth medium only was used as negative controls and inoculum without honey was served as positive controls. The plates were incubated overnight at 37°C. Absorbance was measured by using the microtiter plate reader (Tecan Infinite 200 PRO, Austria) at 540 nm. The MIC_{50} and MIC_{90} were determined by using the following formula as mentioned below (13, 14).

$$\text{Growth inhibition (\%)} = \frac{\text{1-OD of the test well – OD of corresponding negative control}}{\text{OD of bacterial growth control−OD of sterility control}} \times 100$$

Minimum bactericidal concentration (MBC). MBC test was performed after MIC assay via streak plate method. A 20 µL from each well of the microdilution method was taken and plated onto NA plates. Subsequently, the plates were incubated for 24 hours at 37°C. MBC was considered as the lowest antimicrobial concentration that produced no colony growth (13, 14).

Time-kill studies. The inhibitory concentration (2×MIC) of MH, JH, KMH, GH and AH that was chosen for subsequent experiments was 2×MIC, because it was two times the MIC. The effect of MH, JH, KMH, GH and AH on the viability of the cells was determined by time-kill curve studies. By inoculating 100 µL of 1×10^6 CFU/mL of both bacteria into 10 mL NB with and without 2×MIC of each honey. Then, the samples were incubated at 37°C in a shaking water bath (100 rpm) for 9 hours. After incubation time, the samples were collected every 3 hours up to 9 hours. Then, the mean of Log10 CFU/ml over time were plotted for each sample. Subsequently, the log reduction (LR) was calculated for each sample by subtracting the Log_{10} CFU at zero time and the Log_{10} CFU at 9 hours of incubation to determine the TVCs (13, 14).

Biofilm assessment. Different concentrations of MH, JH, KMH, GH and AH; 15%, 30%, 45%, and 60% (w/v) in NB were freshly prepared from a stock solution of 100% (w/v). Both bacteria were adjusted to be 0.5McFarland within 0.05 to 0.10 at 600 nm wavelength using spectrophotometer. Then, 200 µl of the culture was dispensed into wells of microtiter plate and incubated at 37°C for 48 hours. After biofilms were formed, 100 µL of planktonic cells were removed and replaced with 100 µL of each honey concentrations. Then, the plates were incubated for overnight (18 hours). Biofilm without honey treatment was served as a positive control, broth only was employed as a sterility control, and honey with broth was served as a corresponding negative control. Finally, after incubation time was done, the media were then removed by invertip the plate and tapping the plate. The plate was washed three times with PBS to remove free-floating planktonic bacteria and was then drained inverted for drying. The plates were stained with 200 µl of 0.1% crystal violet for 5 minutes. Then, the plates were rinsed under running tap water to remove excess stain and were dried at room temperature before solubilizing the biofilm with 95% of ethanol. Microplate reader (Tecan Infinite 200 PRO, Austria) was used to measure the optical density at 595 nm wavelength. Percentage of biofilm degradation was calculated by following formulas as described below (13, 15).

$$\text{Biofilm (\%)} = \frac{(OD_{595}\text{ of positive control})-(OD_{595}\text{ of individual or (combined) antimicrobial})}{(OD_{595}\text{ of positive control})} \times 100\%$$

Scanning electron microscope (SEM) of single-species biofilm. *P. aeruginosa* and *S. pyogenes* were cultivated in NB for 24 hours at 37°C and adjusted to be equal 0.5McFarland. Centrifugation at 3,500g for 10 min at room temperature was used to collect cells and suspended in NB with MICs of MH, JH, KMH, GH and AH for 24 hours. Inoculums without adding honey were used as a positive control. Pellets were collected, fixed overnight with 2.5% (v/v) glutaraldehyde in 0.01 M phosphate buffer solution (PBS). 0.1 M sodium cacodylate buffer were used to rinse the samples. After that, 1% osmium tetroxide in 0.1 M sodium cacodylate buffer were used to rinse the samples. 0.1 M sodium cacodylate were used again to rinse the samples. Subsequently, 0.01 PBS was used to wash the samples and underwent serial dehydration with ascending concentrations of ethanol and subjected to critical point drying. The samples were coated with platinum, placed onto the copper stage holder and examined by scanning electron microscope (SEM) (JEOL 6360LA, Japan) (13).

Scanning electron microscope (SEM) of mixed-species biofilm. The effects of MH, JH, KMH, GH and AH on mixed-species biofilm was determined using SEM. Briefly, *P. aeruginosa* and *S.*

pyogenes cell suspensions were adjusted to be equal to 0.5 McFarland, 1:1 mixed-species were prepared in sterile NB and 200 µL of this standard, cell suspension was added into microtiter plate and then incubated for 24 hours at 37°C. After incubation time was done, the liquid phase was replaced by 200 µL of MIC of each honey. Biofilm mixed-species without honey treatment was used as a positive control. Then, the plates were incubated for 24 hours at 37°C. Subsequently, all samples were then centrifuged at 3500 rpm for 5 minutes. SEM procedure was followed as described earlier. The samples were then viewed by SEM (13).

RNA extraction for RT-qPCR. A0.5 McFarland of *P. aeruginosa* and *S. pyogenes* cells were treated with MIC of MH, JH, KMH, GH and AH. Meanwhile, positive control was included inoculum without honey. Then, the incubation time was performed at 37ºC for 8 hours in a shaking (100 rpm). Subsequently, one ml of treated and untreated cells was separated and centrifuged at 13,000 rpm for 2 minutes. The supernatant was discarded and the pellet was washed with PBS. Total RNA extracted using kit SV Total RNA Isolation System (Promega, UK) according to the manufacturer's instructions. Total RNA concentrations were examined by ImplenNanoPhotometer® NP80. Total RNA samples were converted to cDNA according to the manufacturer's instructions (Promega, UK). Samples were diluted to 100ng/µl using ultra-pure water. For each reaction, qPCR mastermix was prepared by following the manufacturer's instructions (Promega, UK) and PCR primers were used as shown in Tables 1 and 2. The following PCR protocol was used: denaturation at 95ºC for 2 minutes in one cycle, amplification at 95ºC for 15 seconds in 40 cycles and a final elongation annealing at 60ºC for 1 min in 40 cycles. Densitometry was performed using the Applied Biosystems StepOne Software v2.3. To determine and calculate the level of gene expression, a modified $2^{-\Delta\Delta}$ Ct method was used (13, 16-18).

Statistical analysis. For all assays, all experiments were carried out in triplicate. All data were expressed as mean ± standard deviation. Independent student t-test from (SPSS version 20) was used to compare between treated and untreated groups. The statistical analyses performed were considered significant when $P< 0.05$.

RESULTS

Agar well diffusion assay. Inhibition zone for MH, JH, KMH, GH and AH against *P. aeruginosa* and *S. pyogenes* is mentioned in Tables 3 and 4. All tested honeys were observed to have antibacterial activity against both bacteria. In general, all tested honeys showed a measurable antibacterial activity on both bacteria with different values. MH, JH, KMH, GH and AH showed a significant inhibition zone against both bacteria at 100%, 75%, 50% and 25% concentrations.

Determination of MICs, $MICs_{90}$, $MICs_{50}$ and MBCs. As shown in Table 5, the MIC value for MH, JH, KMH, GH and AH against planktonically grown *P. aeruginosa* was 12.5%, 25%, 20%, 20% and 20% (w/v) respectively. The MBC value for MH, JH, KMH, GH and AH against planktonically grown *P. aeruginosa* was 20%, 50%, 25%, 25% and 50% (w/v) respectively. In addition, the MIC_{90} for MH, JH, KMH, GH and AH against planktonically grown *P. aeruginosa* was 20%, 50%, 25-50%, 25% and 50% (w/v) respectively. The MIC_{50} for MTH against planktonically grown *P. aeruginosa* was 12.5%, 20%, 20-25%, 20% and 25% (w/v) respectively.

From Table 6, the MIC value for MH, JH, KMH, GH and AH against planktonically grown *S. pyogenes* was 12.5%, 25%, 20%, 20% and 20% (w/v) respectively. The MBC value for MH, JH, KMH, GH and AH against planktonically grown *S. pyogenes* was 20%, 50%, 25%, 50% and 50% (w/v) respectively. In addition, the MIC_{90} for MH, JH, KMH, GH and AH against planktonically grown *S. pyogenes* was 25%, 50%, 25-50%, 25-50% and 50% (w/v) respectively. The MIC_{50} for MTH against planktonically grown *S. pyogenes* was 20%, 20%, 20-25%, 20% and 25% (w/v) respectively.

Time-kill studies. The total number of *P. aeruginosa* cells significantly decreased when exposed to 2×MIC MH, JH, KMH, GH, and AH. However, *P. aeruginosa* incubated with 2×MIC MH, JH, KMH, GH, and AH demonstrated rapid loss of viability. Therefore, after exposure to 2×MIC of MH, JH, KMH, GH and AH, *P. aeruginosa* resulted in 1.7-$\log_{10}$, 1.3-$\log_{10}$, 1.5-$\log_{10}$, 1.4-$\log_{10}$ and 1.2-$\log_{10}$ reduction in CFU/ml compared to untreated cells at 6 hours incubation ($P<0.05$) respectively. *P. aeruginosa* incubated with 2×MIC MH, JH, KMH, GH, and AH demonstrated that the greatest bactericidal activ-

Table 1. Gene specific primers of *P. aeruginosa* used for RT-qPCR analysis

Gene name	Amplicon Size (bp)	Annealing temp (C°)	Direction	Primer sequence (5' → 3')	References
*fli*A	132	55	Forward	CTCCAATTGAGCCTCGAAGA	(13, 19)
			Reverse	TTCGTTGTGACTGAGGCTGG	
*fli*C	121	55	Forward	GCTTCGACAACACCATCAAC	(13, 19)
			Reverse	AGCACCTGGTTCTTGGTCAG	
*flh*F	127	54	Forward	CGAGCCTGAACGTGAAGAAT	(13, 19)
			Reverse	GCCTCGTCCAGCTTAGTCA	
*fle*N	137	56	Forward	GAGCCGTATACGAGGCATTC	(13, 19)
			Reverse	GTGTTGGACCAGTCGTTCG	
*fle*Q	134	54	Forward	AAGGACTACCTGGCCAACCT	(13, 19)
			Reverse	CCGTACTTGCGCATCTTCTC	
*fle*R	109	55	Forward	ACAGCCGCAAGATGAACCT	(13, 19)
			Reverse	TGGATGGCGTTGTCGAGTT	
*rpo*D*	146	53	Forward	GCGACGGTATTCGAACTTGT	(13, 19)
			Reverse	CGAAGAAGGAAATGGTCGAG	

*Reference gene

Table 2. Gene specific primers of *S. pyogenes* used for RT-qPCR analysis

Gene name	Amplicon Size (bp)	Annealing temp (C°)	Direction	Primer sequence (5' → 3')	References
Sof	873	57	Forward	ACTTAGAAAGTTATCTGTAGGG	(13, 19)
			Reverse	TCTCTCGAGCTTTATGGATAG	
*sfb*I	960	55	Forward	AACTGCTTTAGGAACAGCTTC	(13, 19)
			Reverse	CCACCATAGCCACAATGCT	
*scp*A	622	55	Forward	GCTCGGTTACCTCACTTGTCC	(13, 19)
			Reverse	CAATAGCAGCAAACAAGTCACC	
*fts*Y	97	54	Forward	TCGAAAATTCTTTGGCCTGT AT-	(13, 19)
			Reverse	CAAACGTGTTGTGCCAGA	
*glr**	797	54	Forward	ATGGATACAAGACCAATTGG	(13, 19)
			Reverse	TCATAAGGTGACATGCTCCAC	

*Reference gene

Table 3. Antibacterial activity (Inhibition Zone (mm) ± SD) of all tested honeys at different concentrations against *P. aeruginosa*

Honey samples	100%	75%	50%	25%
MH	25.3 ± 0.6	21.4 ± 0.1	18.6 ± 0.2	14.6 ± 0.3
JH	19.2 ± 0.4	17.2 ± 0.4	14.1 ± 0.6	13.0 ± 1.0
KMH	25.1 ± 0.6	21.3 ± 0.5	17.1 ± 0.4	11.6 ± 0.3
GH	20.2 ± 0.4	16.0 ± 0.6	12.1 ± 0.5	11.5 ± 0.2
AH	19.7 ± 0.5	18.0 ± 0.4	12.1 ± 0.6	11.4 ± 0.1

Table 4. Antibacterial activity (Inhibition Zone (mm) ± SD) of all tested honeys at different concentrations against *S. pyogenes*

Honey samples	100%	75%	50%	25%
MH	25.1 ± 0.5	21.2 ± 0.1	18.4 ± 0.3	14.1 ± 0.4
JH	18.4 ± 0.5	16.1 ± 0.2	13.1 ± 0.5	12.0 ± 0.8
KMH	24.1 ± 0.2	20.1 ± 0.3	16.7 ± 0.3	10.8 ± 0.7
GH	19.4 ± 0.2	15.4 ± 0.1	11.7 ± 0.4	10.6 ± 0.5
AH	18.6 ± 0.7	17.2 ± 0.2	11.6 ± 0.3	10.3 ± 0.3

Table 5. MIC, MIC_{90}, MIC_{50} and MBC of all tested honeys against *P. aeruginosa*

Honey samples	MIC % (w/v)	MIC_{90} % (w/v)	MIC_{50} % (w/v)	MBC % (w/v)
MH	12.5%	20%	12.5%	20%
JH	25%	50%	20%	50%
KMH	20%	25-50%	20-25%	25%
GH	20%	25%	20%	25%
AH	20%	50%	25%	50%

Table 6. MIC, MIC_{90}, MIC_{50} and MBC of all tested honeys against *S. pyogenes*

Honey samples	MIC % (w/v)	MIC_{90} % (w/v)	MIC_{50} % (w/v)	MBC % (w/v)
MH	12.5%	25%	20%	20%
JH	25%	50%	20%	50%
KMH	20%	25-50%	20-25%	25%
GH	20%	25-50%	20%	50%
AH	20%	50%	25%	50%

ity at 9 h incubation with >3-log_{10} killing unit for MH and JH and with >2.5-log_{10} for KMH, GH and AH. The change in cell count in *P. aeruginosa* treated and untreated cells was statistically significant (Fig. 1).

The number of *S. pyogenes* cells decreased after following treatment with 2×MIC MH, JH, KMH, GH, and AH with 1.7-log_{10}, 1.3-log_{10}, 1.3-log_{10}, 1.5-log_{10}, and 1.3-log_{10}, reduction in CFU/ml (≈99% killing) respectively at 6 h. The mean difference between treated and untreated *S. pyogenes* cells was statistically significant (P <0.05). However, 2×MIC MH, JH, KMH, GH, and AH achieved a 2.5-log_{10}, 2.3-log_{10}, 2.2-log_{10}, 2.4-log_{10}, and 2.7-log_{10} reduction (≈99% killing) in *S. pyogenes* population at 9 h (Fig. 1).

Sub-inhibitory concentrations of all tested honeys decreased the biofilm biomass. The average of optical density (OD) for control sample and tested sample of biofilm mass was calculated. The effect of MH, JH, KMH, GH and AH on biofilm biomass varied depending on the MH, JH, KMH, GH and AH concentrations. A statistically significant (P<0.05). When MH, JH, KMH, GH and AH were used at the 15% (w/v), the optical density of *P. aeruginosa* biofilm biomass was reduced to 1.8, 2.1, 2.2, 2.3, and 2.3 respectively compared to untreated biofilm. However, at 30% (w/v) MH, JH, KMH, GH and AH, the optical density of *P. aeruginosa* biofilm biomass was reduced to 0.9, 1.5, 1.6, 1.7, and 1.8 respectively compared to untreated biofilm. Meanwhile, the optical density of *P. aeruginosa* biofilm biomass was reduced to 0.5, 1.0, 1.1, 1.3, and 1.4 respectively compared to untreated biofilm at 45% (w/v). At 60% (w/v) MH, JH, KMH, GH and AH was more effective at reducing the optical density of *P. aeruginosa* biofilm biomass by 0.3, 0.5, 0.6, 0.8 and 0.8 respectively compared to untreated biofilm (Fig. 2).

In the presence of 15%, 30%, 45% and 60% (w/v) MH, JH, KMH, GH and AH concentrations, the optical density of established *S. pyogenes* biofilms was significantly (P < 0.05) decreased compared to untreated biofilm. After MH, JH, KMH, GH and AH was used at the 15% (w/v), the optical density of established *S. pyogenes* biofilms was reduced to 1.7, 1.9, 2.1, 2.2, and 2.2 respectively compared to untreated biofilm. However, at 30% (w/v) MH, JH, KMH, GH and AH, the optical density of established *S. pyogenes* biofilms was reduced to 0.8, 1.4, 1.5, 1.6, and 1.7 respectively compared to untreated biofilm. Meanwhile, at 45% (w/v) MH, JH, KMH, GH and AH, the optical density of established *S. pyogenes* biofilms was reduced to 0.4, 0.9, 1.0, 1.2, and 1.3 respectively compared to untreated biofilm. In addition, at 60% (w/v) MH, JH, KMH, GH and AH was more effective at reducing the optical density of established *S. pyogenes* biofilms by 0.2, 0.4, 0.5, 0.7, and 0.7 respectively compared to untreated biofilm. It was observed that the lowest concentration of MH, JH, KMH, GH and AH prevented *S. pyogenes* to establish biofilm was found to be 15% (w/v). Remarkably, MH was the most effective in preventing formation of *S. pyogenes* and *P. aeruginosa* biofilm. The inhibiting effect of MH, at low concentrations (15%), on the formation of *S. pyogenes* and *P. aeruginosa* biofilm was greater than that of the other honeys (Fig. 2).

Scanning electron microscope (SEM) of single-species biofilm. SEM micrographs of untreated *P. aeruginosa* cells showed that the cells appeared to be rod-shaped with regular structure and hundreds of bacterial cells are connected by a substantial amount of extracellular matrix. Extensive structural changes in biofilms were seen following treatment with all honeys and loss of viability was found, in addition to loss of biofilm structure. SEM images provided reasonable evidence of damage and disruption of

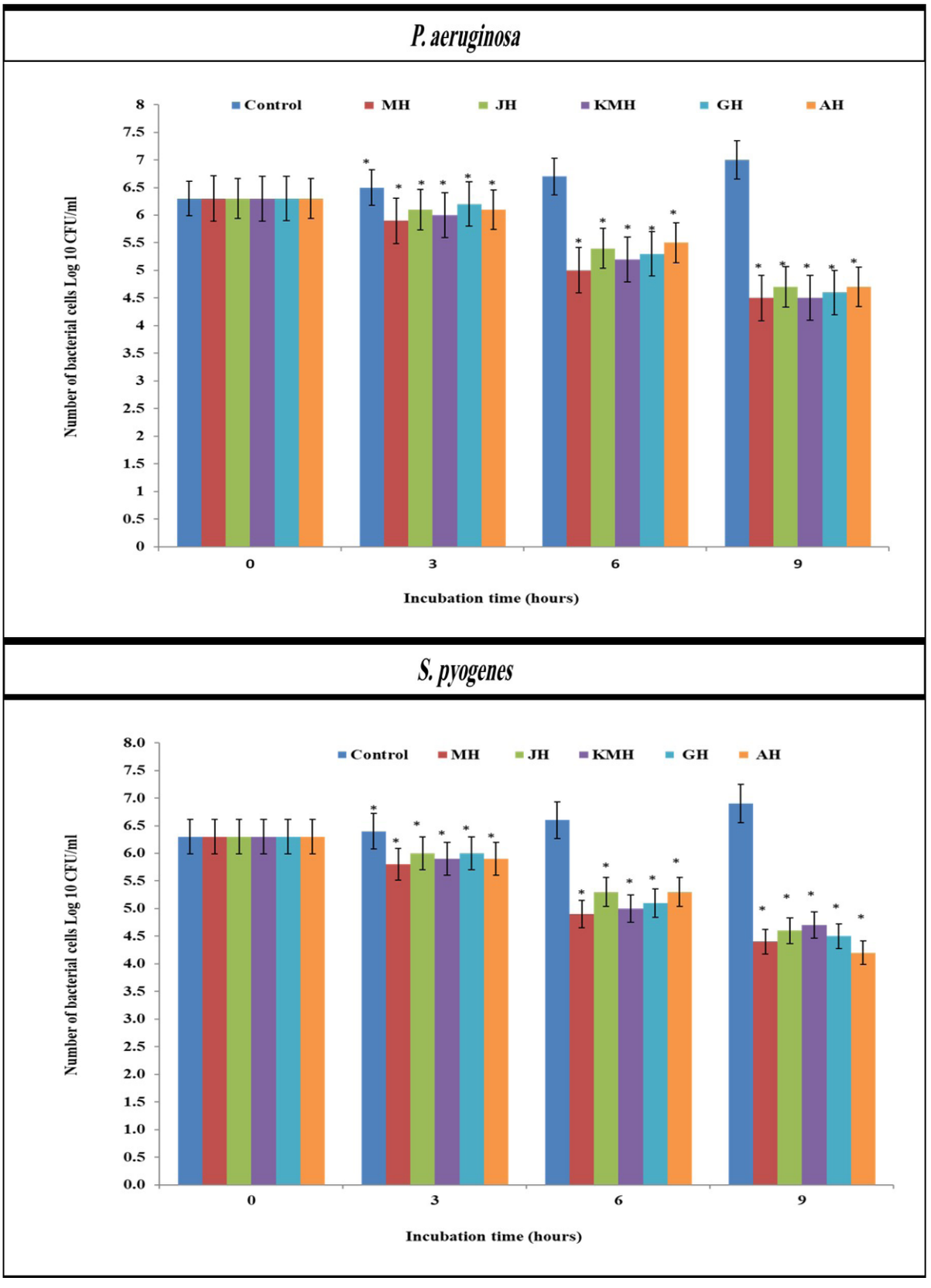


Fig. 1. Time-kill studies of *P. aeruginosa* and *S. pyogenes* after exposed to all honeys. Asterisks; *P<0.05 indicate statistically significant difference between treated and control samples.

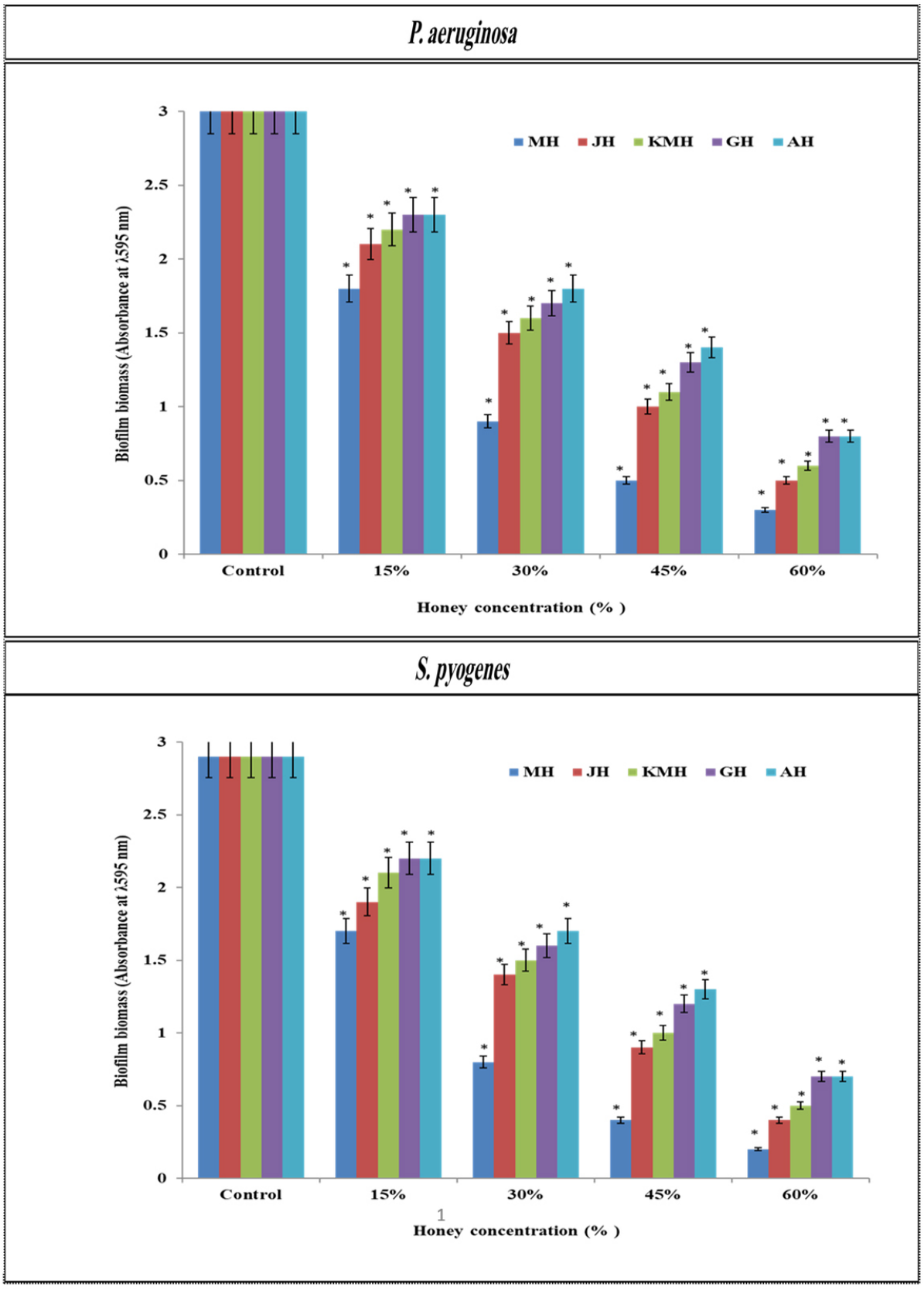


Fig. 2. Biofilm formation of *P. aeruginosa* and *S. pyogenes* grown with and without all honeys. Asterisks; * $P<0.05$ indicate statistically significant difference between treated and control samples.

the integrity of biofilm after exposure to all honeys. In addition, rough cell surfaces were observed after treated with all honeys (Fig. 3).

SEM micrographs of untreated *S. pyogenes* demonstrated the regular cocci with chain structure and *S. pyogenes* biofilm shows numerous cocci cells and diverse thickness connected each other. When *S. pyogenes* following treatment with all honeys the density of biofilm formed were reduced compared to untreated. In addition, several morphological changes, such as changes of cocci shape, abnormal cell division and ruptured cell structure were observed after exposed to all honeys (Fig. 3).

Scanning electron microscope (SEM) of mixed-species biofilm. SEM showed that the surface structure and morphology of mixed-species biofilms formed by *P. aeruginosa* and *S. pyogenes* without honey treatment. The control group of mixed-species biofilms showed the typical multilayer growth of bacterial biofilms, while the group treated with MH, JH, KMH, GH and AH demonstrated that exhibited a reduction of mixed-species biofilm, reduction of cell density, and decrease extracellular matrix compared to control cells for both bacteria. Altogether, the findings provide evidence that MH, JH, KMH, GH and AH have a potent antibiofilm action against the mixed-species biofilm (Fig. 3).

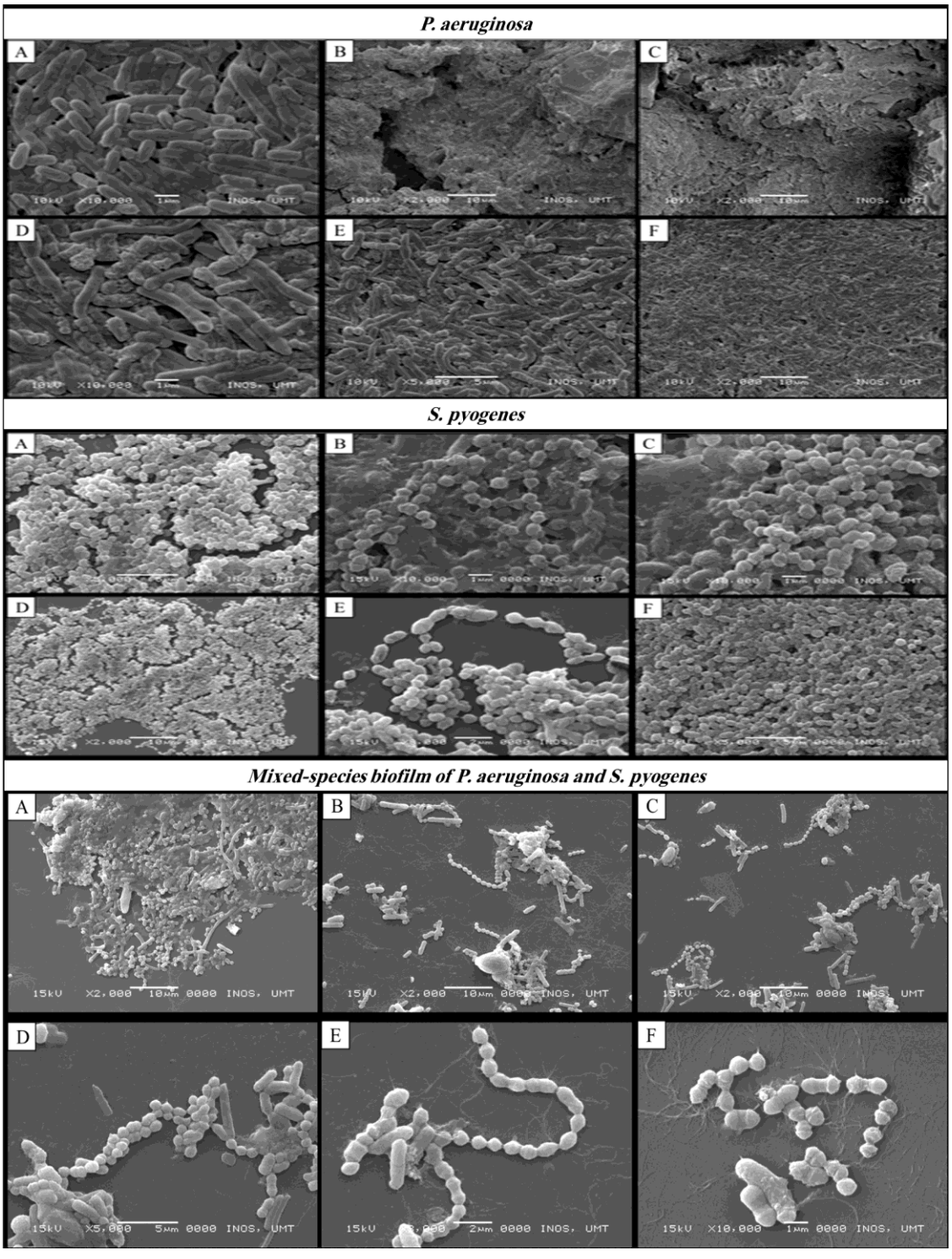
Characterization of virulence factor activity indicated that honeys were able to reduce activity of several key virulence factors. In the present study, RT-qPCR was used to assess and compare the expression of six genes in *P. aeruginosa* that have been previously shown to be involved in the flagella regulon proteins, biofilm formation, motility and virulence of the microorganism and four genes in *S. pyogenes* that have been previously shown to be involved in the fibronectin binding proteins, surface adhesins and biofilm formation after exposure to all honeys.

Effects of five tested honeys on the mRNA expression of *P. aeruginosa*. Following treatment of *P. aeruginosa* with MIC of MH, JH, KMH, GH and AH there were significant reductions ($P<0.05$, $P\leq 0.01$ and $P\leq 0.001$) in the relative abundance of mRNA for *fli*A, *fli*C, *flh*F, *fle*N, *fle*Q and *fle*R when compared to untreated cells. When *P. aeruginosa* treated with MH, the fold change ranged from 3.2-fold to 6.3-fold (for *fle*R, *fli*A, *flh*F, *fle*Q, *fli*C and *fle*N respectively). Also, when *P. aeruginosa* treated with JH, the fold change ranged from 2.7-fold to 4.5-fold (for *fli*A, *fle*R, *flh*F, *fle*Q, *fli*C and *fle*N respectively). In addition, when *P. aeruginosa* treated with KMH, the fold change ranged from 1.9-fold to 4.1-fold (for *fle*R, *fli*A, *fle*Q, *flh*F, *fle*N, and *fli*C respectively). Furthermore, when *P. aeruginosa* treated with GH, the fold change ranged from 1.5-fold to 3.8-fold (for *fle*R, *fli*A, *fle*Q, *flh*F, *fle*N, and *fli*C respectively). When *P. aeruginosa* treated with AH, the fold change ranged from 1.3-fold to 2.8-fold (for *fle*R, *fli*A, *fle*Q, *flh*F, *fle*N, and *fli*C respectively). Comparing MH honey treated *P. aeruginosa* cell samples with JH, KMH, GH and AH, the expression of mRNA transcripts for each gene tested with MH was decreased more than other honeys (Fig. 4).

Effects of five tested honeys on the mRNA expression of *S. pyogenes*. The major genes encoding the surface adhesins for *scpA, ftsY, sfbI,* and *sof* in *S. pyogenes* were downregulated after exposure to MIC of MH, JH, KMH, GH and AH. Following treatment of *S. pyogenes* with MIC of MH, JH, KMH, GH and AH there were significant reductions ($P<0.05$, $P\leq 0.01$ and $P\leq 0.001$) in the relative abundance of mRNA for *scpA, ftsY, sfbI,* and *sof* when compared to untreated cells. When *S. pyogenes* treated with MH, the fold change ranged from 4.4-fold to 6.8-fold (for *scpA, ftsY, sfbI,* and *sof* respectively). Also, when *S. pyogenes* treated with JH, the fold change ranged from 3.6-fold to 5-fold (for *scpA, ftsY, sfbI,* and *sof* respectively). In addition, when *S. pyogenes* treated with KMH, the fold change ranged from 3.7-fold to 6.1-fold (for *scpA, ftsY, sof* and *sfbI* respectively). When *S. pyogenes* treated with GH, the fold change ranged from 3.4-fold to 4.8-fold (for *scpA, ftsY, sfbI,* and *sof* respectively). Whereas, when *S. pyogenes* treated with GH, the fold change ranged from 2.8-fold to 4.3-fold (for *scpA, ftsY, sfbI,* and *sof* respectively). Comparing MH honey treated *S. pyogenes* cell samples with JH, KMH, GH and AH, the expression of mRNA transcripts for each gene tested with MH was decreased more than other honeys (Fig. 4).

DISCUSSION

Antibacterial activity of honey has been broadly discussed among researchers worldwide. It is postulated to be closely on several factors such as, os-



Single-species biofilm of *P. aeruginosa*: Control (A), *P. aeruginosa* treated with MH (B), JH (C), KMH (D), GH (E), and AH (F). Viewed at 10,000×, 5,000× and 2,000× magnification. Scale bar 1µm, 5µm and 10µm.

Single-species biofilm of *S. pyogenes:* Control (A), *S. pyogenes* treated with MH (B), JH (C), KMH (D), GH (E), and AH (F). Viewed at 10,000×, 5,000x and 2,000× magnification. Scale bar 1µm, 5µm and 10µm.

Mixed-species biofilm of *P. aeruginosa* and *S. pyogenes*: Control (A), *P. aeruginosa* and *S. pyogenes* exposure to MH (B), JH(C), KMH (D), GH (E) and AH (F). Viewed at 10,000×, 5,000× and 2,000× magnification. Scale bar 1µm, 2µm, 5µm and 10µm.

Fig. 3. SEM of single and mixed-species biofilm of *P. aeruginosa* and *S. pyogenes* after exposure to all honeys

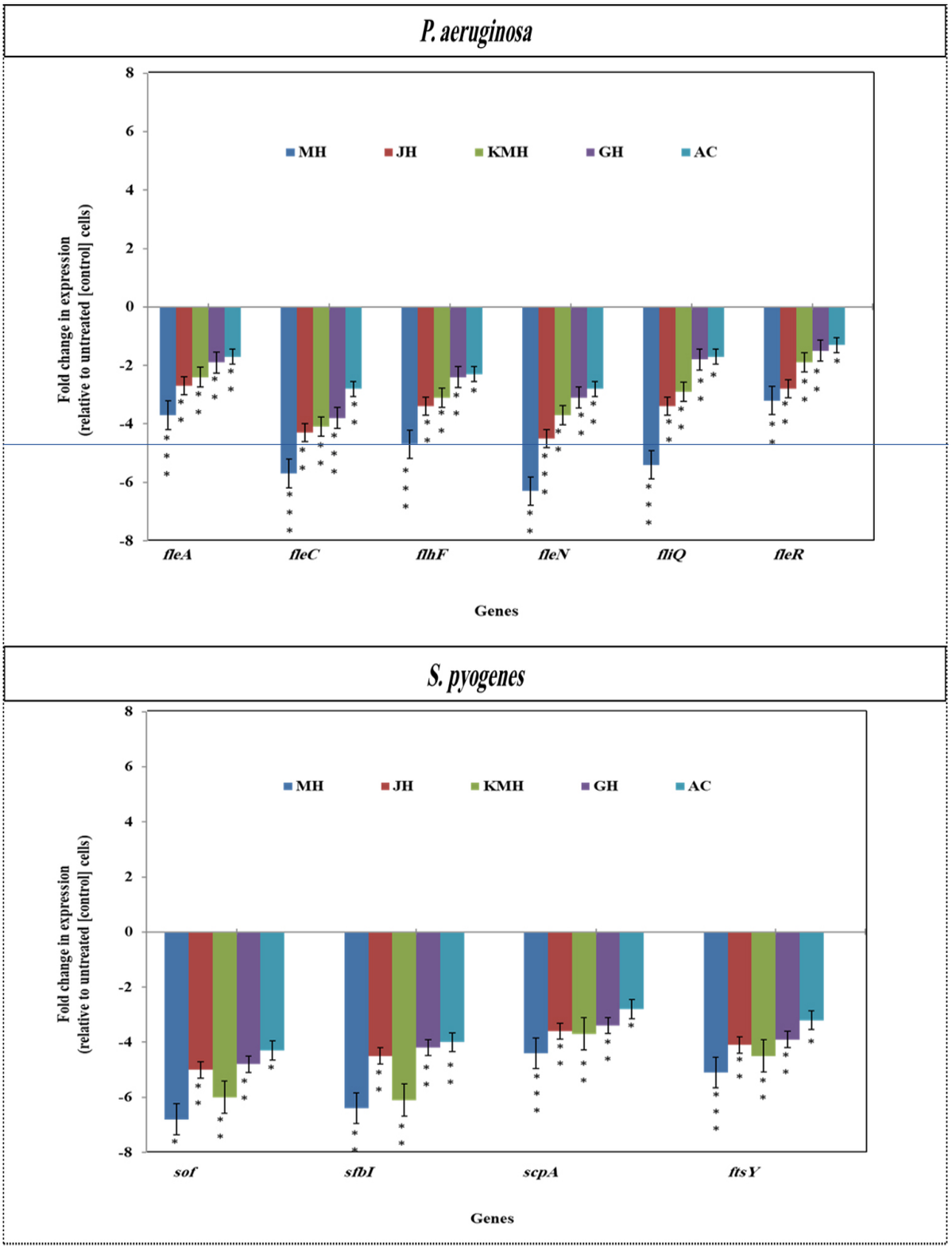


Fig. 4. Changes in gene expression profiles of *P. aeruginosa* and *S. pyogenes* after treated with all tested honeys as determined by RT-qPCR. Mean values of fold changes (± SD) are shown in relation to untreated *S. pyogenes* cells. Asterisks; * $P<0.05$; ** $P \leq 0.01$; and *** $P \leq 0.001$ indicate statistically significant difference in the expression of each gene between treated and untreated samples.

molarity, pH and other major constituents such as phenolic acids and flavonoids (20). Previous study showed that 25% concentration of honey exhibited lower antibacterial action (21). The similarly or divergence of results might be due to several reasons such contain different level of active compounds including phenolic acids and flavonoids (22). Limitations of some antibacterial assay such as agar well-diffusion test were reported including the insensitivity in detecting low level of antimicrobial activity, variation in the experimental conditions and permeability of non-polar components (23, 24). Agar well-diffusion test may not be the most appropriate method to evaluate the antibacterial activity of honey. Micro-broth dilution was performed to determine the MIC for antibacterial activity of honey toward all the tested bacteria (25). The lowest concentration of honey solution needed to inhibit 99% of bacterial growth is considered to be MIC. The lowest concentration of honey required to kill at least 99% of the tested bacterial strains is defined as MBC (25). In the current study, the MIC values ranging from 12.5% to 20% against both bacteria and the MBC values ranging from 20% to 50% against both bacteria. Previous studies showed that the MIC for Algerian, Manuka and Egyptian clover honeys against *P. aeruginosa* was at 20% and MBC was at 25% (25-28). Recently, other studies revealed that MIC for Manuka honey on *S. pyogenes* was at 20% and MBC was at 25% (29, 30). A previous study showed that the MIC for Manuka honey against *P. aeruginosa* was at 12% and MBC was at 16% (31). Time-kill studies were used to determine the bactericidal or bacteriostatic actions of antimicrobials (32). It is investigated by plotting $\log_{10}$ CFU/mL over time (13). The $\log_{10}$ CFU/ml for *P. aeruginosa* and *S. pyogenes* exposure to all honeys were seen at 9 hours which is about >3-$\log_{10}$ of both bacteria were killed. All tested honeys were able to decrease biofilm biomass in both bacteria. However, this study found that the higher concentration of all honeys was necessary to complete elimination of established biofilm. Regarding to the results obtained for *P. aeruginosa* and *S. pyogenes* biofilms, a significant reduction was observed after 24 hour's exposure to all honey at all concentrations were used. In the current study, SEM revealed that the morphological changes of cells, cells destruction, cells lysis and biofilm disruption in both bacteria following treatment with all honeys. In addition, SEM images showed that the treated mixed-species biofilm presented several damaged cells in both bacteria compared to untreated mixed-species biofilm. The previous study demonstrated that the structure of *P. aeruginosa* was influenced using Manuka honey (33). A study by Enany et al. (2015) pointed that Sidr honey disrupted the cell of *S. aureus* (34). As demonstrated by RT-qPCR, a number of genes *fli*A, *fli*C, *flh*F, *fle*N, *fle*Q and *fle*R have been previously shown to be involved in the process of microcolony, biofilm formation and motility in *P. aeruginosa* (13, 16). Also, a set of genes have been previously shown to play an important role in the adhesion and biofilm formation and quorum-sensing network of *S. pyogenes*, such as the *sof, sfbl, emm13, scpA* and *ftsY* genes (29). The current results revealed that all selected genes in both bacteria were downregulated following exposure to all honeys. Our results are in agreement with those of (16, 31), who reported downregulation of multiple genes involved in microcolony, motility and biofilm formation in *P. aeruginosa* strain following exposure to manuka honey. Study by Maddocks et al. (2012) reported that downregulation of *sof,* and *sfbl* genes in *S. pyogenes* after exposure to Manuka honey (29). Previous study showed that five genes; *fleN, fleQ, fleR, fliA* and *fliC* in *P. aeruginosa* and five genes; *sof, sfbl, scpA, ftsY* and *emm13* in *S. pyogenes* were reduced in gene expression following treatment with Tualang honey (13). Another study reported that *ycfR (BhsA)* and *evgA* genes of *E. coli* were upregulated in expression in the range of 2.2-4.19-fold and 1.09-fold respectively after treated with 25% concentration of Egyptian honey (6). Study by Roberts et al. (2014) showed that *fliA, fliC, flhF, fleN, fleQ* and *fleR* genes in *P. aeruginosa* were reduced in gene expression after treated with manuka honey (16). Previous study showed that *tnaA* and *yjfO (bsmA)* genes were downregulated in expression of *E. coli* in the range of 12.5-16.2-fold after treated with 25% concentration of Egyptian honey (6). It was noticed that all these studies that mentioned above are in agreement with our results. This indicates that the honey-induced alterations in the expression of this group of genes are most probably due to particular molecules contained in honey and not only due to their sugar content. Previous study suggested that the osmotic action of sugar combined with hydrogen peroxide and bee-derived antibacterial peptide defensin-1 is crucial for the antibiofilm activity of honey (35). In addition, this change in expression pattern may indicate variations in the phytochemical

components and/or differences in the antimicrobial mechanisms of all honey on both bacteria (30). It is evident that honey is effective at inhibiting the growth of both bacteria, causing abnormal cell by reducing structural integrity to the point of cell lysis as mentioned in SEM results. To our knowledge, this is the first attempt to compare the impacts of Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) with that of Manuka honey (MH) on the tested organisms at both structural and molecular levels.

CONCLUSION

This is the first attempt study to compare the impacts of Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) with that of Manuka honey (MH) on the tested organisms at both structural and molecular levels. A reduction of *P. aeruginosa* and *S. pyogenes* cell growth in both planktonic and biofilm state was observed with all honey treatment. Comparing all honeys tested, for planktonic and biofilm cultures, Manuka honey (MH) had a higher effect on both bacteria. In this study, results indicate that the JH, KMH, GH, and AH may represent promising antibacterial, antibiofilm and anti-virulence agents for treatment and modulation of infections caused by *P. aeruginosa* and *S. pyogenes* compared with MH. Antibacterial and antibiofilm activities of all tested honeys against both bacteria, which were further supported by the morphological and structural investigations. However, understanding the behavior of *P. aeruginosa* and *S. pyogenes* species in polymicrobial biofilms is an important step in the clinical context and for the selection of the most efficient treatment. Because of this, the effect of all honeys was assessed on structure of mixed *P. aeruginosa* and *S. pyogenes* biofilms. The honeys were able to reduce both species in the mixed biofilm and were demonstrated to be a promising alternative for the treatment of infections caused by mixed species biofilms. The use of a natural product such as honey may be used in clinical practice, to prevent or even treat *P. aeruginosa* and *S. pyogenes* infections. This study, suggest that each honey could have a crucial derivatives compound that have the ability to effectively inhibit the biofilms of *P. aeruginosa* and *S. pyogenes.*

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ARTICLES FOR FACULTY MEMBERS

CULTURAL HERITAGE

Title/Author	Antioxidant properties and characterization of Heterotrigona itama honey from various botanical origins according to their polyphenol compounds / Shamsudin, S., Selamat, J., Abdul Shomad, M., Ab Aziz, M. F., & Haque Akanda, M. J.
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Research Article

Antioxidant Properties and Characterization of *Heterotrigona itama* Honey from Various Botanical Origins according to Their Polyphenol Compounds

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Stingless bee honey is a good source of antioxidants, which is attributed to the phenolic compounds. The type and concentration of phenolic compounds in honey can be affected by botanical origin. Therefore, in this study, *Heterotrigona itama* honey from three botanical origins (gelam, acacia, and starfruit) was evaluated for its antioxidant activity and profile of phenolic compounds. *Apis mellifera* honey was used as a comparison. Antioxidant activity and profile of phenolic compounds in honey were determined using spectrophotometric and chromatographic methods, respectively. The total phenolic content (TPC), total flavonoids content (TFC), free radical scavenging activity (IC_{50}), and ferric reducing antioxidant power (FRAP) of *H. itama* were ranged between 52.64 and 74.72 mg GAE/100 g honey, 10.70–25.71 mg QE/100 g honey, 11.27–24.09 mg/mL, and 77.88–164.88 μmol $FeSO_4.7H_2O$/100 g honey, respectively. The findings showed that the antioxidant activity and phenolic and flavonoid contents in *H. itama* honey were significantly higher than *Apis* honey. Benzoic acid and taxifolin were found as the predominant phenolic acid and flavonoid in all samples. However, chrysin was significantly highest in *Apis* honey than stingless bee honey. This result suggested that chrysin can be used as a chemical marker to distinguish *Apis* honey from stingless bee honey. Gallic acid and ellagic acid were found as the chemical marker for gelam honey, salicylic acid, benzoic acid, and 4-hydroxybenzoic acid for starfruit honey while ferulic acid for acacia honey.

1. Introduction

Honey is produced by stingless bees and honeybees mainly from the nectar of plants or plant sap [1]. It has been documented that honey is a complex substance and contains over 200 components, which may be either produced during the maturation process of honey, added by bees, or derived from plants [2]. The main components of honey are sugar and water. It also comprises important minor components such as organic acids, amino acids, vitamins, minerals, enzymes, and phenolic compounds. The composition of honey and phenolic compounds primarily depends on the botanical origin [3, 4] and external factors such as harvesting season, environment, storage, and processing method. Phenolic compounds are the most important antioxidant in honey that are responsible for the honey's therapeutic properties. Thus, honey has been used in traditional and modern treatments to treat human illnesses. In modern

treatment, honey has been used to treat diseases associated with oxidative stress such as diabetes mellitus, hypertension, atherosclerosis, cancer, and Alzheimer's [5]. In addition, phenolic compounds also can affect the organoleptic properties (color, taste, or flavor) of honey [2]. In a recent study, phenolic compounds have been used as a chemical marker to differentiate honey from different botanical and geographical origins [6]. For instance, abscisic acid was reported as a potential floral marker for two Polish unifloral honey [7], and quercetin was suggested as a marker for sunflower honey [8].

Phenolic compounds (phenolic acids and flavonoids) are the secondary components of plants [9] that transfer to honey through nectar, pollen, or propolis by bees [10]. They are responsible for the most antioxidant activity in honey [11]. In recent years, there has been a growing interest in identifying phenolic compounds in stingless bee honey as it has been reported to have higher antioxidant activity and is considered as a potential source of antioxidants compared to *Apis mellifera* honey, which is good for human health and well-being [12]. Biluca et al. [13], da Silva et al. [14], Sousa et al. [15], and Olivera et al. [16] have identified numerous phenolic compounds in *Melipona and Trigona* honey. All the studies are from America Latin. However, in Malaysia, there is very limited scientific data on the profile of phenolic compounds in stingless bee honey. Ranneh et al. [17] investigated Malaysian stingless bee honey and Tualang honey. They found gallic, caffeic, syringic, catechin, cinnamic, p-coumaric, apigenin, and 4-hydroxybenzoic as major compounds. Recently, Majid et al. [18] reported six phenolic compounds (chlorogenic acid, p-coumeric acid, epicatechin, rutin, catechin, and protocatechuic acid in unifloral and multifloral honey produced by *Heterotrigona itama* from Johor. Moreover, there are no available studies on phenolic compounds in stingless bee honey from acacia, gelam, and starfruit. Therefore, more studies need to be conducted to explore and identify more phenolic acids and flavonoids present in stingless bee honey from different botanical origins. In this study, three types of stingless bee honey (acacia, gelam, and starfruit) that are commonly consumed in Malaysia were investigated. The main goal of this present study was to determine the phenolic profiles of stingless bee honey and *Apis* honey by identifying their phenolic acids and flavonoids, correlate antioxidant activity with phenolic compounds, and verify the botanical origin according to the specific phenolic compounds using multivariate statistical method.

2. Materials and Methods

2.1. Materials. Methanol (MeOH), hydrochloric acid (HCl), formic acid (CH_2O_2), acetic acid (CH_3COOH), sodium acetate (CH_3COONa), sodium Carbonate ($NaCO_3$), sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH), ferric tripyridyltriazine (Fe^{3+}-TPTZ), iron (III) chloride hexahydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, Folin–Ciocalteu reagent, and amberlite XAD-2 resin were purchased from Fisher Scientific (Pittsburg, PA). Phenolic compound standards (gallic acid, 3, 4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, syringic acid, (-)-epicatechin, (+-)-catechin hydrate, p-coumaric acid, ferulic acid, ellagic acid, benzoic acid, salicylic acid, trans-cinnamic acid, vanillic acid, caffeic acid, taxifolin, myricetin, quercetin hydrate, (+-)-naringenin, and chrysin) were obtained from Sigma Chemical Co. (St. Louis, MO). For HPLC analysis, all chemicals and solvents were of HPLC grade.

Figure 1: Source of honey.

2.2. Honey Samples. Natural stingless bee honey produced by *H. itama* from acacia (*Acacia mangium*), starfruit (*Averrhoa carambola* L.), and gelam (*Meleleuca cajaputi* Powell) were obtained from three different farms that cultivated acacia, gelam, and starfruit trees located in Johor, Terengganu, and Pahang, respectively (Figure 1). The samples were collected during the flowering season between August and November 2016. *A. mellifera* honey (acacia) was used as a comparison in this study. The botanical origin of the honey samples was determined based on the location of beehives and the availability of the floral source on the farm. The samples were collected using a vacuum pump and filtered to remove foreign materials. They were stored in airtight containers and kept at 4°C until further analysis. The details of honey samples used are summarized in Table 1.

2.3. Total Phenolic Content. The total phenolic content (TPC) was determined using Folin–Ciocalteu assay according to the method described by Garjanovic et al. [19] with some modifications. A 200 μL of honey solution (0.05 g/mL methanol) was added to 1 mL of Folin–Ciocalteu reagent in a test tube. Then, the mixture was allowed to stand at room temperature. After 6 min, 800 μL of sodium carbonate solution (7.5% Na_2CO_3) was added to the mixture. Then, the mixture was left to stand for 2 h in a dark room at room temperature. The absorbance of the mixture was measured at 740 nm using a UV-visible spectrophotometer (GENESYS™ 10S, Thermo Fisher Scientific, MA). The calibration curve was prepared using gallic acid at concentrations between 10 and 50 µg/mL ($r^2 = 0.9999$). Results were expressed as mg gallic acid equivalents (GAE) per 100 g. The analysis was performed in triplicate.

Table 1: Botanical origin of the honey samples used in this study.

Bee species	Type of honey (common name)	Botanical origin (scientific name)	Location
Heterotrigona itama	*Acacia*	*Acacia mangium*	Johor
	Gelam	*Meleleuca cajaputi* Powell	Terengganu
	Starfruit	*Averrhoa carambola* L.	Pahang
Apis mellifera (as a comparison)	*Acacia*	*Acacia mangium*	Johor

2.4. Total Flavonoids Content. The total flavonoid content (TFC) was measured according to the method suggested by Kamboj et al. [20] with minor modifications. One milliliter of honey solution (0.25 g/mL methanol) was mixed with 0.3 mL of 5% sodium nitrite ($NaNO_2$). The mixture was left to stand for 5 min at room temperature. Afterward, 0.3 mL of 10% aluminum chloride (Al_3Cl) was added, and the mixture was allowed to stand at room temperature for 6 min. Finally, 2 mL of 1 M of sodium hydroxide (NaOH) was added to the mixture. The absorbance of the mixture was measured at 510 nm using a UV-visible spectrophotometer (GENESYS™ 10S, Thermo Fisher Scientific, MA). Quercetin was used as a standard to plot the calibration curve at the concentrations of 10–50 μg/mL ($r^2 = 0.98$). The results were expressed as mg quercetin equivalent (QE) per 100 g. The analysis was carried out in triplicate.

2.5. Free Radical Scavenging Activity. The scavenging activities of the honey samples were measured by DPPH assay as described by Meda et al. [21] with slight modifications. A 400 mg of honey sample was mixed with different amounts of methanol (3, 6, 9, 12, and 15 mL) separately. Then, 0.75 mL of honey in methanol was mixed with 1.5 mL of DPPH solution. The mixture was left in the dark area for 15 min at room temperature. The absorbance was measured against a blank solution containing water with a UV-visible spectrophotometer (A GENESYS^{TM} 10S, Thermo Fisher Scientific, MA) at 517 nm. The antiradical activity of the sample was calculated using the following formula:

$$\text{antiradical activity}\,(\%) = \left[\frac{(A_c - A_s)}{A_c}\right] \times 100\%, \quad (1)$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample. Each honey sample was analyzed in five dilutions. The antiradical activity was expressed as IC_{50} (the concentration of honey solution needed to reduce the concentration of DPPH in the solution to 50% of its initial concentration). The concentration of honey sample required to scavenge 50% of DPPH (IC_{50}) was determined from the plotted graph of scavenging activity against the honey dilutions.

2.6. Ferric Reducing Antioxidant Power. The reducing ability of the sample was carried out according to the procedure described by Khalil et al. [22] with slight modifications. The method was based on the reduction of Fe^{3+}-TPTZ to a blue-colored Fe^{2+} FRAP reagent. It was prepared by mixing 0.3 M of acetate buffer (pH 3.6), 10 mM of TPTZ, and 20 mM $FeCl_3.6H_2O$ in a ratio of 10:1:1 at 37°C. A 200 μL of the honey sample (0.05 g/mL) in methanol was added to 1.5 mL of FRAP reagent in a test tube. After 10 min of incubation at 37°C in a water bath, the absorbance was measured using the UV-visible spectrophotometer (GENESYS™ 10S, Thermo Fisher Scientific, MA) at 593 nm. The antioxidant potential of the sample was determined from a standard curve using ferrous sulfate heptahydrate $FeSO_4.7H_2O$ at concentrations between 10 and 100 μg/mL ($r^2 = 0.98$). Triplication of the test was performed, and the results were expressed as the mean average.

2.7. Extraction of Phenolic Compounds Using XAD-2 Resin. The extraction of phenolic compounds was prepared according to the method described by Kassim et al. [23] and Ferreira et al. [24] with some modifications. A 10 g of honey sample was mixed with 100 mL of acidified water (pH 2; 0.02 M HCl) and 150 g of Amberlite XAD-2 resin (Fluka Chemei; pore size = 9 nm; particle size = 0.3–1.2 mm). The mixture was then homogenized for 10 min. The mixture was poured into a column (35 × 2 cm) and washed with 100 mL of acid solution (0.02 M HCl) to remove all sugars and other polar constituents in honey. Subsequently, 300 mL of deionized water was used to rinse the mixture in the column. The phenolic fractions were eluted with 100 mL of methanol and evaporated using a rotary evaporator until dry at 40°C. Methanol was used based on its ability to extract more phenolic compounds as reported by Kassim et al. [23]. The residues were dissolved in 250 μL methanol for HPLC analysis.

2.8. HPLC Analysis. The detection of phenolic compounds was performed using high-performance liquid chromatography equipped with a diode array detector (HPLC-DAD). The column used was a reversed-phase C18 column, ACE (4.6 × 250 mm, particle size 5 μM) from Altmann Analytik GmbH & Co. KG (Munich, Germany). Approximately 10 μL of the phenolic extract was injected into the HPLC, and the separation was performed using 0.25% formic acid and 2% methanol in water (solvent A) and methanol (solvent B). The gradients used were: 10% methanol (B) for 15 min, 40% methanol (B) for 20 min, 45% methanol (B) for 30 min, 60% methanol (B) for 50 min, 80% methanol (B) for 52 min, 90% methanol (B) for 60 min, and followed by isocratic elution with 90% methanol (B) for 65 min. Then, the gradient was changed to 10% methanol for 68 min until 73 min. The flow rate used was 1 mL/min. Phenolic compounds were detected using UV absorption spectra monitored at 290 nm and 340 nm. The phenolic compounds were identified by comparing the chromatographic retention times with the

standards. The calibration curve of the standards was used to determine the concentration of the phenolic compounds in the extracts [23].

2.9. Statistical Analysis. All analyses were conducted in triplicate, and the data were expressed as means ± standard deviation. Analysis of variance (ANOVA) and Turkey's test at the 95% confidence level were performed using MINITAB software (State College, PA). Correlations between antioxidant activity and TPC, TFC, IC_{50}, and FRAP were obtained by Pearson's correlation analysis. Multivariate analysis was performed using MINITAB software (State College, PA).

3. Results and Discussion

3.1. Antioxidant Properties of Honey

3.1.1. Total Phenolic Content and Total Flavonoids Content. The antioxidant capacity of honey is usually measured by evaluating the contents of phenolic and flavonoids as they are the key compounds responsible for the antioxidant activity of honey [13]. TPC values in the honey samples ranged between 52.64 and 74.62 mg GAE/100 g honey (Table 2). A significant difference ($p < 0.05$) was observed in TPC values between *H. itama* (gelam and starfruit honey) and *A. mellifera* honey. Gelam honey showed the significantly highest value of TPC, while acacia honey obtained the lowest value. However, no significant differences ($p > 0.05$) were observed among *H. itama* honey from different botanical origins. In contrast, Abu Bakar et al. [25] demonstrated the TPC value of *H. itama* from Malaysia was higher than our results. The TPC values varied between 435.69 and 516.07 mg GAE/100 g honey. Recently, Imtiazah et al. [26] reported 368.11 mg GAE/100 g honey total phenolic content. However, Tufail Ahmad et al. [27] reported 5.86 mg GAE/100 g honey total phenolic content in Malaysian stingless bee honey produced by *Geniotrigona thoracica*, which was lower than our results. Ranneh et al. [17] also reported lower TPC in stingless bee honey collected from the forest in Kedah and Johor. The values varied between 228.09 and 235.28 mg GAE/kg honey. Ismail et al. [28] reported 33.2–60.2 mg GAE/100 g honey of total phenolic content, which agreed with our study. In comparison to other countries, the results found in this study were comparable to those found by Silva et al. [29] and da Silva et al. [14], where the TPC values were ranged between 1.30 and 66 mg GAE/100 g honey for stingless bee honey from Paraiba and Amazona, Brazil. Furthermore, Silva et al. [29] also reported the higher TPC values in nine *Melipona subnitida* honey from two semiarid regions, Paraiba, Brazil.

The TFC values of *H. itama* honey and *Apis* honey are shown in Table 2. The highest TFC value (25.71 mg QE/100 g honey) was found in starfruit honey, followed by gelam (20.67 mg QE/100 g honey), acacia (10.70 mg QE/100 g honey), and *Apis* (7.02 mg QE/100 g honey) honey. A significant difference ($p < 0.05$) was observed in all honey samples investigated. In comparison with Malaysian stingless bee honey, Ranneh et al. [17] and Selvaraju et al. [30] reported lower TFC where the values ranged between 97.88 and 101.5 mg CE/kg and 10.18 and 12.68 mg CE/kg, respectively. In a recent study, Imtiazah et al. [26] also demonstrated a lower TFC in Malaysian stingless bee honey (64.25 mg CEQ/kg honey). However, higher TFC was found in multifloral stingless bee honey collected in Sabah and Kelantan with values between 43.2 and 65.9 mg QE/100 g honey [28]. The results from this study were consistent with those reported for stingless bee honey from Alagoas, Brazil, 11.69–49.50 mg QE/100 g honey [31]. Nevertheless, a higher TFC value was found in six species of *Melipona* honey from Brazil [32] and a lower TFC value in *Melipona beecheii* honey from Cuba [11].

Overall, stingless bee honey had significantly higher TPC and TFC as compared to *Apis* honey. A low concentration of phenolic acids and flavonoids may contribute to the low TPC and TFC values of *Apis* honey as shown in Table 3. In addition to that, the difference can be explained by the different bee species and the way honey is produced by two different bees [33]. While the variation observed between stingless bee honey is due to the different botanical origins of the honey as the botanical origin has a direct association with the phenolic compounds present in honey [34]. Comparison of TPC and TFC data with similar honey from Malaysia and other regions could not be done as there is no related publication available.

3.1.2. The Free Radical Scavenging Activity and Ferric Reducing Antioxidant Power. Antioxidant activities of honey samples were estimated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. The free radical scavenging activity of honey samples was expressed as IC_{50}, indicating the amount of antioxidant needed to reduce the initial concentration of DPPH solution by 50%. Honey with a low value of IC_{50} has greater antioxidant activity than honey with a high value of IC_{50} [35]. Results of the free radical scavenging activity of honey samples are presented in Table 2. The IC_{50} value of *H. itama* honey varied between 11.27 and 24.09 mg/mL with the gelam honey having the lowest value. Thus, gelam honey had greater antioxidant activity than starfruit and acacia honey, but no significant difference ($p > 0.05$) was observed among them. Aljadi and Kamaruddin [36] also reported that gelam honey (honeybee honey) had higher antioxidant activity than coconut honey. Kek et al. [37] reported 26.63 mL/g in Malaysian stingless bee honey produced by *H. itama*. While Ismail et al. [28] reported a range of 10.6–19.7 mg/mL, which was consistent with our results. When compared to the stingless bee honey from Brazil, the IC_{50} value in this study was lower than the results reported (25.39–51.55 mg/mL). While the IC_{50} value of *H. itama* honey (11.27–24.09 mg/mL) was lower than *Apis* honey (53.65 mg/mL). A similar finding was reported by Duarte et al. [31]. However, there was no significant difference found among the honey samples except between gelam and *Apis* honey. In contrast, Alvarez-Suarez et al. [12] reported a higher IC_{50} in *M. beecheii* honey than *Apis* honey.

The ferric reducing antioxidant power (FRAP) analysis was performed to measure the ability of phenolic

Table 2: The antioxidant properties (total phenolic content, total flavonoid content, DPPH IC_{50}, and FRAP) of *Heterotrigona itama* and *Apis mellifera* honey.

Parameters	*Heterotrigona itama*			*Apis mellifera*
	Acacia	Gelam	Starfruit	*Acacia*
Total phenolic content (mg GAE/100 g honey)	58.39 ± 4.14^{ab}	74.72 ± 6.88^{a}	70.83 ± 1.03^{a}	52.64 ± 2.45^{b}
Total flavonoid content (mg QE/100 g honey)	10.70 ± 0.71^{c}	20.67 ± 0.23^{b}	25.71 ± 0.08^{a}	7.02 ± 0.06^{d}
DPPH(IC_{50}) (mg/mL)	21.41 ± 3.80^{ab}	11.27 ± 2.40^{b}	24.09 ± 1.77^{ab}	53.65 ± 2.55^{a}
FRAP (μmol $FeSO_4.7H_2O$/100 g honey)	77.8 ± 2.39^{b}	141.68 ± 4.94^{a}	164.88 ± 11.79^{a}	72.78 ± 1.45^{b}

*Data are expressed as mean ± standard deviation (SD). The small letters in the same raw (a–d) indicate significant differences at the level of $p < 0.05$ between honey samples from different botanical origins. GAE = gallic acid equivalent, QE = quercetin equivalent, and DPPH (IC_{50}) = free radical scavenging activity.

Table 3: Correlation matrix between total phenolic content (TPC) and total flavonoids content (TFC) and antioxidant activity (FRAP and DPPH (IC_{50})) of *Heterotrigona itama* and *Apis* honey.

Variables	TPC	TFC	DPPH (IC_{50})	FRAP
TPC	1.000			
TFC	0.922**	1.000		
DPPH(IC_{50})	−0.813**	−0.658**	1.000	
FRAP	0.919**	0.991*	−0.588**	1.000

*Significant at $p < 0.05$; **Not significant at $p < 0.05$. TPC = total phenolic content, TFC = total flavonoids content, DPPH = free radical scavenging activity, and FRAP = ferric reducing antioxidant power.

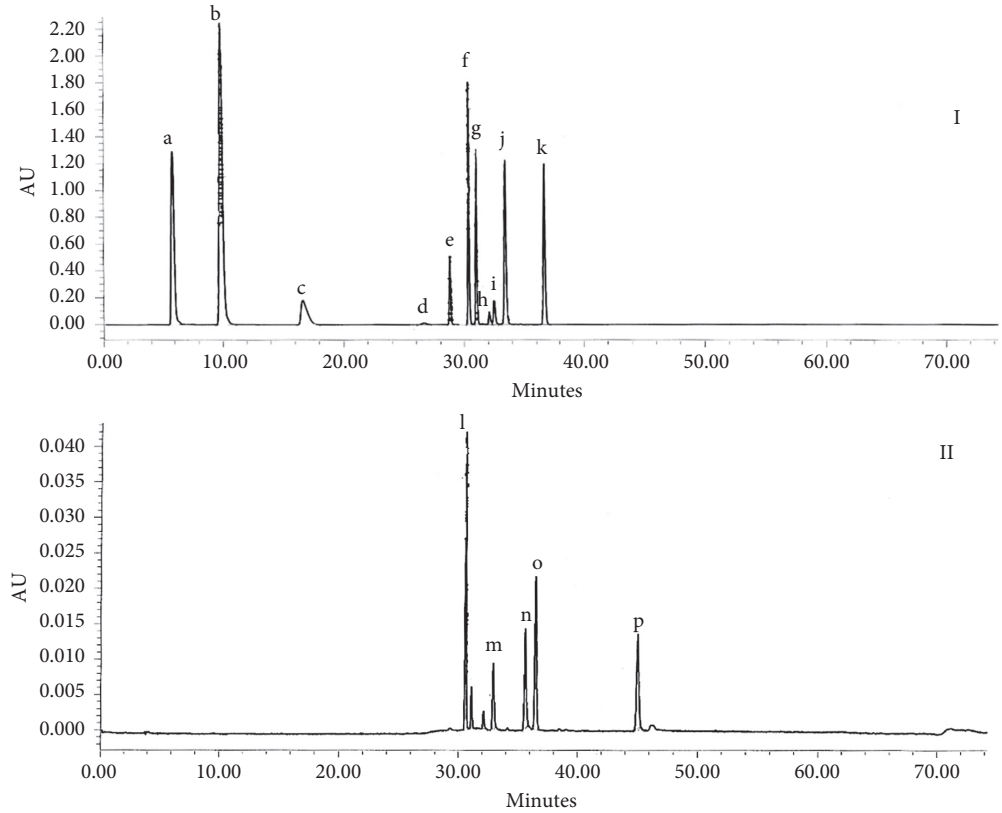
compounds as an antioxidant to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). From the results in Table 2, the FRAP values of all honey samples varied between 72.78 and 164.88 μmol $FeSO4.7H_2O$/100 g honey, where both gelam and starfruit honey displayed significantly ($p < 0.05$) higher FRAP values than those from acacia and *Apis* honey. Among the analyzed honey samples, starfruit honey showed the highest FRAP value, which indicated that starfruit honey possesses a stronger reducing power than other honey samples. The values of FRAP in this study were higher than those reported by Alvarez-Suarez et al. [12] on Cuban honey (*M. beecheii*) of 38.54 μmol $FeSO4.7H_2O$/100 g honey but lower than those reported by Chan et al. [38] on Malaysia honey (*Trigona* spp.) of 3,630.18–7,477.03 μmol $FeSO4.7H_2O$/kg honey and by Nweze et al. [39] on Nigerian honey of 417.36–439.15 μmol $FeSO4.7H_2O$/100 g honey. In recent studies, Majid et al. [23] and Tufail Ahmad et al. [27] reported higher FRAP values of Malaysian stingless bee honey ranging between 283.80 and 1401.80 μM Fe (II)/100 g honey and 27.18 μmol $FeSO4.7H_2O$/g honey, respectively.

The variation in IC_{50} and FRAP values in the honey samples analyzed could attribute to the different amounts and types of phenolic compounds [40] presented in the honey samples as the samples used in this study belonged to different botanical origins. Therefore, honey from different botanical origins possesses distinct antioxidant activity [14].

3.2. Correlation Analysis. The Pearson's correlation showed a significant correlation between FRAP and TFC (0.991). This indicates that flavonoids are the greatest contributor to the reducing power of honey. Nevertheless, no significant correlation was observed between TPC and TFC (0.922), between DPPH (IC_{50}) and TPC (−0.813) and TFC (−0.658), and between FRAP and TPC (0.919) and DPPH (IC_{50}) (−0.588). The findings from this study also suggest that the antioxidant activity of honey is not only influenced by the phenolic and flavonoids content but also other antioxidant compounds that partially contribute to the antioxidant activity of honey. As known, honey contains a wide variety of significant antioxidant compounds such as organic acids, amino acids, enzymes, carotenoids, Maillard reaction products, and proteins [34, 41, 42]. Duarte et al. [30] also reported a significant correlation between the FRAP and flavonoids content in Africanized and stingless bee honey from Alagoas, Brazil. Other studies also demonstrated a significant correlation between the FRAP and flavonoids content, suggesting flavonoids as one of the key components responsible for the antioxidant activity of honey [22, 40, 43, 44].

3.3. Limit of Detection and Limit of Quantitation of Phenolic Compounds. The limit of detection (LOD) and limit of quantitation (LOQ) values were calculated based on the 3 ∗ standard deviation of blank response/slope and 10 ∗ standard deviation of blank response/slope, respectively. The LOD of phenolic acids and flavonoids were ranged between 0.30 and 148.33 μg/100 g honey and 0.05–0.55 μg/100 g honey, respectively, while the LOQ of phenolic acids and flavonoids were ranged between 1.25 and 494.38 μg/100 g honey and 0.18–1.85 μg/100 g honey, respectively.

3.4. Phenolic Profile. Phenolic compounds identification was performed using HPLC analysis. The chromatograms of the standard of phenolic compounds and honey samples are shown in Figures 2(a) and 2(b), respectively. Table 4 shows the phenolic compounds present in all honey samples. Sixteen compounds were found in starfruit honey, while 15 compounds were identified in acacia and gelam honey. These results indicate that all honey samples possess almost similar phenolic compound profiles; however, different concentrations were quantified in the honey sample from different botanical origins. Ranneh et al. [17] found 13 phenolic compounds (gallic acid, caffeic acid, caffeic acid phenethyl ester, syringic acid, catechin, apigenin, chrysin, cinnamic acid, 2-hydroxybenzoic acid, kaempferol, p-coumaric acid, quercetin-3-O-rutinosid, and 4-hydroxybenzoic acid) in Malaysian stingless bee honey using LC-ESI-MS/MS. On the contrary, Majid et al. [18] identified 6 phenolic compounds (chlorogenic acid, p-coumaric acid, catechin,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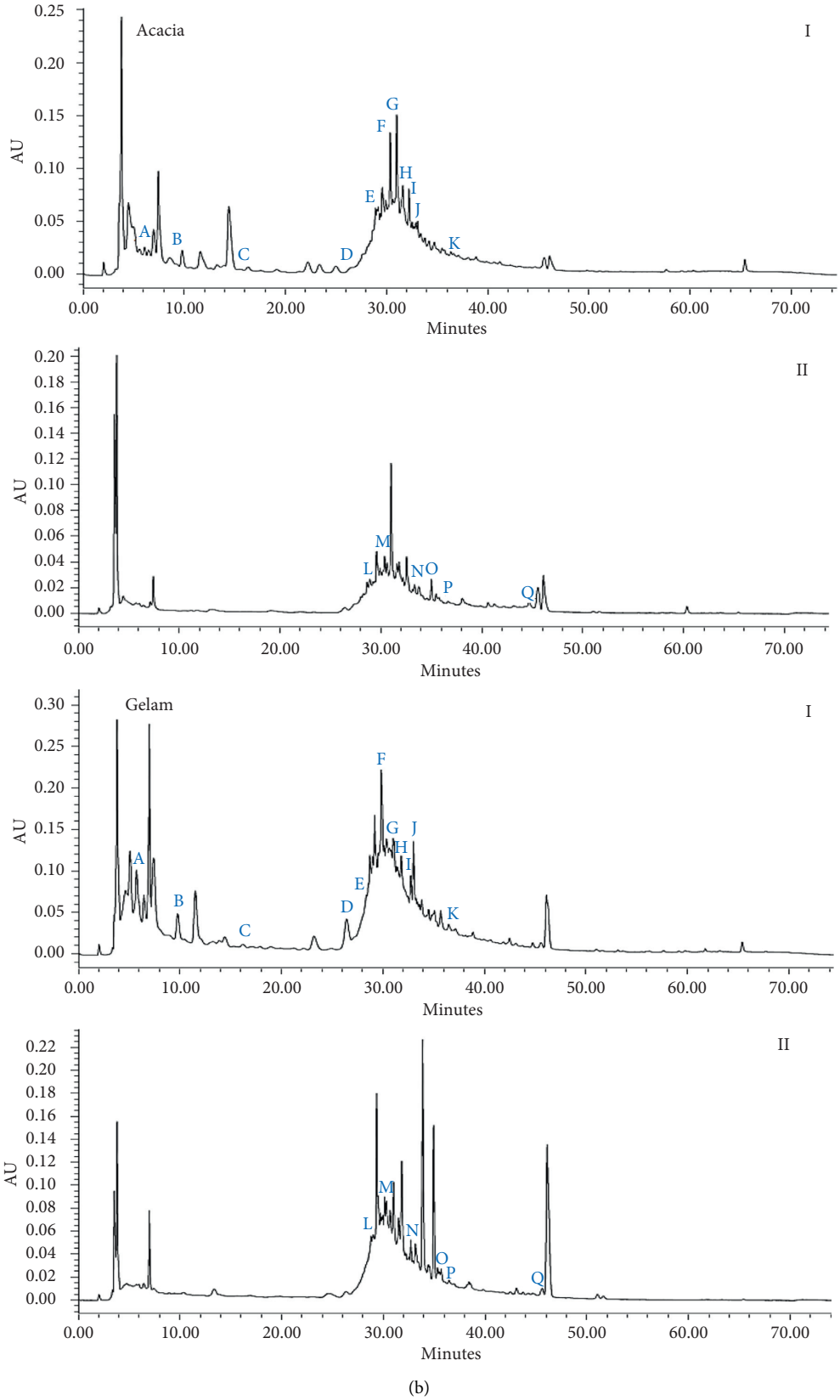

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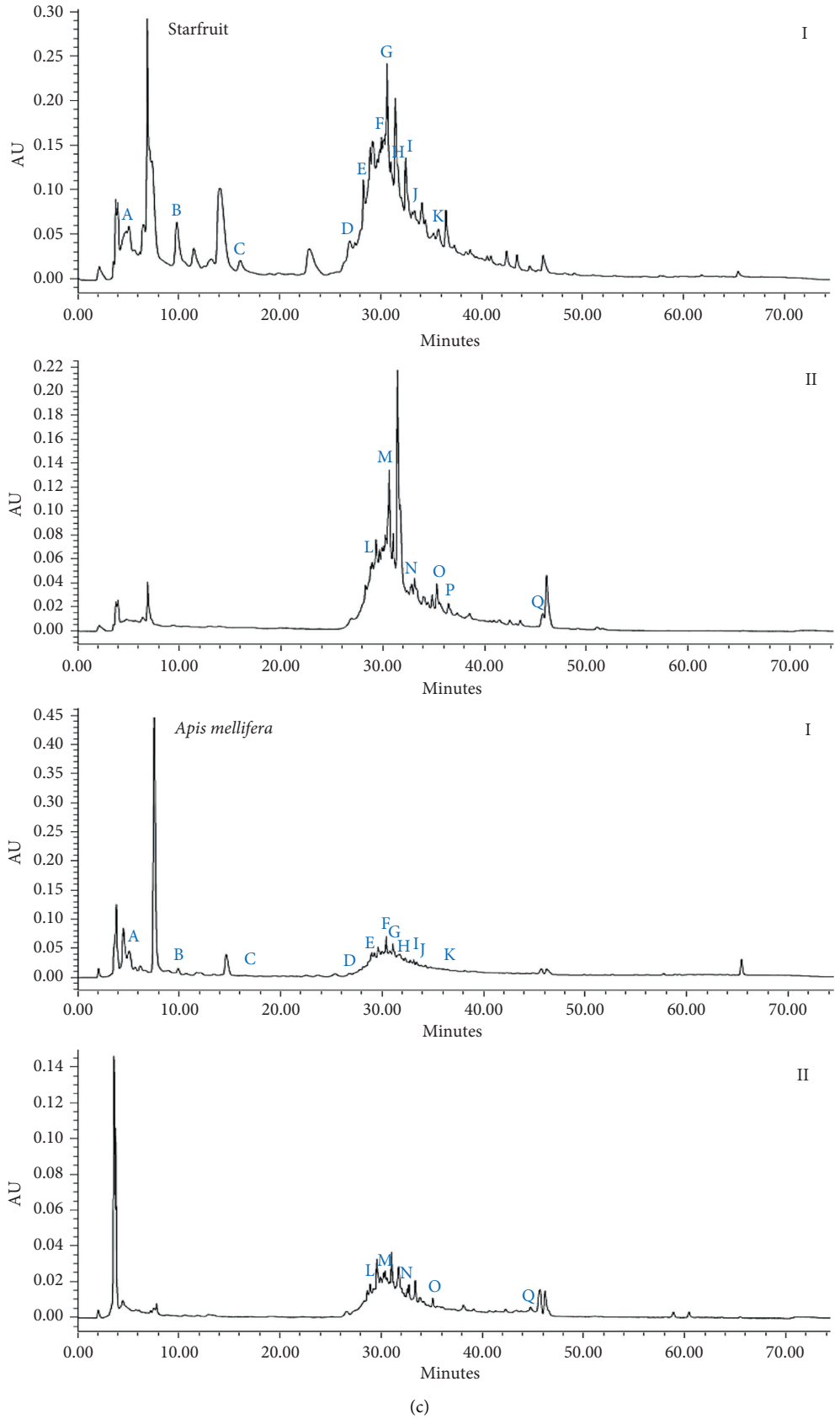

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Figure 2: Chromatogram of standard of phenolic compounds (a) and chromatogram of phenolic acids and flavonoids detected in *Heterotrigona itama* (acacia, gelam, and starfruit) and *Apis* honey (b) at the wavelength of (I) 290 nm and (II) 340 nm. Phenolic acids: A = gallic acid, B = 3,4-dihydroxybenzoic acid, C = 4-hydroxybenzoic acid, D = chlorogenic acid, E = syringic acid, F = p-coumaric acid, G = ferulic acid, H = ellagic acid, I = benzoic acid, J = salicylic acid, and K = trans-cinnamic acid. Flavonoids: L = taxifolin, M = myricetin, N = quercetin, O = naringenin, and P = chrysin.

Table 4: The profile and concentration of phenolic compounds (μg/100 g honey) in *Heterotrigona itama* honey from different botanical origins (acacia, gelam, and starfruit) and *Apis mellifera* (acacia) honey.

Phenolic compounds	Botanical origin of honey			
	Heterotrigona itama			*Apis mellifera*
	Acacia	Gelam	Starfruit	*Acacia*
Phenolic acids				
Ferulic acid	135.01 ± 8.73^{a}	125.69 ± 3.35^{a}	116.54 ± 1.36^{a}	43.18 ± 1.47^{b}
p-Coumaric acid	87.54 ± 10.37^{a}	84.83 ± 0.89^{a}	59.67 ± 0.02^{b}	33.70 ± 0.81^{c}
trans-Cinnamic acid	ND	6.60 ± 0.40^{a}	19.37 ± 0.40^{a}	ND
Salicylic acid	137.72 ± 5.77^{b}	113.00 ± 1.35^{c}	302.38 ± 1.10^{a}	71.04 ± 1.20^{d}
Gallic acid	34.04 ± 0.42^{b}	145.02 ± 3.52^{a}	32.76 ± 0.02^{b}	34.27 ± 0.01^{b}
Ellagic acid	312.89 ± 13.87^{b}	855.30 ± 16.80^{a}	334.77 ± 11.92^{b}	73.25 ± 0.06^{c}
3,4-Dihydroxy benzoic acid	2.32 ± 2.86^{c}	24.84 ± 1.71^{b}	132.81 ± 0.47^{a}	ND
Benzoic acid	$2{,}434.80 \pm 24.80^{c}$	$2{,}808.40 \pm 65.20^{b}$	$12{,}626.00 \pm 14.70^{a}$	738.08 ± 1.58^{d}
4-Hydroxy benzoic acid	42.59 ± 1.12^{b}	34.17 ± 5.40^{b}	419.03 ± 0.89^{a}	ND
Chlorogenic acid	17.63 ± 0.21^{c}	116.41 ± 0.18^{b}	132.14 ± 0.28^{a}	13.10 ± 0.58^{d}
Syringic acid	161.94 ± 3.17^{c}	233.63 ± 5.40^{b}	405.43 ± 0.41^{a}	71.17 ± 4.07^{d}
Flavonoids				
Taxifolin	248.69 ± 3.15^{c}	496.33 ± 4.00^{b}	$1{,}212.70 \pm 3.07^{a}$	76.50 ± 1.63^{d}
Myricetin	11.16 ± 0.09^{d}	45.42 ± 2.47^{a}	29.61 ± 0.01^{b}	18.32 ± 0.13^{c}
Quercetin	11.66 ± 0.01^{c}	15.31 ± 0.36^{b}	29.82 ± 0.08^{c}	11.66 ± 0.04^{c}
Naringenin	14.69 ± 0.73^{a}	ND	15.89 ± 0.24^{a}	ND
Chrysin	6.62 ± 0.04^{d}	12.31 ± 0.10^{c}	21.23 ± 0.01^{b}	66.00 ± 1.45^{a}

*Data are expressed as mean ± standard deviation. The letters in the same row (a–d) indicate significant differences at the level of $p < 0.05$ between honey from different botanical origins. ND = not detected.

protocatechuic acid, epicatechin, and rutin) in *H. itama* honey collected from Johor, Malaysia. Biluca et al. [13] demonstrated the presence of mandelic acid, rosmarinic acid, caffeic acid, aromadendrin, vanillin, isoquercetin, umbelliferone, eriodictyol, sinapaldehyde, syringaldehyde, and carnosol in Brazilian stingless bee honey from different geographical origins. In a different study, Alvarez-Suarez et al. [12] found dihydrocaffeic acid, C-pentosyl-C-hexosyl-apigenin, quercetin deoxyhexosyl hexoside, apigenin trihexoside, kaempferol deoxyhexosyl hexoside, isohamnetin deoxyhexosyl hexoside, isohamnetin, bis-methylated quercetin, apigenin, kaempferol, methyl luteolin, and methyl quercetin in *M. beecheii* from Cuba. All these compounds were not detected in this present study, which could be explained by the difference in the origin of the honey, the bee species, and the method of analysis used.

Benzoic acid was the most abundant phenolic compound found in all honey samples with values ranging between 738.08 μg/100 g honey–12,626.00 μg/100 g honey. Starfruit honey had the highest benzoic acid (12,626.00 μg/100 g honey), followed by gelam (2,808.40 μg/100 g honey), acacia (2,434.80 μg/100 g honey), and *Apis* (738.08 μg/100 g honey) honey. Statistical differences were observed between all honey samples. In a recent study, Braghini et al. [45] reported that carnosol was identified as the major phenolic compound in *Melipona bicolor* honey. In previous studies, fraxin was found abundantly in Ecuadorian stingless bee honey [46] and taxifolin in *Melipona* (*Michmelia*) *seminigra merrillae* honey from Brazil [13]. Regarding the flavonoids content, taxifolin was identified as the major flavonoid (76.50–1,212.70 μg/100 g honey) in all honey samples investigated. However, stingless bee honey (248.69–1,212.70 μg/100 g honey) exhibited significantly higher taxifolin content than *Apis* honey (76.50 μg/100 g honey). Our result was in accordance with those previously found in Amazon honey from *M.* (*Michmelia*) *seminigra merrillae* [14]. On the other hand, luteolin was found as the predominant flavonoid in *Melipona asilvai* and *Melipona quadrifascita* honey; kaempferol in *Melipona anthidioides*, *Melipona scutellaris*, and *M. subnitida* honey; and apigenin in *Melipona mandacaia* honey from Sergipe state, Brazil [32]. Different types and concentrations of phenolic compounds in honey were reported from different studies, which might be attributed to the different botanical origins, bee species, and geographical locations of honey.

In this present study, ferulic acid, salicylic acid, ellagic acid, benzoic acid, 4-hydroxybenzoic acid, taxifolin, myricetin, quercetin, and naringenin were detected in stingless bee honey. To the best of our knowledge, this is the first report of the presence of these compounds in Malaysian stingless bee honey. The findings revealed that stingless bee honey contains a wide range of phenolic compounds. This finding could have a significant impact on the stingless bee industry sustainability as well as promote Malaysian stingless bee honey globally.

Overall, stingless bee honey exhibited more types and higher concentrations of phenolic compounds compared to *Apis* honey. This suggests that stingless bee honey is a good source of natural phenolic acids and flavonoids. These findings are consistent with those reported by Alvarez-Suarez et al. [12] and Guerrini et al. [46]. Phenolic acids and flavonoids have been proven to have antioxidant activity, which has a significant correlation with honey's medicinal properties, and each of them has specific health benefits [47]. Furthermore, stingless bee honey has the potential in treating eye illnesses, wounds, cancer, diabetes mellitus,

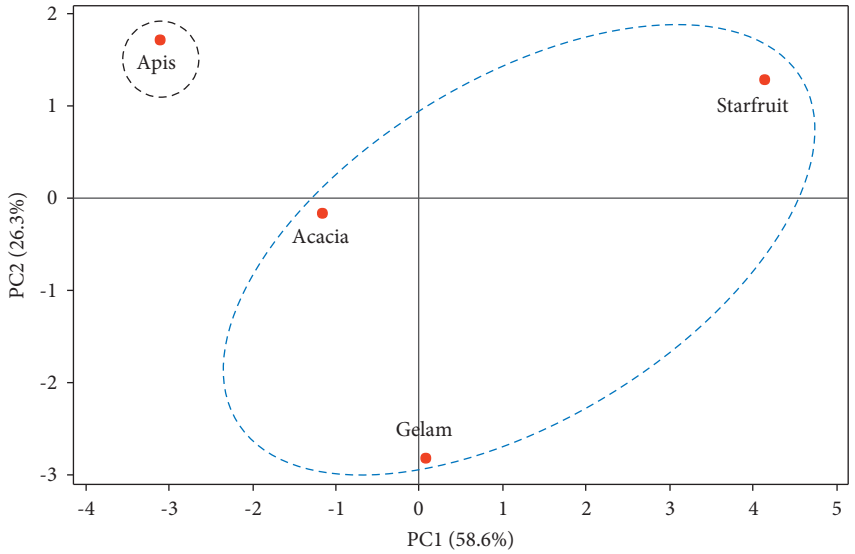

Figure 3: Score plot of stingless bee and *Apis* honey.

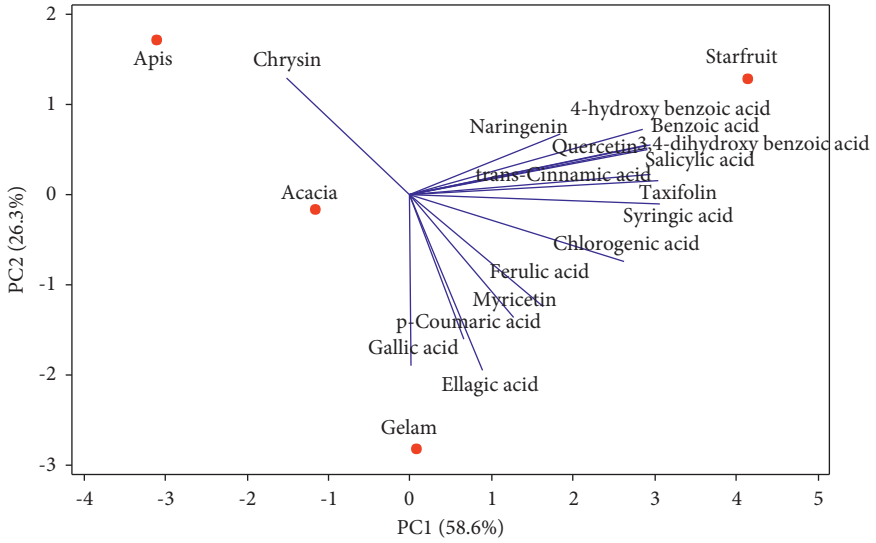

Figure 4: Biplot of phenolic compounds in stingless bee and *Apis* honey.

hypertension, microbial infection, fertility problems, and dysregulated lipid profiles [47]. This indicates that stingless bee has a great potential to be commercialized and consumed by a large population. *Acacia*, gelam, and starfruit honey can be a good choice as an antioxidant source for humans. Since they have high antioxidant content than *Apis* honey, we believe that stingless bee honey has a better effect on human health.

3.5. Multivariate Analysis. Multivariate analysis was performed to determine potential phenolic compounds, which can be used as a chemical marker to distinguish stingless bee honey from *Apis* honey. The principal component analysis (PCA) was used to identify honey based on the concentration of phenolic compounds. To establish the best parameters for classifying honey samples, two principal components with eigenvalues greater than one were extracted. The first principal component (PC1) accounted for 58.6% of the variance, while the second principal component (PC2) accounted for 26.3%. These two components explained 84.9% of the variation in the data. Based on the score plot (Figure 3), all honey samples were scattered away from each other, indicating that all honey samples contained different types and concentrations of phenolic compounds. From the score plot, *Apis* honey was located far away from the stingless bee honey, proving that *Apis* honey was significantly different from the stingless bee honey in terms of the phenolic compounds. This finding suggests that phenolic compounds can distinguish *Apis* honey from stingless bee honey. A biplot was further created to display the relationship between honey samples and phenolic compounds (Figure 4). Then, a loading plot was performed to assess which phenolic compounds contributed significantly to

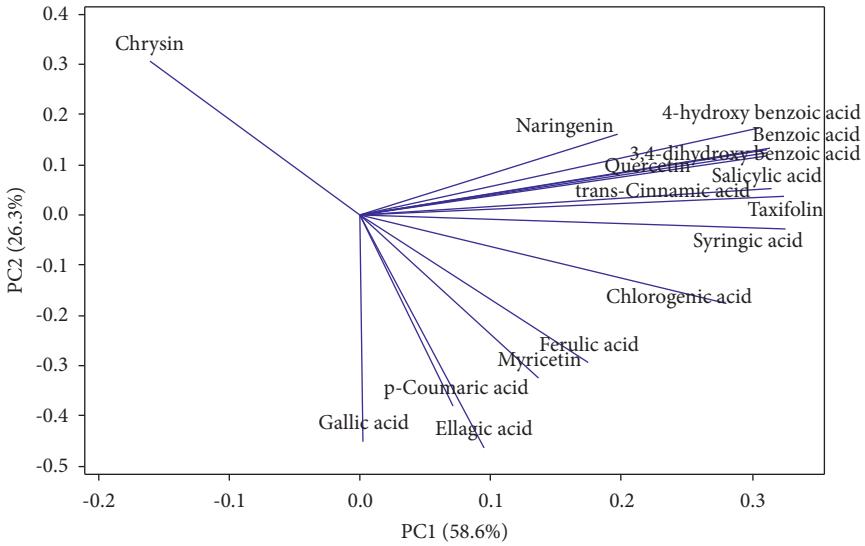

Figure 5: Loading plot of phenolic compounds in stingless bee and *Apis* honey.

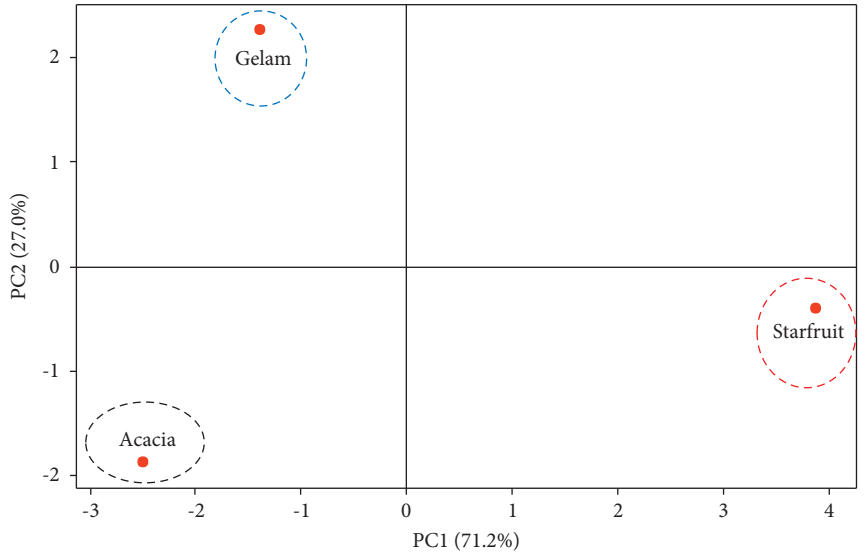

Figure 6: Score plot of stingless bee honey (acacia, gelam, and starfruit).

the separation (Figure 5). According to the PC2, chrysin was the only phenolic compound responsible to separate *Apis* honey from stingless bee honey. As a result, chrysin can be used as a chemical marker to distinguish *Apis* honey from stingless bee honey.

To study the potential to differentiate acacia, gelam, and starfruit honey, data of *Apis* honey were removed, and principal component analysis (PCA) was constructed. The first principal component (PC1) accounted for 71.2% of the variance, while the second principal component (PC2) accounted for 27.0%. These two components explained 98.2% of the variation in the data. Based on the score plot (Figure 6), all stingless bee honey were well separated, indicating that all honey samples were significantly different in phenolic compounds content.

The most influential phenolic compounds responsible for clustering to be identified as a potential marker for all honey samples were determined using biplot (Figure 7(a)) and loading plot (Figure 7(b)). Based on the loading plot, gallic acid and ellagic acid have a strong impact on the

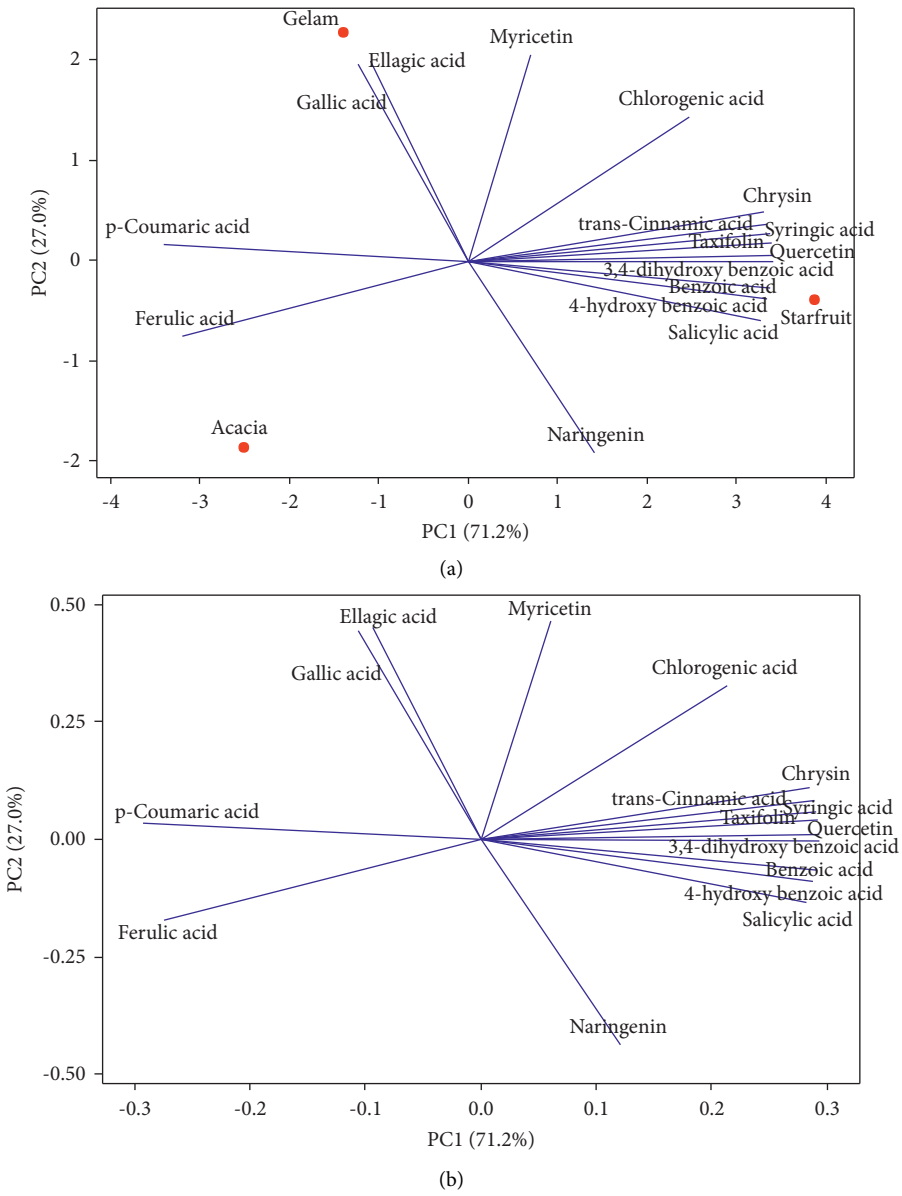

Figure 7: Biplot (a) and loading plot (b) of acacia, gelam, and starfruit honey.

separation of gelam honey. Salicylic acid, benzoic acid, and 4-hydroxybenzoic acid are responsible for the separation of starfruit honey, while ferulic acid is the only one that separated acacia from other honey samples.

4. Conclusion

This study discovered that botanical origin significantly influences antioxidant activity and the composition of phenolic compounds in honey. Flavonoids were shown to have a significant relationship with the antioxidant activity (FRAP) of honey. In addition, HPLC analysis indicated and validated that stingless bee honey is rich in phenolic components as compared to *Apis* honey, showing that stingless bee honey is a valuable source of natural phenolic acids and flavonoids. Furthermore, the findings suggest that the botanical origin has a significant impact on the content of phenolic compounds in honey. A multivariate data analysis was used to find a potential marker that might be used to distinguish honey samples based on their phenolic compounds. The results showed that chrysin successfully distinguished the stingless bee honey from *Apis* honey. Gelam honey was discriminated by gallic acid and ellagic acid, starfruit honey by salicylic acid, benzoic acid, and 4-hydroxybenzoic acid, while acacia honey by ferulic acid. However, the number of samples used in this study was limited. Thus, a further investigation with a large number of samples is required in order to confirm the present results.

Data Availability

Data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors declare that there are no potential conflicts of interest.

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RESEARCH ARTICLE OPEN ACCESS

Evaluation of Antibacterial Activity of Essential Oils of *Melaleuca cajuputi* Powell

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Abstract

***Melaleuca cajuputi* Powell is a tree species belonging to the family Myrtaceae and is widely used in traditional medicine. This study was conducted to investigate the antibacterial activities of essential oils of *M. cajuputi* Powell. Antibacterial activity was tested against Gram positive and Gram negative bacteria using the agar disc diffusion method. The essential oils of *M. cajuputi* were found to exert antibacterial activity against all of the tested bacteria, including *Staphylococcus aureus, Streptococcus pyogenes,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae,* and *Escherichia coli.* The zones of inhibition for *S. aureus, S. pyogenes,* MRSA, *E. coli,* and *K. pneumoniae* were 12.7 mm, 10.7 mm, 10.0 mm, 8.7 mm and 9.3 mm respectively, against 0.714% (w/w) of the essential oils. These results highlighted that Gram negative bacteria are less susceptible to the essential oils of *M. cajuputi*. A large zone of inhibition might be a sign of a leaching antimicrobial agent. These findings suggest that *M. cajuputi* is a potential natural antibacterial agent.**

Keywords: *Melaleuca cajuputi* Powell, essential oils, antibacterial, minimum inhibitory concentration

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INTRODUCTION

Essential oils are highly concentrated, unstable substances found in plants. They have a distinctive fragrance with a high refractive index owing to ethers, aldehydes, terpenes, esters, ketones, phenols, and alcohols.[1] Most essential oils are colorless or pale yellow in color and are liquid at room temperature. Essential oils are a mixture of volatile compounds that exclude saponins, flavonoids, tannins, steroids, terpenoids, and alkaloids.[2] Notably, essential oils are significant in folk herbal medicines, cosmetic products, aromatics, perfumes, and phototherapy. [3] Moreover, they are well known for their antimicrobial properties and are also effective in treating diseases such as carcinoma, Alzheimer's disease, cardiovascular disease, discomfort, pregnancy, and insomnia.[4,5] The secondary metabolites in essential oils play a critical role in plant protection, as they regularly have antimicrobial characteristics.

Melaleuca cajuputi Powell is a member of the Myrtaceae family and is prominently known as gelam, white tree, cajeput oil tree, tea tree, or paper bark tree. *M. cajuputi* is commonly present in swampy ground close to the coasts and is frequently found in tropical countries, such as Malaysia, Indonesia, Vietnam, Thailand, Myanmar, and northern Australia.[6] In Malaysia, it can be found in mangrove swamps, particularly in Peninsular Malaysia. A few *Melaleuca* species harbor essential oils that are broadly utilized as therapeutic products, insecticides, and body care products.[7] Adult *M. cajuputi* grows up to 33 m in height and are characterized by a slender crown. The tree is normally an unattached stem but may develop into collective stems. It can be easily recognized by a pure-white papery bark and thin strips as an outer layer. The leaves are gray-green in color, 4-10 cm long and 2 cm wide, firm, and have a pleasant scent. The flowers are whitish-pink or purple in color when bloom. The seeds are firmly encircled and joined. Stems are enclosed in grayish-brown woody capsules. *M. cajuputi* essential oils are isolated from the leaves via simple steam distillation. These essential oils are either colorless or pale yellow in color and release camphor (menthol)-like aroma with a moderately bitter taste. This odor makes *M. cajuputi* essential oils anti-insecticidal because the aroma expels the mosquitoes. They are also prescribed as mucus expectorant and as medication for bronchitis. In Australia, the leaves are used for various types of ill-treatment for centuries. In Asia, its oil is customarily used to diminish joint discomfort, stiff joints, and rheumatism, and used as a mosquito repellent. Interestingly, water from the boiled leaves could relieve pain and jaundice. Meanwhile, the shoots can be eaten as a salad.[8]

According to a study by Hyldgaard et al, p-cymene is the most abundant compound in Cajaput essential oils.[9] This compound might potentially act as a substitutional impurity, which partly disturbs the cytoplasmic membrane of bacteria. Phytochemical analysis revealed that α-pinene, limonene, αterpinene, and 4-terpineol extracted from *M. cajuputi* leaves have antibacterial, anti-inflammatory, anodyne, and insecticidal properties. These phytochemical compounds are also used as cooking seasonings and aromatic agents in soaps, body care products, cleansers, and fragrances. *M. cajuputi* essential oils are also used to ease dental pain, headaches, seizures, and rheumatoid arthritis, and screen insects.[10,11] In the present study, we focused on the antibacterial properties of the essential oils of *M. cajuputi* against five bacteria.

MATERIALS AND METHODS

Plant material

Information regarding plant collection is listed in Table 1. Plant authentication was performed by a competent botanist from the Universiti Sultan Zainal Abidin. In this study, 21 essential oils of different geographical origins were used.

Extraction of essential oils

The extraction yield of essential oils ranges from 0.2 to 0.3 %. Briefly, the essential oils were extracted from the fresh leaves via steam distillation for 4 h. By utilizing water vapor at atmospheric pressure, the oil are refined from the leaves at a temperature below 100 °C, and the 4-h extraction allows for the isolation and production of essential oil from the crude leaf samples. This process was followed by mixing the sample with distilled water and boiling at 100 °C in a distillation flask. The emulsion of oil and water was permitted

Table 1. Place of plant collection

Voucher No.	Location	latitude, longitude	Types of soil	Soil area	States	District
UniSZA P1	Kg. Gong Badak	5.395461, 103.086865	Bris	Village	Terengganu	Kuala Terengganu
UniSZA P2	Kg. Merabang Panjang	5.487304, 102.985082	Bris	Village	Terengganu	Kuala Terengganu
UniSZA P3	Kg. Merang	5.522293, 102.965654	Bris	Village	Terengganu	Setiu
UniSZA P4	Kg. Pulai Baru	5.373320, 103.065907	Bris	Village	Terengganu	Setiu
UniSZA P7	Sg. Merang	5.524582, 102.941955	Bris	Village	Terengganu	Merang
UniSZA P8	Kg. Lembah Bidong	5.490218, 102.988754	Bris	Village	Terengganu	Setiu
UniSZA P9	Kg. Telaga Papan	5.533344, 102.911413	Bris	Village	Terengganu	Kuala Terengganu
UniSZA P10	Kg. Sekeping, Penarek	5.570487, 102.846866	Bris	Village	Terengganu	Kuala Terengganu
UniSZA P11	Kg. Rhu Tapai	5.515039, 102.978965	Bris	Village	Terengganu	Kuala Terengganu
UniSZA P12	Kg. Beris Tok Ku	5.590235, 102.462125	Bris	Village	Terengganu	Setiu
UniSZA P13	Pantai Bachok	5.920937, 102.462125	Peaty	Waterlogged	Kelantan	Bachok
UniSZA P14	Cherang Ruku	5.886560, 102.487515	Peaty	Waterlogged	Kelantan	Pasir Puteh
UniSZA P15	Kg. Pendas	1.3764218, 103.6366524	Peaty	Waterlogged	Johor Bharu	Tanjung Kupang
UniSZA P16	Damai laut	4.2590101, 100.591164	Peaty	Waterlogged	Perak	Lumut
UniSZA P20	Sg. Jawi	5.192505, 100.504226	Bris	Village	Pulau Pinang	Seberang Perai
UniSZA P21	Rantau Panjang	5.9475900, 101.9570540	Peaty	Waterlogged	Kelantan	Pasir Mas
UniSZA P22	Kerubong	2.2809800, 102. 2340030	Peaty	Waterlogged	Melaka	Melaka Tengah
UniSZA P23	Port Dickson	2.4284720, 101.8946300	Clay	Hill	Negeri Sembilan	Port Dickson
UniSZA P24	Wangsa Maju	3.224553, 101.728333	Clay	Hill	Kuala Lumpur	Kuala Lumpur
UniSZA P25	Sintok	6.459666, 100.498843	Clay	Hill	Kedah	Sintok
UniSZA P26	Shah Alam	3.065988, 101.491633	Peaty	Waterlogged	Selangor	Shah Alam

for 4 h to guarantee the segregation of oil layers and water. The oil yield was transferred into an amber bottle. The proportion of essential oils was determined and stored at room temperature until further use. The oil samples were then analyzed using FTIR and GCMS according to Zainon et al. with some modifications.[12]

Determination of the extraction yield of essential oils

Chemometric analysis was performed using the spectroscopic data to assess the spatial variations of the 21 *M. cajuputi* essential oils. Spectroscopy techniques are used for phytochemical identification and provide significant information regarding the qualitative and quantitative composition of essential oils, as well as their pattern recognition using chemometrics. Hierarchical cluster analysis was used to differentiate the samples. To evaluate the samples, the similarity between the spectral fingerprints was determined using similarity analysis (SA), which is based on correlation coefficients r.

Test organisms

The bacterial species used as test organisms were *Staphylococcus aureus* (ATCC 11632), *Streptococcus pyogenes,* and clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* (ATCC 10536), and *Klebsiella pneumoniae* (ATCC 10031). All stock cultures were obtained from the Faculty of Medicine, Microbiology Laboratory, Universiti Sultan Zainal Abidin.

Preparation of microbial cultures

All tested bacterial strains were cultured in nutrient agar and broth. Bacterial suspensions were prepared by inoculating the nutrient broth with each of the bacterial cultures and incubated overnight at 37 °C.

Antibacterial assay

Antibacterial activity assay was performed using the disc diffusion method. The test organisms were cultured on sterile Petri dishes containing nutrient agar for 18–24 hours at 37 °C. On the next day, the cultures were adjusted to match 0.5 McFarland standards using normal saline. Then, a sterile cotton swab was dipped into each bacterial suspension and streaked onto MHA plates. Blank discs that were already impregnated with essential oil and distilled water (negative control) were placed on the surface of the agar using forceps. A chloramphenicol disk was used as a positive control. The three discs were placed on each plate and labelled correctly. The plates were incubated at 37 °C for 24 h. A clear zone indicated growth inhibition, and the diameter of the zone was measured in millimeters using a ruler. The test was performed in triplicate.[13]

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the *M. cajaputi* essential was determined by performing MIC using a 96-well microtiter

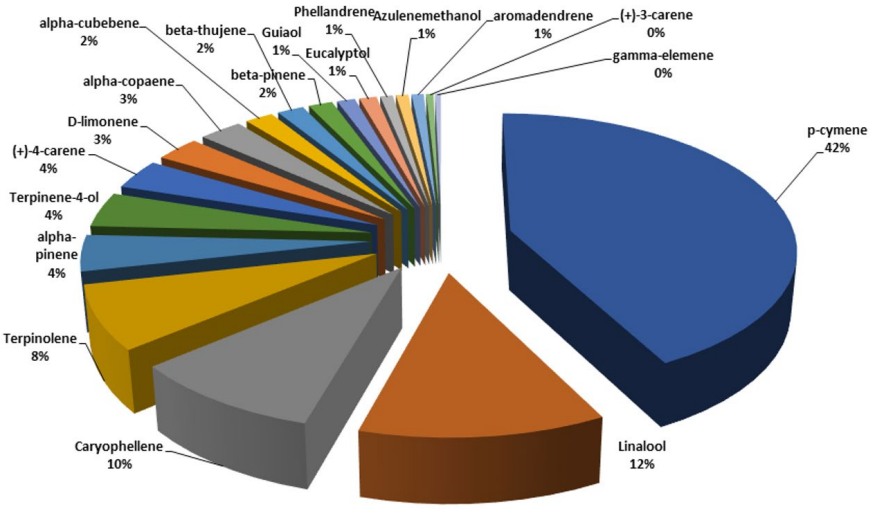

Fig. 1. Fraction of chemical compounds identified from *Melaleuca cajuputi* essential oils in Peninsular Malaysia.

Table 2. Zone of inhibition (mm) for disc diffusion method of the essential oils of *M. cajuputi* against selected bacteria

	Zone of inhibition (mm)				
	S. aureus	*S. pyogenes*	MRSA	*E. coli*	*K. pneumoniae*
Essential oil 0.714% (w/w)	12.7	10.7	10.0	8.7	9.3
Distilled water (negative control)	6	6	6	6	6
Chloramphenicol (positive control)	26.7	29.3	22.0	22.7	26.0

Tests were performed in triplicate.

plate.[14] The highest concentration of essential oils used was 0.714% (w/w). For the positive control, 1 mg/L to 512 mg/L chloramphenicol was prepared. The negative control was prepared using 100 µL of MHB inoculated with the bacteria and 100 µL of 10% methanol. Then, the microtiter plate was incubated for 24 h at 37 °C.

RESULTS

A total of 19 compounds were identified from the essential oils of *M. cajuputi* leaves (Fig. 1). Moreover, the essential oils exhibited antibacterial activity against *S. aureus, S. pyogenes,* MRSA, *K. pneumoniae,* and *E. coli* (Table 2), with inhibition zones ranging from 8.7 to 12.7 mm. Chloramphenicol disk was used as a positive control, and distilled water was used as a negative control. The largest inhibition zone (12.7 mm) was observed against *S. aureus,* followed by *S. pyogenes* (10.7 mm), MRSA (10.0 mm), *K. pneumoniae* (9.3 mm), and E. coli (8.7 mm).

Next, MIC was used to measure the efficacy of the extracts against the tested bacterial strains. MIC was for the tested bacteria, which showed a zone of inhibition and were susceptible to the essential oils of *M. cajuputi* in the earlier antibacterial assay using the disc diffusion method. Results showed that the essential oils of *M. cajuputi* showed promising antibacterial activities. The MIC of the essential oils against *E. coli* and *K. pneumoniae* was 0.714%. Similarly, the MICs against Gram negative bacteria (*S. aureus, S. pyogenes,* and MRSA) were also 0.714% (Table 3).

Table 3. MIC value of *M. cajuputi* essential oils

Tested bacterial strains	MIC (%)
S. aureus	0.714
S. pyogenes	0.714
MRSA	0.714
K. pneumoniae	0.714
E. coli	0.714

Tests were performed in triplicate.

DISCUSSION

The phytochemical compounds found in *M. cajuputi* essential oils were determined using FTIR and GC-MS. Nineteen compounds, namely p-cymene, linalool, caryophellene, terpinolene, alpha-pinene, terpinene-4-ol, (+)-4-carene, D-limonene, alpha-copaene, alpha-cubebene, beta-thujene, beta-pinene, guiaol, eucalyptol, azulenemethanol, phellandrene, aromadendrene, (+)-3-carene, and gamma-elemene, were identified. The highest compounds found in *M. cajuputi* essential oils were p-cymene, followed by linalool and caryophellene. p-Cymene is the main antimicrobial compound in *M. cajuputi* essential oils. Several studies have suggested that this monoterpene possesses antibacterial, antiviral, and antifungal activities.[15] Furthermore, previous studies have shown that linalool has anxiolytic, anti-cholesterol, and antibacterial activities.[16-17] Aelenei et al. demonstrated that linalool alone or in combination with antibiotics showed antibacterial activity against Gram positive

and Gram negative[18]. Meanwhile, the antimicrobial effect of β-caryophyllene was previously examined against human pathogenic bacterial and fungal strains. The results showed that β-caryophyllene demonstrated selective antibacterial activity against *S. aureus* and had a more pronounced antifungal activity than kanamycin.[19] Therefore, our results showed that *M. cajuputi* essential oils were effective in inhibiting Gram positive and Gram negative bacteria.

Essential oils can inhibit the growth of various types of pathogens because of the existence of natural substances produced by plants.[20] The phytochemical composition of essential oils is heterogeneous and comprises 20–60 different bioactive compounds.[21] Essential oils and their components are hydrophobic, which makes them promising antimicrobial agents. This characteristic allows them to be separated from lipids, which are constituents of the cell membrane of bacteria and mitochondria. Thus, the disruption of cell structures renders the cell membrane more permeable, which causes the leakage of critical molecules and ions from the bacterial cell. As a result, the bacteria eventually die. Some compounds aim for the efflux mechanisms in Gram negative bacteria to regulate drug resistance.[22]

In this study, we determined the antibacterial activities of essential oils of *M. cajuputi* against Gram positive (*S. aureus, S. pyogenes,* and MRSA) and Gram negative (*K. pneumoniae* and *E. coli*) bacteria using the disc diffusion and MIC assays. The results revealed that the essential oils of *M. cajuputi* intensively inhibited Gram positive bacteria compared to Gram negative bacteria, wherein the inhibition zones against Gram positive bacteria were greater than those in Gram negative bacteria. This is due to the more comprehensible cell walls of Gram positive bacteria. These findings are of great significance, especially in cases of *S. aureus* and clinical isolates of MRSA that are prominent for being resistant to some antibiotics. In addition, these organisms have the ability to produce several types of enterotoxins that can cause serious infections, leading to sepsis or death.[23] The efficacy of essential oils depends on the structure of the target bacteria. According to a study by Swamy et al. essential oils easily penetrate the bacterial cell membranes and destabilize cellular architecture.[24] The disruption of the membrane integrity is caused by an increase in bacterial cell membrane permeability, resulting in the leakage of cellular components, loss of ions, and disruption of many cellular activities. Gram negative bacteria have an advanced tolerance toward hydrophobic antimicrobial substances because their outer membrane encloses hydrophilic lipopolysaccharides, which block macromolecules and hydrophobic substances like those found in essential oils. This differentiating character of the cell wall makes Gram positive bacteria more sensitive to distinctive substances than Gram negative bacteria. Therefore, Gram negative bacteria are ordinarily less sensitive than Gram positive bacteria. Interestingly, our results were similar to that reported by Al-Abd et al., wherein *M. cajuputi* flower and leaf extracts were found to have a wide range of antimicrobial potential against Gram positive bacteria.[25] However, they did not observe inhibition zones against the Gram negative bacteria tested. The differences in these findings are probably due to the distinctive solvent types that were used to extract phytocomponents from plant materials.[26] Bioactive phytocomponents present in plants are proven and confirmed to be simulated using extraction approaches and extraction solvent systems[27,28].

Conventional medication practices in old-world human cultures worldwide have shown that plants are beneficial sources of potent antimicrobial agents. For this reason, scientific studies have been conducted on the antimicrobial activities of plant extracts against various types of microorganisms, which have arisen in the evolution of alternative plant-based antimicrobial pharmaceutical medicines.[29,30,31,32] Extracts and some natural phytoconstituents found in the Myrtaceae family have been reported to have anticancer, antimicrobial, antioxidant, and anti-inflammatory properties.[33,34,35,36] Qualitative determination of phytochemicals showed the presence of flavonoids, saponins, and condensed tannins in all parts of this plant.[37,38] The monoterpene content of essential oils is mainly composed of melaleucol, β-caryophyllene, terpinolene, g-terpinene, and plathyllol.[39,40] Therefore, the antibacterial activity of the essential oils M. cajuput used in this study corresponded with their phytochemical contents.

CONCLUSION

Essential oils of *M. cajuputi* possess antibacterial properties against diverse clinical isolates and can be used as a medication for several bacterial diseases. Nonetheless, further studies are required to explore their efficiency in suppressing the growth of pathogenic microorganisms.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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ARTICLES FOR FACULTY MEMBERS

CULTURAL HERITAGE

Title/Author	Evaluation of in vitro bioactivity of Melaleuca cajuputi Powell essential oil against Aedes aegypti (L.) and Aedes albopictus (Skuse) / Bakar, A. A., Ahmad, H., Sulaiman, S., Omar, B., & Ali, R. M.
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Evaluation of *in vitro* Bioactivity of *Melaleuca cajuputi* Powell Essential Oil against *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse)

(Penilaian Bioaktiviti *in vitro* Minyak Pati *Melaleuca cajuputi* Powell terhadap *Aedes aegypti* (L.) dan *Aedes albopictus* (Skuse)

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ABSTRACT

The aim of this study was to evaluate the in vitro *activity of* Melaleuca cajuputi *essential oil leaf extract against* Aedes aegypti *and* Aedes albopictus. *The essential oil of the* M. cajuputi *was obtained via steam hydro-distillation and analyzed by GC-MS for the chemical constituents. GC-MS analysis showed that the essential oil of* M. cajuputi *contained 13 compounds with two major chemical constituents, 2-propenoic acid (29.55%) and caryophyllene (20.04%). Adulticidal and larvicidal bioassay was evaluated following WHO guidelines. Larvicidal bioassays were conducted with the 10, 50, 80, 100, and 120 mg/L* M. cajuputi *essential oil against late 3rd instar of* Ae. aegypti *and* Ae. albopictus *which give* LC_{50} *120.99 mg/L and 222.58 mg/L, respectively. Adult mortality was observed after 24 h with the* LC_{50} *of 0.029 mg/cm² for* Ae. aegypti *and 0.028 mg/cm² for* Ae. albopictus. *In adulticidal bioassay*, M. cajuputi *essential oils showed effective results at 0.04 mg/cm² concentrations, with values for* KT_{50} *of 77.71 min for* Ae. aegypti *and 69.49 min for* Ae. albopictus. M. cajuputi *essential oils exhibited moderate toxicity effects against the larva and adults of* Aedes *species and may be used as an alternative to chemical insecticide.*

Keywords: Adulticidal; Aedes*; essential oil; larvicidal;* Melaleuca cajuputi

ABSTRAK

Tujuan kajian ini adalah untuk menilai aktiviti ekstrak daun minyak pati Melaleuca cajuputi *secara* in vitro *terhadap larva* Aedes aegypti *dan* Aedes albopictus. *Minyak pati* M. cajuputi *diperoleh secara stim penyulingan-hidro dan dianalisis menggunakan GC-MS untuk menentukan komponen bahan kimia. Analisis GC-MS menunjukkan kandungan komponen kimia minyak pati* M. cajuputi *terdiri daripada 13 sebatian dengan dua unsur kimia utama iaitu asid 2-propenoik (29.55%) dan kariofilena (20.04%). Bioasai nyamuk dewasa dan larva dijalankan mengikut garis panduan WHO. Bioasai larva telah dijalankan dengan minyak pati* M. cajuputi *berkepekatan 10, 50, 80, 100, dan 120 mg/L terhadap instar 3* Ae. aegypti *dan* Ae. albopictus *yang memberikan nilai* LC_{50} *masing-masing, ialah 120.99 mg/L dan 222.58 mg/L. Kadar mortaliti nyamuk dewasa diperhatikan selepas 24 jam dan nilai* LC_{50} *yang diperoleh bagi* Ae. aegypti *dan* Ae. albopictus *masing-masing, ialah 0.029 mg/cm² dan 0.028 mg/cm². Melalui ujian bioasai adultisid yang dijalankan, minyak pati* M. cajuputi *menunjukkan hasil yang efektif pada kepekatan 0.04 mg/cm² dengan nilai* KT_{50} *ialah 77.71 min bagi* Ae. aegypti *dan 69.49 min bagi* Ae. albopictus. *Minyak pati* M. cajuputi *menunjukkan kesan ketoksikan sederhana terhadap larva dan dewasa spesies* Aedes *dan dapat digunakan sebagai insektisid alternatif terhadap insektisid bahan kimia.*

Kata kunci: Adultisidal; Aedes*; bioaktiviti; larvisidal;* Melaleuca cajuputi*; minyak pati*

INTRODUCTION

The *Ae. aegypti* mosquito has emerged as one of the most dangerous vectors, as it is able to transmit Yellow fever, dengue, chikungunya, and Zika. Dengue is a serious arboviral disease of the Africa, America, and Asia. The spread of dengue fever and Yellow fever in many parts of the world can be directly attributed to the proliferation and adaptation of these mosquitoes to breeding and living close to human populations and settlings. The secondary vector, *Ae. albopictus*, which was originally confined to Asia and known as Asian Tiger mosquito, has also contributed to the spread of the dengue fever, chikungunya, and Yellow fever as well. Yellow fever, which has a 400-year history, occurs only in tropical areas of Africa and the Americas. In Malaysia, dengue fever (DF) and dengue hemorrhagic fever (DHF) are the most common vector-borne diseases throughout the year across the nation, with occasional cases of chikungunya in some areas. However, both DF and DHF disease have a significant impact in shaping the socio-economic development and the formation of a government and local authorities' policy (Packierisamy 2015). Dengue fever in Malaysia was first reported by Skae (1902). By the early 1970s, DHF had spread all of Malaysia and has since caused a significant health burden to the population. Until now, the most effective way to control vector-borne diseases has been relying on chemical insecticides. Chemical insecticides are shown to be effective in controlling the spread of the diseases during the outbreak

by suppressing and eliminate the number of vector population (Lee et al. 2015). Organophosphates such as temephos, fenthion, and insect growth regulators such as diflubenzuron and methoprene are generally used for the control of mosquito larvae, whilst pyrethroids such as permethrin and deltamethrin, are used to control vector population of adult mosquitoes (Goindin et al. 2017). However, their repeated use has disrupted natural biological control systems and has led to outbreaks of insect species and the widespread development of resistance, leading to undesirable effects on non-target organisms and human health concerns (Yang et al. 2002). Therefore, there is an urgent need to develop an alternative pesticide which is environmentally safe, friendly and effective and thus has the potential to replace synthetic pesticides (Tapondjou et al. 2005).

In recent decades, research on the interactions between plants and insects has shown the potential use of plant metabolites for this purpose (Kamaraj et al. 2010). Indeed, in many cases plants have a history of use as home remedies to kill or repel insects (Kim et al. 2010). Hence, activities towards the exploring and screening of the potential of new plants and its chemical components in their insecticidal properties have increasingly been carried out among researchers worldwide (Abu Bakar et al. 2018). Chemicals derived from plants have been projected as weapons in future mosquito control programs, as they have been shown to function as general toxicants, growth and reproductive inhibitors, repellents and oviposition-deterrents (Sukumar et al. 1991). Currently, the screening of natural products has received the attention of researchers around the world (Kebede et al. 2010), and many specific compounds have been isolated from plant extracts or essential oils and tested for their specific roles as insecticides (Maciel et al. 2010; Pavela 2009). The aim of this present study was to evaluate the effectiveness of *M. cajuputi* essential oil against *Aedes* spp.

Materials and Methods

MOSQUITO REARING

The *Ae. aegypti* (L.) and *Ae. albopictus* used were from laboratory strain reared in the Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia. The mosquito colonies were maintained at 30-33ºC and 60%-80% relative humidity. All larvae stages were provided with powdered food (a mixture of grounded dried cow liver, cat biscuit, vitamin B complex, powder milk, yeast). Larvae transformed into pupae stage will be transferred into the bowl containing water and placed in screened cages until they emerged into adults. The adults were provided with 10% sucrose solution on saturated cotton pads. For the bioassay testing, the larvae used were late 3rd instar and/or early 4th instar and the adult was 3-5 days old.

PLANT MATERIALS

Plant leaves were collected from Mukim Telong, Kandis (5.9785463 North: 102.4265952 East) Bachok, Kelantan, Malaysia. The plant specimens were sent to Forest Research Institute of Malaysia (FRIM) in Kepong Selangor for species confirmation and kept at the Herbarium of Forest Biodiversity Division, with sample number PID 351016-23.

EXTRACTION OF THE ESSENTIAL OILS

The *M. cajuputi* essential oils were obtained from the extraction of leaves by performing a steam distillation process. The processing includes air-dried samples at room temperature for 5-7 days and later grounded to small particles. The grounded leaf particles were placed into the distillation flask (500 mL) and filled with distilled water (60-70°C). The flask left to boil slowly at 100ºC until distillation process completed. The distillation process completed when the essential oils had settled at the bottom layer of the water. The water layer was slowly drawn off and the remaining oil was transferred and kept in an airtight amber bottle in the refrigerator at 4°C.

PREPARATION OF THE SOLUTIONS

The solution concentrations for the larvicidal and adulticidal bioassay were prepared according to WHO (2005). For the larvicidal bioassay, essential oil was diluted to 10% (v/v), and five concentrations of 10, 50, 80, 100, 120 mg/L were prepared in 200 mL solutions using acetone as a solvent. 0.01% acetone and untreated tap water were used for positive and negative control, respectively. The solution for the adulticidal bioassay was prepared by dissolving essential oils in 70% ethanol with final concentrations of 0.026, 0.03, 0.034, and 0.04 mg/cm^2. Whatman No 1. filter papers (12 cm × 15 cm), were used for the paper-impregnated solutions. The paper was soaked in 2 mL of each concentration and allowed to dry at room temperature (27-30ºC). The prepared impregnated papers were then kept in aluminum foil and stored in a refrigerator at 4ºC. Control used a paper consisting of 70% ethanol.

BIOASSAY

Larvicidal Bioassay Twenty larvae were placed in a plastic cup with 200 mL of aqueous suspension of tested essential oils at various concentrations. Five replicates per concentration were run simultaneously with a set of positive and negative controls. The number of dead larvae in each cup was counted after one and 24 h following exposure. The larvae are considered dead if they were immobile and do not respond to any mechanical touches after 24 h. Larval mortality was recorded at 24 h post exposure. During observation, food was not supplied to the larvae.

Adulticidal Bioassay 25 adult females (3-5 days old) used in the bioassays were from batches of non-blood-fed

mosquitoes. They were introduced into holding tubes then transferred to the exposure tube with a piece of treated filter paper. After 1 h in the exposure tube, mosquitoes were returned to the holding tube and provided a 10% sucrose. Malathion was used as positive control. Knockdown (1 h) and mortality (24 h) were recorded. According to WHO guidelines, a mosquito was scored dead or knockdown/ moribund when the legs or wings were impaired and the mosquito was unable to fly.

ANALYSIS OF THE ESSENTIAL OILS

Qualitative analysis of the chemical constituents was carried out using GCMS analysis under the following conditions: Alltech 15897 AT-1MS capillary column (30 m×0.25 mm ID×0.25 μm, film thickness); held at 60°C for 1 min, raised to 150°C at a rate of 6°C/min, raised to 240°C at a rate of 10°C/min, and held for 6 min; 250°C injector temperature; carrier helium gas at a flow rate of 1.0 mL/min; 300:1 split ratio. Diluted oil (0.1 μL, 1:10, v/v, in dichloromethane) automatically injected into the system using a splitless mode. The oil components were identified by using GC-MS libraries (NIST107.LIB and WILEY229.LIB). The percentage of the identified component was computed *via* a total ion chromatogram (TIC).

STATISTICAL ANALYSIS

The data of mean larval mortality were subjected to probit analysis for calculating LC_{50} and LC_{90} as followed:

$$\% \text{ mortality} = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \text{control mortality}} \times 100$$

Other statistics data were calculated at 95% confidence limits of upper confidence limit and lower confidence limit by using SPSS. Results with the P value less than 0.05 were statistically significant. The lethal concentrations (LC_{50} and LC_{90}) were calculated by probit analysis (Finney 1971).

RESULTS AND DISCUSSION

In this present study, we focused on the larvicidal and adulticidal properties of *M. cajuputi* (Powell) plant essential oils against the dengue vectors *Ae. aegypti* and *Ae. albopictus*. Various studies have been carried out by other researchers on the insecticidal properties of plant essential oils. *M. cajuputi* is a member of Myrtaceae family and an indigenous plant of Australia (Southwell & Lowe 1999). In Malaysia, it is commonly found in swampy ground near the coasts (Corner 1997). The Aboriginal people of Australia use the leaves for numerous kinds of ill-treatment for centuries. In Asia, local people used its oil traditionally to relieve joint pain, stiff joints, and rheumatism, and as a mosquito repellent. In the Malay community it is known locally as 'gelam' or 'kayu putih', and the plant is highly adaptable in a wide range of conditions. GCMS analysis of the *M. cajuputi* essential oil compounds showed a total of 13 identified components representing 92.16% of total constituents (Table 1). The major constituents of the essential oil were 2-propenoic acid (29.55%) and caryophyllene (20.04%). All compounds detected under RT ranged from 11.206 min to 18.365 min. Studies by Sakasegawa et al. (2003) on various types of *Melaleuca* spp. demonstrated that *M. cajuputi* collected from the residential area at Palembang (Indonesia) had four similar compounds to those of our *M. cajuputi* specimen, i.e. caryophyllene, α-Humulene, δ-Cadinene, and (-)-Spathulenol. However, their major constituents of the essential oil were 1,8-cineole (31.78%), α-Terpineol (11.88%), and (-)-Globulol (10.38%), and total percentages of detected chemical compounds were 82.03%, with 25 identified chemical constituents. Similar studies by Kim et al. (2006) reported the differences in the chemical components of *M. cajuputi* essential oil leaf from six sampling locations. Minor components vary in terms of both structure and proportion but there were no differences in major components. Reasons for the differences and variety of the essential oil composition

TABLE 1. Chemical constituents of *M. cajuputi* essential oil

Peak	Chemical components	Retention time (RT)	Composition (%)*
1	Caryophyllene	11.206	20.04
2	α-Humulene	11.663	2.92
3	α-Gurjunene	11.919	2.29
4	β-Selenene	12.118	3.02
5	α-Selinene	12.228	3.60
6	δ-Cadinene	12.531	3.26
7	(-)-Spathulenol	13.326	8.95
8	2-Propenoic acid	13.702	29.55
9	1-Naphtalenol	13.900	1.80
10	T-Muurolol	14.076	5.33
11	α-Cadinol	14.262	7.54
12	Juniper camphor	14.455	1.37
13	Ethanone	18.365	2.49
	Total		92.16

*relative percentage of total essential oil compositions

could be due to geographical location, components of soil minerals/quality, the maturity of the plant, seasonal diversity, genetic diversity, and the specific parts of the plants used for essential oil isolation (Anwar et al. 2009). The results of the larvicidal activity of the *M. cajuputi* essential oil are presented in Table 2. At the lowest concentration of 10 mg/L, both *Aedes* spp. showed very minimal mortality of 3% and 1%, respectively. Within 24 h post-exposure, mortality gradually increased to 55% and 23% at the highest concentration of 120 mg/L. The *M. cajuputi* essential oil was more effective against *Ae. aegypti* larvae populations compared to *Ae. albopictus*. There was a significant difference in mortality between *Ae. aegypti* and *Ae. albopictus* ($p < 0.05$ p=0.04). A similar study by Jayakumar et al. (2016) reported that eucalyptus essential oil was the most effective among various plants tested against larvae and pupae of *Culex quinquefasciatus* with the LC_{50} values were 186.77 mg/L and 206.08 mg/L, respectively. In comparison, *M. cajuputi* essential oil in the present study gives lower LC_{50} of 120.99 mg/L against *Ae. aegypti* larvae. The potential of larvicidal properties in Myrtaceae plants essential oil was also recorded by Dias et al. (2015) against the *Ae. aegypti* larvae. The plants obtained from the Chapada das Mesas National Park, Brazil showed effectiveness with lethal concentrations (LC_{50}) ranging from 230 to 292 mg/L. As known that botanical derivatives products are very unique, their results vary and exhibit a broad spectrum of bioactivities against different mosquito species at different concentrations. This has highlighted the need for further testing on the composition of tested essential oils.

Many plants essential oils have shown effectiveness when tested against mosquito larvae. However, most studies have failed to demonstrate adulticidal effects. Therefore, from the potential results obtained in the larvicidal efficacy, we tested the effects of the essential oils on the adults' mosquito. The results of adulticidal activity of essential oil are presented in terms of mean knockdown and mortality of *Ae. aegypti* and *Ae. albopictus* (Table 3). At the highest concentration of 0.04 mg/cm^2, both *Aedes* spp. gives 100% mean mortality within 24 h post exposure. Early exposure at the lowest concentration 0.026 mg/cm^2, *Ae. albopictus* showed higher mean knockdown of 12.50 ± 0.58 compared to *Ae. aegypti* 6.50 ± 0.58. However, the mean knockdown of *Ae. aegypti* gradually increased during 1 h observation. The mean mortality was also gradually increased within 24 h post-exposure in both *Aedes* spp. tested populations. The statistical data of LC_{50} 95% confidence limits were also calculated. The results of the susceptibility test showed that 0.04 mg/cm^2 *M. cajuputi* essential oil exhibited the highest activity for *Ae. aegypti* (LC_{50}= 0.029 mg/cm^2) and *Ae. albopictus* (LC_{50}=0.028 mg/cm^2). It showed that mortality rates were at 100%. This suggests that essential oil does not possess insecticidal properties at lower doses or at less than 0.04 mg/cm^2. There was no significant difference in the mean mortality among two mosquito species ($p>0.05$).

The different findings among two tested population species between *Ae. aegypti* and *Ae. albopictus* have comprehended the multifactorial contributions to mortality and survival of some mosquito species in certain conditions. According to a previous study (Brady et al. 2013), on the adult survival rate in *Aedes* spp., the *Ae. albopictus* has a higher survival rate than *Ae. aegypti* in the laboratory and field settings with ranges of important factors such as temperature and other environmental factors. In contrast, *Ae. albopictus* population in our study was more susceptible than *Ae. aegypti*. These small findings provide insight into the susceptibility/survival update status of both tested population species in this present study.

The biological properties of the plants are known to be dependent on their composition and products (essential oils, fixed oils, latex, resins, or extracts) (Dias et al. 2015). Among the numerous plant-derived products, essential oils are considered to have the most variable

TABLE 2. Larvicidal bioassay of *M. cajuputi* essential oil against *Aedes* sp mosquito larvae

Mosquitoes	Dose (mg/L)	[1]Mean Mortality ± SD	Mortality (%)	LC_{50} (mg/L) (CI 95%)	LC_{90} (mg/L) (CI 95%)	χ^2	df
[2]*Ae. aegypti*	10	0.6 ± 0.55	3				
	50	3.0 ± 0.71	15				
	80	5.0 ± 1.00	25	120.99	287.76	2.266	2
	100	7.8 ± 0.84	39	(107.67-146.24)	(210.68-562.37)		
	120	11.0 ± 1.58	55				
	*Control	0	0				
[2]*Ae. albopictus*	10	0.2 ± 0.45	1				
	50	0.8 ± 0.84	4				
	80	2.8 ± 1.3	14	222.58	659.84	0.506	2
	100	3.6 ± 2.3	18	(162.05-607.11)	(332.84-6358.33)		
	120	4.6 ± 1.14	23				
	*Control	0	0				

*Control (=acetone 0.1%) [1 2]Significantly difference at $p<0.05$ (p=0.04)

TABLE 3. Mean of knockdown and mortality of *M. cajuputi* essential oils against *Aedes* sp adult mosquitoes

Concentration (mg/cm^2)	*Ae. aegypti* (N=100)		*Ae. albopictus* (N=100)	
	Mean knockdown ± SD	[a]Mean mortality ± SD	Mean knockdown ± SD	[b]Mean mortality ± SD
0.026	6.50 ± 0.58	7.50 ± 0.58	12.50 ± 0.58	8.50 ± 1.29
0.03	9.75 ± 0.56	12.50 ± 2.89	13.75 ± 0.96	16.25 ± 0.50
0.034	18.00 ± 0.82	20.75 ± 0.82	15.25 ± 2.63	21.25 ± 0.50
0.04	20.00 ± 1.83	25.00 ± 0.00	22.75 ± 1.71	25.00 ± 0.00
Malathion 5%	24.75 ± 0.50	25.00 ± 0.00	25.00 ± 0.00	25.00 ± 0.00
Ethanol 70%	0	0	0	0
LC_{50} (CI 95%)	0.029 (0.023-0.033)		0.028 (0.027-0.029)	

CI = confidence intervals

[a, b] Not significantly difference at *p>0.05* (p=0.156)

composition because of multifactorial factors such as soil, climate, altitude, age and part of the plant used (Barbosa et al. 2012; Feitosa et al. 2009). Many current types of research have recognized plant extracts and plant essential oil as important resources for botanical insecticides. Different part of plants contains a mixture of phytochemicals and secondary metabolites with distinctive biological activities (Ghosh et al. 2012). The diversity in the mosquitocidal activity of plant extracts is probably due to the differences in their active compounds, genetic characteristics of plant species and its harvested conditions (Sujatha et al. 1988).

The values of Knockdown Time (KT) in adulticidal bioassay are presented in Table 4 and a graph line (Figure 1). The values of KT_{50} were obtained from a probit analysis calculation using SPSS (v.24). Generally, the KT values of both *Aedes* spp. were lower with the increase of dose concentration. At the lowest concentration of 0.026 mg/cm^2, KT_{50} value for *Ae. aegypti* was 87.53 min (73.67-148.96 min) and highest concentration of 0.04 mg/cm^2 gives the KT_{50} of 77.71 min (64.05-168.20 min). On the other hand, KT_{50} values of *Ae. albopictus* did not show the same pattern as *Ae. aegypti*. The highest value of KT_{50} was 92.59 min (78.88-124.43 min) at a concentration of 0.034 mg/cm^2 instead of 0.026 mg/cm^2. However, the lowest KT_{50} recorded was 69.61 min (61.15-101.75 min) at the highest concentration of 0.04 mg/cm^2. A positive control using 5% malathion have the lowest KT_{50} for *Ae. aegypti* and *Ae. albopictus* of 36.09 min (33.71-38.45 min) and 35.78 min (34.33-37.24 min), respectively. The results of regression analysis showed that the KT_{50} values of *M. cajuputi* were negatively correlated with concentration (mg/cm^2), and both *Ae. aegypti and Ae. albopictus* have a regression coefficient (R^2) of more than 0.5, 0.6686 and 0.5336, respectively (Figure 1).

In this present study, results of *M. cajuputi* essential oil showed significant larvicidal activity. Previous studies on the effectiveness of *M. cajuputi* essential oils in aerosol form (Abu Bakar et al. 2012; Azlinda et al. 2009) have shown significant effects against adult mosquito dengue vectors. In general, essential oil act as contact insecticides, with a neurotoxic mode-of-action targeting gamma-aminobutyric acid (GABA) and octopamine synapses and acetylcholinesterase (Regnault-Roger et al. 2012). The mode of action of essential oils of mosquito larvae is not known, but the earlier studies stated that the plant chemicals initially affect the midgut epithelium, gastric caeca and Malpighian tubules in mosquito larvae

TABLE 4. KT_{50} values of *M. cajuputi* essential oils against *Aedes* sp adult mosquitoes

Concentration (mg/cm^2)	*Ae. aegypti*		*Ae. albopictus*	
	KT_{50} (CI 95%)	Regression ± S.E	KT_{50} (CI 95%)	Regression ± S.E
0.026	87.53 (73.67-148.96)	0.05 ± 0.02	82.30 (71.26-118.55)	0.04 ± 0.01
0.03	82.07 (71.36-117.0)	0.05 ± 0.01	76.139 (68.93-91.46)	0.06 ± 0.01
0.034	79.56 (70.10-97.09)	0.04 ± 0.01	92.59 (78.88-124.43)	0.03 ± 0.01
0.04	77.71 (64.05-168.20)	0.04 ± 0.01	69.49 (61.15-101.75)	0.05 ± 0.01
Malathion 5%	36.09 (33.71-38.45)	4.99 ± 0.34	35.78 (34.33-37.24)	5.13 ± 0.30

CI=confidence intervals

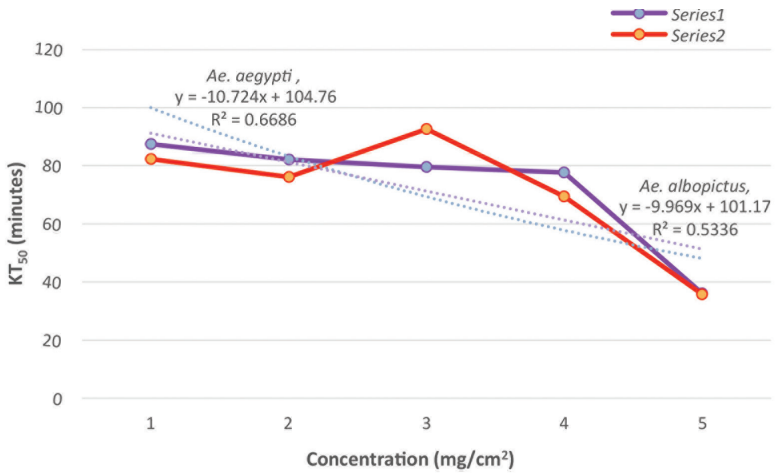

FIGURE 1. Relationship of KT_{50} of adulticidal activity of *M. cajuputi* essential oils against *Ae. aegypti* and *Ae. albopictus* mosquito adults

(David et al. 2000; Rey et al. 1999). According to a recent report by Govindaraju et al. (2016), other possible targets of essential oils are transient receptor type ion channels, acetylcholinesterase, and receptors tyramine, octopamine and GABA. Instead of synthetic insecticides, plant compounds could lead to the development of potent natural mosquitocidal products. Due to the volatile nature of plant essential oils, their insecticidal products are easily degraded, which leads to a lower level of risk to the environment than synthetic insecticides. Masetti (2016) has suggested in his report that essential oil based products should be effective under a wide range of field conditions (organic matter in the water, salinity, temperature, pH) and different levels of susceptibility among mosquito species should also be considered. The variations in mortality and lethal concentration could be due to insecticidal ingredients of plants, time of collection and season (Sosan et al. 2001).

CONCLUSION

The findings of this study have demonstrated a moderate effect on larval and adult mortality induced by *M. cajuputi* essential oil against *Ae. aegypti* and *Ae. albopictus*. In brief, the essential oil from *M. cajuputi* leaves may be explored as a potential bio-insecticide and could be used as an alternative to chemical and synthetic insecticides. However, further studies are needed to assess the effects of individual compounds in both laboratory and semi-field conditions on larval and adult mortality and the mechanisms involved.

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Green facile synthesis of cajuput (*Melaleuca cajuputi* Powell.) essential oil loaded chitosan film and evaluation of its effectiveness on shelf-life extension of white button mushroom

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ABSTRACT

This study reports first time investigation on efficacy of cajuput essential oil loaded chitosan nanoparticle (CjEO-CSNP) on shelf-life of white button mushroom (*Agaricus bisporus*) stored at 4±1 °C for 7-days. CjEO-CSNP was characterized through scanning electron microscopy, X-ray diffraction, and dynamic light scattering. The nanoparticles exhibited spherical shapes with average particle size 43.17–97.03 nm. The nanoencapsulation efficiency and loading capacity were ranged between 45.86 and 92.26% and 0.69–8.87%, respectively. The release study confirmed that CjEO-CSNP showed biphasic release patterns at different pH. Positive results were unveiled when the effect of CjEO-CSNP on shelf-life of mushroom was validated by analyzing the visual appearance and firmness. Further, CjEO-CSNP prevented weight loss and respiration rate, and improved the antioxidant activity of mushrooms. CjEO-CSNP also exhibited high safety profile (LD_{50}= > 1200 mg/Kg body weight) without altering the sensory quality of coated mushrooms. Overall, CjEO-CSNP might be used as promising candidate to lengthen the shelf-life of button mushroom.

1. Introduction

Agaricus bisporus, popularly known as 'white button mushroom' has been accepted as one of the most cultivated and extensively consumed edible mushrooms, accounting for 30% of total mushroom production in the world due to its delicious taste, good quality protein, high dietary fibre, vitamins, minerals, and polyphenols (Muszyńska, Kała, Sułkowska-Ziaja, Krakowska, & Opoka, 2016). The increasing consumption of mushroom has converted the harvest and cultivation of this mushroom into an economically important trade globally. However, during post-harvest storage, mushroom experiences a series of quality deterioration such as discolouration, water loss, texture changes, microbial attacks, change in enzymatic activity, and nutrient as well as flavour loss (Fattahifar, Barzegar, Gavlighi, & Sahari, 2018). In order to maintain the postharvest quality and to extend their shelf-life, there has been drawing attention toward the use of nanoemulsion based polymeric coating incorporating essential oils (EOs) (Guo, Yadav, & Jin, 2017). Depending upon the selection of incorporating material and characteristics, the packaging can be categorized into three classes: intelligent, chemoactive, and bioactive packaging (Mukurumbira, Shellie, Keast, Palombo, & Jadhav, 2022). Intelligent packaging refers to a packaging that can monitor the condition of packaged food or the environmental changes surrounding the food. In chemoactive packaging, chemical compound is used as an active agent, whereas in bioactive packaging, antimicrobial compounds like EO and their bioactive compound, mostly derived from the plants are used as active agent in the packaging material (Sharma, Barkauskaite, Jaiswal, & Jaiswal, 2021). The incorporation of EO with strong antimicrobial and antioxidant properties in coating can extend mushroom's shelf-life by forming a thin layer on their surface, and protects them from mechanical injury, water loss, and oxidation by limiting gaseous exchange (Pires, de Souza, & Fernando, 2018). Moreover, the capacity to release the EO in a controlled way is another advantage, where only effective dose of EO is released on the food surface, resulting in no significant changes in the organoleptic properties (Donsì & Ferrari, 2016; Haghighi et al., 2019; Chaudhari, Singh, Das, & Dubey, 2021).

Among different coating carriers, chitosan (CS, obtained by deacetylation of chitin) have an innumerable potential for being use in

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bioactive coating owing to its intrinsic properties viz., non-toxic, biocompatible, biodegradable, cheap, renewable, edible, and film forming ability (Pei et al., 2022). Additionally, CS based bioactive packaging are rather tough and transparent with good biological properties (antimicrobial and antioxidant), and therefore, EO is incorporated in CS with the aim to check the microbial and non-microbial mediated spoilage of perishable food products (Khodayari et al., 2019). Some previous studies have also confirmed the application of CS coating containing natural antimicrobials and antioxidant compounds on lengthening the shelf-life of different perishable foods.

Melaleuca cajuputi Powell. (Family: Myrtaceae) is a native *Melaleuca* species found in Australia and adjoining areas like New Guinea, Indonesia, and Malaysia. It is a multi-purpose tree, but famous for its EO (CjEO), that has been used for the treatment of different diseases. Interestingly, the food preservative potential of *M. cajuputi* EO against aflatoxigenic fungal isolates and lipid oxidation of stored maize samples was confirmed in our previous study (Chaudhari, Singh, Das, Kujur, & Dubey, 2022). However, as far as we are concerned, no comprehensive study have been carried out to investigate the effectiveness of bioactive coating made from CS with incorporated CjEO on preservation of mushrooms during storage.

The present study was, therefore, attempts to prepare the bioactive CS coating enriched with CjEO (CjEO-CSNP), and to evaluate their effect on the shelf-life of white button mushrooms based on quality attributes, weight loss, respiration rate, and antioxidant activities. Finally, the safety profile assessment on mice model and sensory analysis were conducted in order to assess its safety, consumers' acceptance, and promising application in food packaging.

2. Materials and methods

2.1. Materials

CS with low molecular weight and deacetylation degree >75% was purchased from Hi-media Laboratory (Mumbai, India). The other chemicals viz., glacial acetic acid (99.7% purity), Tween-80 (extra pure), tripolyphosphate (TPP, 85% purity), 2,2-diphenyl-1-picrylhydrazyl (DPPH, 98.5% purity), 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, 98.5% purity), Folin-Ciocalteu's reagent (extra pure), and sodium carbonate (99.5% purity) were supplied by SRL, Co., Ltd. (Mumbai, India). All other chemicals were of analytical grade and used as received.

2.2. Isolation and characterization of essential oil (CjEO)

For the isolation of CjEO, 500 g mature leaves of *M. cajuputi* (harvested in February 2021) was subjected to hydro-distillation using a Clevenger-type apparatus equipped with a 5-L round bottom flask and boiled for 3 h at 75 °C. The obtained oil was dried over anhydrous sodium sulfate, and stored in a dark amber glass vial at 4 °C.

The chemical profile of CjEO was analyzed by GC–MS analysis (model TRACE-1300 series, Agilent Technologies) equipped with a flame-ionization detector (FID), and TG-5 capillary column (30 m × 0.25 mm × 0.25 μm) following the procedure reported previously (Chaudhari et al., 2022). The operating conditions were programmed as follows: carrier gas: helium (1 mL/min); injector temperature: 250 °C; detector temperature 220 °C; column temperature: 60 °C for 2 min, then increased to 250 °C at heating rate of 5 °C/min; and mass spectra ionization energy: 70 eV. The sample injection volume was 0.2 μL from the solution (1:100 of the oil in hexane). The identification of components were carried out by comparing their retention indices (RI) and mass spectra relative to *n*-alkanes (C_9–C_{33}), and/or via matching with those available in the literature (Adams, 2007).

2.3. Preparation of CjEO-loaded chitosan nanoparticle (CjEO-CSNP)

The nanoparticles was formulated by ionic-gelation technique according to the method described previously (Yoksan, Jirawutthiwongchai, & Arpo, 2010). Firstly, CS solution was produced by dispersing 1.8 g of CS (1.5%, *w/v*) into deionized water containing glacial acetic acid (1%, *v/v*) with continuous stirring at 25 °C for overnight. Then, Tween 80 (1.239 mL, *v/v*) was added as an emulsifier (due to non-ionic nature) to the CS mixture. Meanwhile, different amount of CjEO mixed with dichloromethane (2 mL) were added to the CS solution, and homogenized by using a high speed homogenizer (IKA-1, Germany) at 10,000 × g for 10 min, so as to achieve the desired ratios (1:0.0, 1:0.2, 1:0.4, 1:0.6, 1:0.8, and 1:1.0, *w/v*) of oil-in-water emulsion. The emulsion without EO was used as control. Thereafter, 0.4% TPP (*w/v*) was added into the solution under continues stirring (45 min) to induce ionic-gelation. The formed nanoparticles were collected through centrifugation followed by washing with deionized water. At the end, sonication was performed to get fine particles, and the resulting suspensions were instantly freeze-dried using lyophilizer (Alpha 1–2 LD plus, Sydney, Australia) at −65 °C for 72 h. Before drying, the suspensions were kept in deep freezer for 24 h. During freeze-drying, the flasks containing nanoparticles were sealed with the valves of lyophilizer caps and frozen at −80 °C for 30 min. Thereafter, main drying phase was performed for 72 h at −62 °C and a pressure of 0.01 m bar. The resulting dried powders were used for solid state studies and characterization.

2.4. Determination of percent nanoencapsulation efficiency (NE) and loading efficiency (LE)

The NE and LE of CjEO-CSNP were determined by UV–visible spectrophotometer. Briefly, the fresh nanoemulsion dispersion sample (consisting of 0.5 mL CjEO-CSNP and 2.5 mL acetonitrile) were centrifuged at 10,000 × *g* for 10 min to obtain a supernatant with free EO. The absorbance of the sample was measured at 224 nm and the amount of CjEO was calculated by using the calibration curve ($R^2 = 0.996$) of CjEO obtained in acetonitrile. The NE and LE were then calculated by the following formula:

$$\%\,\mathrm{NE} = \left(\frac{\text{Total amount of loaded CjEO}}{\text{Initial amount of CjEO}}\right) \times 100$$

$$\%\,\mathrm{LE} = \left(\frac{\text{Total amount of loaded CjEO}}{\text{Mass of freeze} - \text{dried nanoparticles}}\right) \times 100$$

2.5. Physico-chemical characterization of nanoparticles

2.5.1. Morphological and crystallographic properties of nanoparticles

The morphological characterization of the CSNP and CjEO-CSNP was done by scanning electron microscopy (Evo-18 researcher, Zeiss, Germany). Prior to analysis, 5 mg of lyophilized nanoparticles were separately diluted with 10 mL deionized water, and an aliquot of suspension was placed on a clean cover-slip and air dried at room temperature. The samples were fixed on copper stubs, sputter coated with gold (Q150R-ES, Lewes, UK), and examined at an accelerating voltage of 20 kV.

The crystallographic property of CS before and after loading of test EO was obtained by X-ray diffraction (XRD) analysis (Bruker D8 Advance). The XRD patterns of the samples (CS, CSNP, and CjEO-CSNP) were acquired by a scanning rate of 0.02°/ min over the diffraction angle (2θ) ranged between 5 and 50°.

2.5.2. Particle size, ζ potential, and polydispersity index (PDI) of nanoparticles

The mean particle size, ζ potential, and PDI of the nanoparticles were determined by dynamic light scattering (DLS) using a Zetasizer (Nano-ZS, Malvern, UK) at room temperature. Before analysis, the lyophilized nanoparticle samples were diluted with deionized water in a ratio of

1:100 (*w/v*) to avoid scattering effects. Each reading was performed in triplicates, considering stabilization duration of 2 min.

2.6. Release kinetics of CjEO-CSNP

The release behaviour of CjEO from CSNP was investigated in acetate (pH 3) and phosphate buffer saline (pH 7) following Kujur, Kumar, Singh, and Prakash (2021) with some modifications. In brief, 20 mg lyophilized CjEO-CSNP was separately placed in a centifuge tube and incubated at room temperature under different pH solutions. At definite time intervals (0, 24, 48, 72, 96, 120, 144, and 168 h), the incubated sample was centrifuged and 1 mL of supernatant was withdrawn. At the same time, an equal amount of fresh buffer was then replaced in the mixture, and the same procedure was repeated for the subsequent sampling. The resulting supernatant was analyzed at a wavelength of 224 nm and cumulative release percentage (CR%) was calculated by the following formula:

$$CR\% = \sum_{t=0}^{t} \frac{Rt}{Ro} \times 100$$

where,

Rt = cumulative amount of released CjEO at each sampling time.

Ro = initial amount of the encapsulated CjEO in the sample.

2.7. Effect of CjEO-CSNP on shelf-life of mushroom

Fresh mushrooms (*A. bisporus*) were obtained from a local farm of Ghazipur, Uttar Pradesh (India) at button stage and transferred to the laboratory, followed by storage at 4 °C. After 24 h, the mushrooms were sorted based on color, size, and tissue intactness. Thereafter, mushrooms were divided into two groups and each group contained 12 mushrooms: one group was coated with CSNP film (control), while the second group was coated with CjEO-CSNP film (considered as treatment). The coating procedure was carried out at room temperature under aseptic conditions, and packed in commercial propylene boxes. Finally, the samples were incubated at low temperature for a week, and quality was evaluated at 3 and 7 days.

2.8. Determination of weight loss and respiration rate

The weight loss of the mushrooms before and after coating was determined by weighing the polystyrene boxes (containing 12 mushrooms each) at the beginning of the experiment (day-0) and during the incubation (day-3 and day-7) using an analytical balance. The weight loss was calculated using the following equation:

$$Weight\ loss\ (\%) = \left(\frac{Wi - Wf}{Wi}\right) \times 100$$

where,

wi = initial weight of mushroom.

wf = final weight of mushroom.

The respiration rate of the samples was determined using a headspace analyzer. On each storage time, four mushrooms with approximately uniform size from the control and treatment groups were placed in closed plastic containers and kept at 4 °C for 1 h. Then, the concentration of CO_2 was measured employing a needle connected to a gas analyzer. Respiration rates (RR) were calculated from the following equation:

$$RR\left(mgCO_2Kg^{-1}s^{-1}\right) = \left(\frac{YCO_2 \times V}{100 \times W \times T}\right)$$

where,

YCO_2 = concentration fraction increment (%).

V = free volume (mL).

W = weight of mushroom (Kg).

T = testing time (s).

2.9. Determination of antioxidant activity and total phenolic content (TPc)

The antioxidant activities of the control and treated mushrooms were executed using two complementary tests, namely DPPH and ABTS as reported by Jiang, Luo, and Ying (2015) with minor modifications. Prior to evaluation, the methanolic extracts of the mushroom samples were prepared following the methodology reported by Raju, Jenny, Merin Saju, and Rajkumar (2021) with slight modifications. Briefly, 10 g well milled mushroom were extracted in 100 mL of methanol using a soxhlet apparatus for 4 h. The extract was cooled at room temperature (25 ±2 °C) and then filtered using Whatman no. 40 filter paper. The obtained extract was concentrated to 40 mL using a rotary evaporator and then stored in dark bottles under refrigeration to avoid any possible degradation and used for further analysis.

For DPPH, 0.5 mL of the methanolic extract of control and treated mushrooms were mixed with 2.5 mL of the methanolic solution containing 0.04% DPPH. The resulting mixtures were incubated at room temperature under dark condition for 30 min. Then, absorbance of the sample was recorded at 517 nm against a blank (methanolic DPPH). The radical scavenging capacity (RSC) was calculated from the following formula:

$$RSC(\%) = \left(\frac{Ablank - Asample}{Ablank}\right) \times 100$$

where,

Ablank = absorbance of methanolic-DPPH solution.

Asample = absorbance of the sample containing mushroom extract.

During ABTS assay, initially a 7 mM stock solution was prepared by dissolving ABTS in absolute alcohol. Next, 2.45 mM potassium persulfate was added to this stock solution and the mixture was allowed to stand under dark condition for overnight. The $ABTS^{\bullet+}$ solution was diluted with ethanol until its absorbance reach to 0.70±0.02 at 734 nm. Then, 2 mL of diluted $ABTS^{\bullet+}$ solution was added to 0.5 mL of methanolic extract of control and treated mushroom. The absorbance was read after 6 min of reaction at 734 nm. The RSC was calculated as above. The TPc was determined using Folin-Ciocalteu's reagent following the protocol described by Louis et al. (2021). The Folin-Ciocalteu's reagent was prepared by dissolving 10 g sodium tungstate and 2.5 g sodium molybdate in a conical flask containing 70 mL deionized water. Thereafter, 5 mL 85% phosphoric acid and 10 mL concentrated HCl were added to the mixture. The solution was stirred over a magnetic stirrer (100 rpm) for 10 h under dark condition followed by the addition of 15 g lithium sulfate, 5 mL deionized water, and 1 drop of bromine. The sample was then cooled at room temperature and brings to 100 mL with water. For determination of TPc, 5 g well grinded mushroom tissue from both control and treated samples were homogenized with 20 mL of 70% ethanol and centrifuged at 5000 × g for 10 min. Then, 1 mL of the obtained supernatant was mixed with 1 mL of Folin-Ciocalteu's reagent and 10 mL of 7% sodium carbonate, and brought to 25 mL with double deionized water. The absorbance was read after 1 h of incubation at 760 nm. The TPc was expressed as mg gallic acid/g fresh weight (FW).

2.10. Determination of consumers' acceptability

The consumers' acceptance of coated mushrooms was evaluated based on odor, cap color, surface uniformity, and overall acceptability by a panel of ten trained assessors following Nasiri, Barzegar, Sahari, and Niakousari (2018) with modifications. The assessors were asked to sort the samples according to their preference using a 5-point hedonic-scale (5 = excellent, 4 = good, 3 = acceptable, 2 = poor, and 1 = extremely poor). The sample was blind-coded and sensory evaluation

was done at initial (day-0) to last day (day-7) of the storage.

2.11. Oral acute toxicity assessment of CjEO-CSNP

The oral acute toxicity study of CjEO-CSNP was performed on a population of Swiss albino male mice (*Mus musculus* L.; average weight 32 g). The animals were obtained from the Central Animal House, Banaras Hindu University (Varanasi, India) and randomly divided into ten groups containing ten mice each. All the mice were fasted overnight from food prior to dosing with the CjEO-CSNP. Then, different doses (200–2000 mg/Kg body weight) of CjEO-CSNP were given orally to the mice. The treated mice were monitored for any signs of toxicity closely for 1 h, intermittently for 4 h, and 24 hourly for the next 14 days. By the end of this period, dead mice were counted to determine the median lethal dose (LD_{50}, the dose that kills 50% of test mice) using the Probit analysis. At the end of the experiments, all mice were sacrificed by intraperitonial injection of pentobarbital sodium at a dose of 80 mg/Kg body weight. The animal care and all the experimental protocols were performed strictly according to the guidelines defined by the Institutional Animal Ethics Committee (IAEC) of the Banaras Hindu University.

2.12. Statistical analysis

All the tests were carried out in triplicate, and results were reported as the mean ± standard error (SE). One-way ANOVA with the SPSS 16.0 statistical analysis program was used to analyze the data of statistical significance. Significance was defined at $p < 0.05$ by post-hoc Tukey's B multiple comparison.

3. Results and discussion

3.1. Isolation and characterization of essential oil

The yield of the hydro-distilled EO obtained from *M. cajuputi* leaves was 4.5 mL/Kg fresh weight. The EO was a light yellow liquid with characteristic strong aroma. GC–MS analysis was performed to quantify the main volatile components of EO, which was responsible for their biological activities. The results obtained in our previous investigations revealed the presence of α-pinene (49.24%), bornyl acetate (21.07%), and camphor (11.70%) as the predominant components of EO (Chaudhari et al., 2022). The percent composition of other constituents such as 1,8-cineole, isocamphane, p-cymene, limonene, fenchone, α-terpineol, and p-menthenol were ranged from 0.83 to 0.20%. These results were contradicted with the findings of Septiana et al. (2020), who reported 1,8-cineole, α-terpineol, caryophyllene, α-pinene, and γ-terpinene as the major components of cajuputi EO obtained from Indonesia. The main components and their percentage were also differed from those reported in other investigations on the chemical composition of same EO (Bua et al., 2020). They reported 1,8-cineole as the major component followed by (-)-α-pinene, (-)-1S-β-pinene, α-terpineol, c-terpinene, and (+)-limonene. This variability in the chemical composition of EO could be attributed to several factors including geographical location, season, genetic factors, soil composition, collection time, plant parts used, age, and methods of extraction (Dung, Kim, & Kang, 2008; Zhao et al., 2021).

3.2. Preparation of CjEO-CSNP

In this study, ionic-gelation technique was employed for the fabrication of CjEO-CSNP using CS as a wall material and TPP as a cross-linker. The technique is primarily based on the ionic interaction between the positively-charged amino-sugar monomeric units of CS and negatively-charged polyanions of TPP (Di Santo, D'Antoni, Rubio, Alaimo, & Pérez, 2021). The encapsulation of CjEO into CS was achieved by dissolving low molecular weight CS into glacial acid solution, resulting in oil-in-water emulsion followed by droplet formation and solidification via inter and intramolecular cross linkage between $-NH_3^+$ groups of CS and $P_3O_{10}^{5-}$ groups of TPP under continuous stirring at ambient temperature (Pan et al., 2019; Chaudhari et al., 2020).

It is worth noting that the concentration of coating material and cross-linking agent are the two main factors that direct the success of nanoparticle synthesis. Fan, Yan, Xu, and Ni (2012) envisage that the formation of stable nanoparticle is possible only at precise concentration of polymer and cross-linking agent. This fact has been also demonstrated in a study by Hosseini, Zandi, Rezaei, and Farahmandghavi (2013), who reported that the concentration of CS and TPP needs to be kept below 1.5% and 1%, respectively to achieve stable nanoparticles, beyond this, micro/ macro-particles are formed. For this reason, herein, 1.5% CS (*w/v*) and 0.4% TPP (*w/v*) was used. Besides this, ionic-gelation technique is particularly sensitive to pH condition and appropriate pH of the solution is huge requirement for the successful loading of EO into CS. This is due to the fact that CS is insoluble in water, and requires acidic pH, therefore glacial acetic acid was used, which quaternizes CS into soluble polycationic forms that effectively forms cross-linkages with TPP, resulting in formation of stable nanoparticles. Hasheminejad, Khodaiyan, and Safari (2019) using ionic-gelation technique also encapsulated clove EO into CS nanoparticles and achieved good encapsulation efficiency and loading capacity as well as controlled release profile for loaded EO. Jardim, Siqueira, Báo, and Parize (2022) also synthesized CS nanoparticles incorporating quercetin with good colloidal stability, high encapsulation efficiency, and sustained release profile using the same technique.

This is the first attempt involving preparation of CjEO-CSNP using ionic-gelation technique. The encapsulation of EO by ionic-gelation is advantageous over other encapsulation techniques viz., coacervation, spray-drying, emulsification, electro-spinning, and electro-spraying because this is one of the easiest ways to develop CS nanoparticles reported so far in the literature. Its success is mainly due to its one shot synthesis, cost-effectiveness, non-toxicity, controllable, and most importantly that does not requires any toxic chemicals and high temperatures, enabling the loading of EO that usually undergo oxidation under extreme environmental conditions (Bugnicourt & Ladavière, 2016; Chaudhari et al., 2021).

3.3. Determination of percent NE and LE

The results of NE and LE of CSNPs containing different ratios of CjEO are given in Fig. 1A & B. The results indicated that both NE and LE were significantly ($p < 0.05$) increased with increasing the initial CjEO content, reaching maximum at the ratio of 1:1.0 (*w/v*) of CS to EO. The NE and LE values ranged from 45.86 to 92.26% and 0.69–8.87%, respectively. These values were on the same range and even slightly greater than those obtained by our previous study with the same method of encapsulation for allspice EO in CS (Chaudhari, Singh, Das, & Dubey, 2022). Similar results were also described for the encapsulated *Cymbopogon citratus* and *Chelidonium majus* EOs, respectively into chitosan nanomatrices, where increasing trend of encapsulation efficiency and loading capacity were recorded with respect to the ratio of chitosan to EOs (Kumar, Singh, & Prakash, 2022; Hesami et al., 2022). The results verify that a very small amount of EO was lost during of nano-encapsulation. Considering the maximum NE and LE, the weight ratio of CS to CjEO of 1:1.0 (*w/v*) was selected to be the optimum formulae for the characterization and investigations of prepared nanoparticles.

3.4. Physico-chemical characterization of nanoparticles

3.4.1. Morphological and crystallographic properties of nanoparticles

The SEM photographs of the characterized nanoparticles are shown in Fig. 2A & B. The images clearly demonstrated that both CSNP and CjEO-CSNP were spherical in shape without coalescence, confirming good structural integrity. The diameter of the CSNP (without CjEO) was ranged between 13.57 and 37.87 nm; which was increased (43.17–97.03 nm) with the addition of CjEO. These differences of size might be

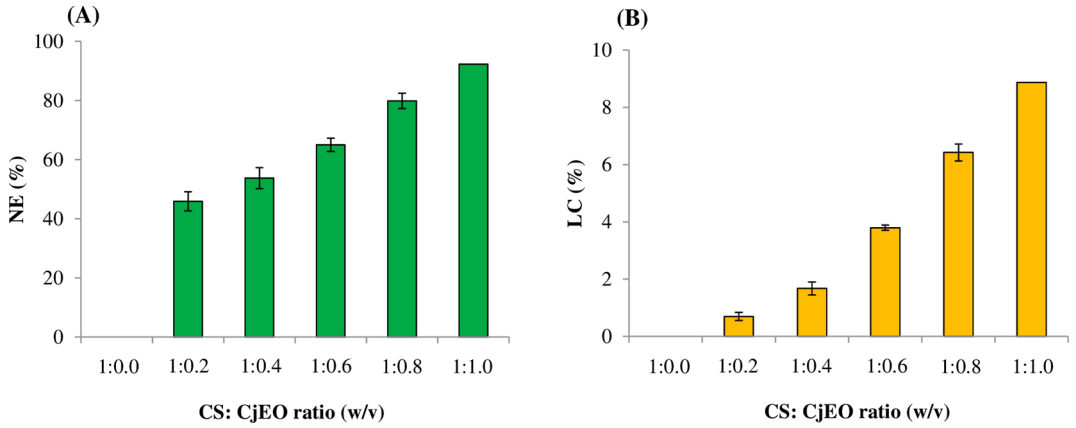

Fig. 1. (A) Percent nanoencapsulation efficiency (NE), and (B) loading efficiency (LE) of CjEO-CSNP prepared at different ratios (1:0.2–1:1.0, *w/v*) of CS:CjEO. Values are presented as mean ± standard error.

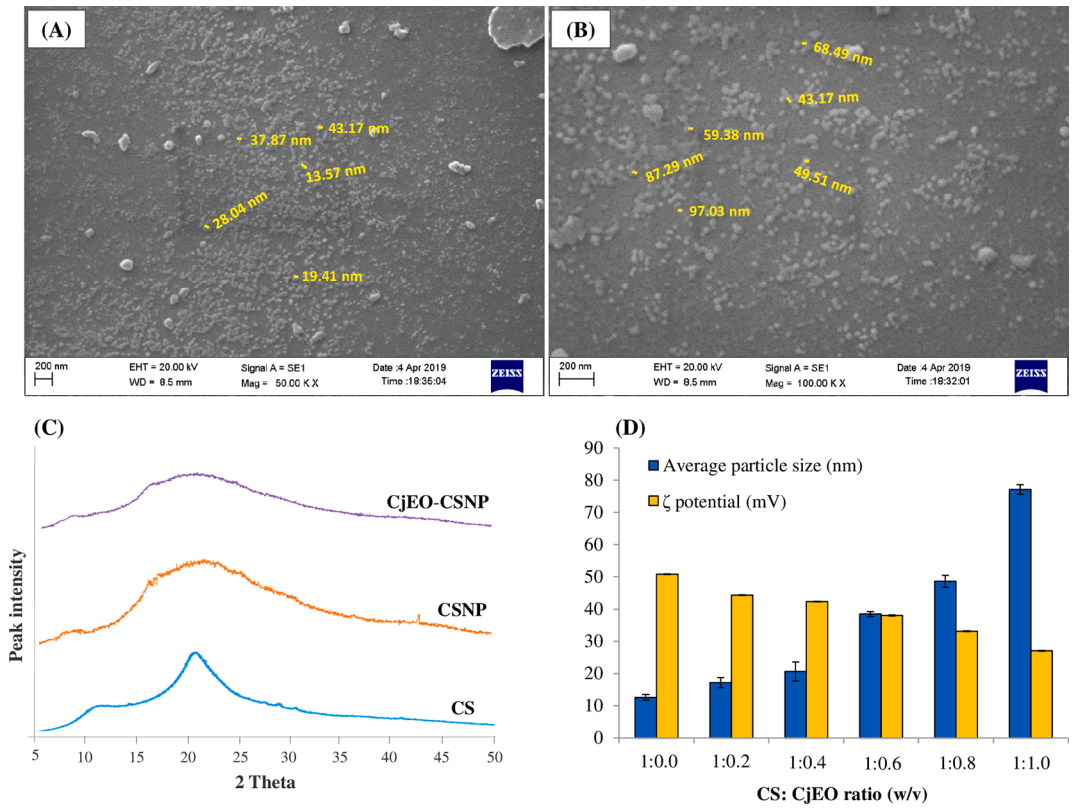

Fig. 2. (A) SEM image of CSNP, (B) SEM image of CjEO-CSNP, (C) XRD spectra of CS, CSNP, and CjEO-CSNP, and (D) particle size distribution and ζ potential of CSNP and CjEO-CSNP prepared at different ratios (1:0.0–1:1.0, *w/v*) of CS: CjEO. Values are presented as mean ± standard error.

attributed to the successful loading of CjEO into the CS core. The same results showing particle size below 100 nm for the CS nanoparticles loaded with clove EO and cellulose nanofiber loaded with *Salvadora persica* extract have been achieved by Hadidi, Pouramin, Adinepour, Haghani, and Jafari (2020), and Ahmadi et al. (2019).

The crystallographic profiles of CS powder, CSNP, and CjEO-CSNP determined by XRD analysis are presented in Fig. 2C. As can be seen from XRD graph, pure CS shows two strong peaks: one at 10° and another at 20.14°, assuring its high degree of crystallinity, which is similar to those reported by others (Shetta, Kegere, & Mamdouh, 2019). However, the intensity of the diffraction spectra seem to have changed significantly after the addition of TPP and CjEO in CSNP and CjEO-CSNP, respectively, interpreting destruction of the native crystalline structure. This disparity might be attributed to a modification in the arrangement of molecules in the crystal lattice induced by ionic cross-linkage between CS and other ingredients such as TPP and CjEO, which is ideal for the successful encapsulation of test EO into CS and their application in food system. These results confirmed that the CjEO was encapsulated in the CS matrix.

3.4.2. Particle size, ζ potential, and polydispersity index (PDI) of nanoparticles

Stability is considered as one of the most significant attributes of the nanoparticles, particularly when applying as food packaging material. The packaging material should tolerate physical hassle without failing to protect the packaged food products from spoilage and to maintain its shelf-life during long term storage. The stability of the developed nanoparticles was evaluated by investigating parameters viz., particle

size distribution, ζ potential, and PDI through DLS. As it can be inferred, the average particle size of the CSNP was 12.57±0.92, which was significantly ($p < 0.05$) increased (17.16±1.60–77.13±1.48 nm) with increasing the ratio of CS:CjEO (1:0.2–1:1.0, respectively) (Fig. 2D). Although, a significant increase in mean particle size was noted with increasing the CS to EO ratios, still showed the size below 100 nm. This result is consistent with those recorded by Das, Singh, Chaudhari, Dwivedy, and Dubey (2021) during encapsulating linalool into CS. The authors reported that average particle size of linalool loaded CS nanoparticle as 80.1 nm, however, our results verified slightly smaller particle size (77.13±1.48 nm). The lower mean particle size indicates the optimal stability.

In general, ζ potential is used to employ information on the magnitude of the surface charge to assess the stability of the particles in the suspension. The nanoparticles with ζ potential value greater than +30 mV or more negative than −30 mV is regarded as stable (López-Meneses et al., 2018). The ζ potential of CSNP was +50.83±0.09 mV, but this value was decreased significantly ($p < 0.05$) from +44.33±0.10 to +27.06±0.10 mV with the addition of CjEO (Fig. 2D). This reduction was positively related with the mass ratio of CS:CjEO. The observed decline in the ζ potential value could be ascribed to the adsorption of CjEO on the particle surfaces, which brings about masking the free $-NH_3^+$ groups of CS polymer (Arya, Vandana, Acharya, & Sahoo, 2011). There was also a report showed that the ζ potential decreased from +49.9 to +38.7 mV with the addition of different concentrations of *Cinnamomum zeylanicum* EO into chitosan solution (Mohammadi, Hosseini, & Hashemi, 2020). They also reported that the reduction of ζ potential reflected the loading of EO decreased the scattering of nanoparticles in the aqueous solution. The nanoparticles showed positive ζ potential, probably because of the occurrence of high proportion of protonated amine groups on the surface of CjEO-CSNP films. A similar finding was shown by Cai et al. (2022), who reported a positive ζ potential for chitosan nanoparticles loaded with *Ocimum basilicum* EO. The authors also predicted that the positive ζ potential of nanoparticles might be associated with the protonation of amine groups under acidic conditions. The values obtained for both the CSNP and CjEO-CSNP were similar with the reference for ζ potential, which indicates that nanoencapsulation technique used was suitable for the preparation of stable nanoparticles without aggregations.

PDI is generally employed to collect the information on particle size distribution and uniformity in the suspension. The PDI values between 0.1 and 0.25 indicate a narrow size distribution, while>0.5 refers to a broad size distribution (Hasani, Rad, Hosseini, & Noghabi, 2015). Our results revealed that PDI of both the samples (CSNP and CjEO-CSNP) was in the range of 0.25 to 0.11 (Fig. 3A), which is an indicative of a narrow and uniform size distribution. The results were consistent with the study of Niu et al. (2022), who has obtained a similar range of PDI (0.23–0.29) during co-encapsulating chlorogenic acid and cinnamaldehyde EO in Pickering emulsion stabilized by CS nanoparticles.

3.5. Release kinetics of CjEO-CSNP

The release profiles of CjEO from CSNP investigated in two different buffer systems is presented in Fig. 3B. The results showed a two-step pattern with an initial quick release (up to 82.92% and 73.42%, respectively, $p < 0.05$) in the first 24 h of assay followed by a controlled release (reaching a plateau). The fast discharge at this stage may be related to the diffusion of poorly entrapped or near-to-surface adhered CjEO molecules, because high dissolution velocity of the CS nears the exterior (Hosseini et al., 2013). On contrary, the release at subsequent stage might be attributed to the concentration gradient of CjEO between CSNP and media, as well as the inability of the buffers to break the compact structure of polymer (Hasheminejad et al., 2019). Nevertheless, the release rate was greater in acetate buffer than the phosphate buffer. This may be explained by the fact that acetate buffer can cause better swelling and dissolution of CS wall than phosphate buffer, possibly due to the ionic-repulsion between the protonated amine groups of CS (Soltanzadeh, Peighambardoust, Ghanbarzadeh, Mohammadi, & Lorenzo, 2021). The greater release in acetate buffer was also thought to be attributed to the deprotonation of the amine groups of CS and alteration of electrostatic interaction between CS and TPP (Konecsni, Low, & Nickerson, 2012). These results are in line with those obtained by others for β-cyclodextrin/CS-nanoparticles entrapping cinnamon EO (Matshetshe, Parani, Manki, & Oluwafemi, 2018). Further, the sustained release of essential oils compounds to the treated food samples from CSNP does not require direct contact, which is advantageous, since this might allow the reduction of undesirable sensory attributes that may occur in the mushrooms.

3.6. Effect of CjEO-CSNP on shelf-life of mushroom

The visual appearance of mushrooms is one of the most significant parameters for evaluating their freshness and quality during storage. During post-harvest condition, the color of mushrooms steadily turns to brown, possibly due to enzyme oxidation, and microbial proliferation, ensuing in the loss of overall nutritional quality and the shelf-life (Liu, Liu, Zhang, Kan, & Jin, 2019). The effect of bioactive coating on the visual appearance and firmness during 7-days of storage are shown in Fig. 4. For all storage times, the maximum freshness was recorded in the control samples. After 3 days of incubation, an apparent browning of the mushrooms coated with CSNP were noted, which were increased significantly ($p < 0.05$) at 7th day, while intact morphology and negligible browning (as observed from naked eyes) were noted in mushrooms coated with CjEO-CSNP, indicating that CS coating loaded with CjEO

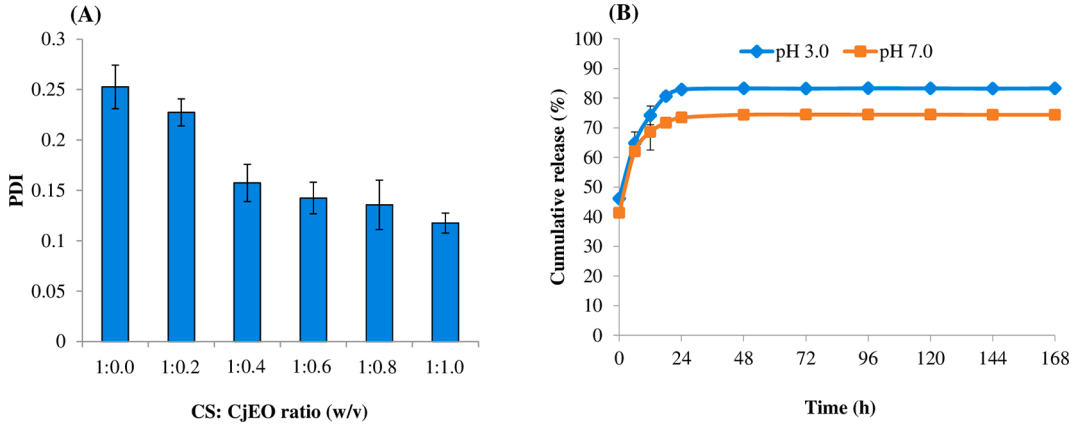

Fig. 3. (A) Polydispersity index (PDI) of CSNP and CjEO-CSNP prepared at different ratios (1:0.0–1:1.0, *w/v*) of CS:CjEO, and (B) *In vitro* release profiles of CjEO-CSNP in different buffer systems. Values are presented as mean ± standard error.

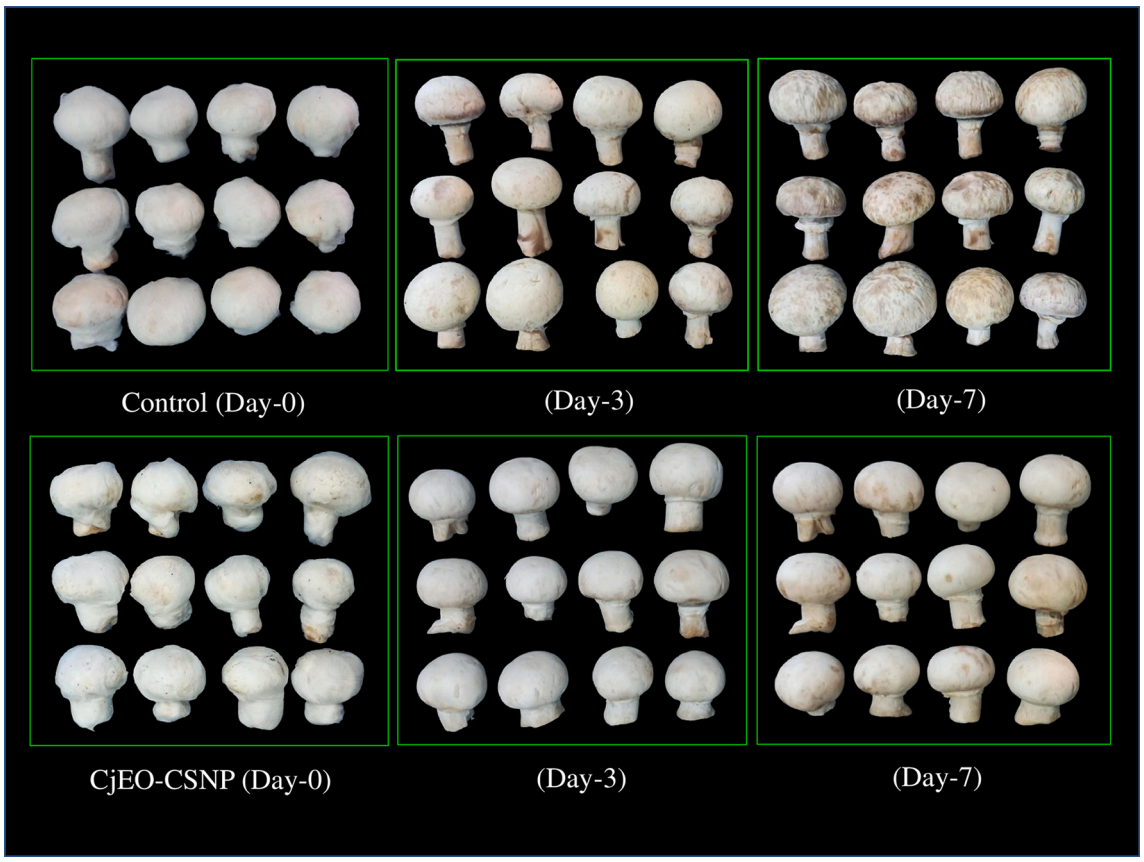

Fig. 4. Effect of CjEO-CSNP coating on the visual appearance and firmness (shelf-life) of mushrooms during storage.

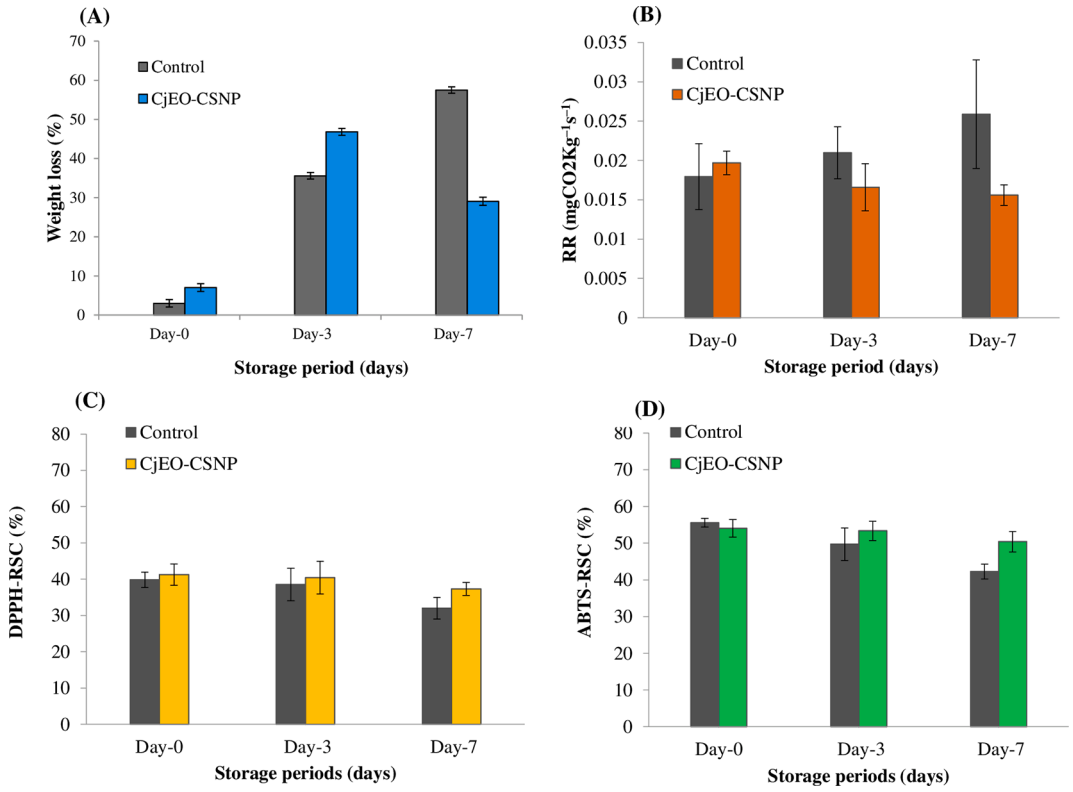

Fig. 5. Effects of CjEO-CSNP coating on (A) weight loss, (B) respiration rate, (C) DPPH, and (D) ABTS radical scavenging capacity of mushrooms during storage. Values are presented as mean ± standard error.

could retain the freshness of mushrooms. This can be ascribed to the greater water vapour barrier property and poor rate of respiration as compared with CSNP. Similar results have been confirmed by Valizadeh, Behnamian, Dezhsetan, and Karimirad (2021) and Wang et al. (2021), during investigating the effect of turmeric and lemon EOs blended in CS and CS/zein films, respectively on the quality of white mushrooms. The results showed that CjEO-CSNP may be a potential agent in retaining the freshness and shelf-life of the coated mushrooms over a longer period of storage.

3.7. Determination of weight loss and respiration rate

The changes in weight loss between CSNP and CjEO-CSNP coated mushrooms during incubation periods are shown in Fig. 5A. Results clearly showed that the weight loss was significantly ($p < 0.05$) increased during the storage time in all mushroom samples. This may be likely due to the phenomenon of exudation during storage leading to continuous moisture loss from mushrooms to surrounding environment. However, in comparison with controls (35.58% and 57.50%), the mushrooms coated with CjEO-CSNP showed lower weight loss (46.81% and 29.06%) after 3 and 7^{th} day of storage, respectively, indicated that prepared nanoparticles retained a good water holding performance. This might be primarily associated with the film forming property of CS that can form a thin barrier on the mushrooms surface, reducing the loss of water to the external environment during storage. This result is in accordance with the study of Nasiri, Barzegar, Sahari, and Niakousari (2017) for mushrooms coated with tragacanth gum loading *Zataria multiflora* EO. The effect of polymeric coating on reduction of weight loss was also confirmed by Ksouda et al. (2019) during investigating the preservative ability of sodium alginate coating enriched *Pimpinella saxifraga* EO on cheese samples.

Besides weight loss, the RR is another very important index related to the quality and metabolism of mushrooms. The results presented in Fig. 5B, clearly evident that the respiratory rate of the control group (CSNP) was significantly ($p < 0.05$) greater than that of CjEO-CSNP treated groups, which might possibly be due to the gaseous barrier effect of the coating. This result can be explained by the fact that EO loaded film were more effective in blocking the passage of gas (CO_2) from inside the package to the outside (Qin et al., 2015). These results agree with the earlier studies that the coating with EO loaded CS films possibly will reduces the respiration rate of strawberry and mushrooms (Wang et al., 2021). The above results indicated that the coating have the ability to reduce the RR of mushrooms, possibly due to the creation of an internal modified atmosphere and decreasing the exchange of gases between the environment and coated mushrooms.

3.8. Determination of antioxidant activity and TPc

Oxidative degradation is one of the main causes of mushrooms quality deterioration during storage. Besides using antioxidant compounds like EO in food itself, the other novel strategy is to use antioxidant compounds in packaging material (Priyadarshi, Kumar, Deeba, Kulshreshtha, & Negi, 2018). For this reason, the RSC of CjEO-CSNP (used as coating material of mushrooms) was confirmed by DPPH and ABTS assays. The RSC of mushrooms coated with CjEO-CSNP during different storage periods are evaluated presented in Fig. 5C & D. As can be seen from the graph, the methanolic extract of mushroom coated with CSNP showed a RSC of 39.85, 38.51, and 31.99%, while that of extract coated with CjEO-CSNP showed a significantly ($p < 0.05$) greater activity of 41.25, 40.39, and 37.31% during 0, 3, and 7-days of storage, respectively through DPPH assay (Fig. 5C). Similarly, during ABTS assay, mushroom coated with CSNP showed a RSC of 55.57, 49.73, and 42.26%, while extract coated with CjEO-CSNP showed significantly ($p < 0.05$) higher activity (54.06, 53.35, and 50.39%) during respective days of storage (Fig. 5D). CjEO-CSNP coated mushrooms showed slightly greater RSC, which might be attributed to the proton absorbing capacity of residual free $-NH_2$ groups of CS, and loaded EO itself, resulting in formation of stable non-radical molecule (Yen, Yang, & Mau, 2008; Siripatrawan & Harte, 2010). Similar results have been described by Jiang, Feng, and Li (2012) and Nasiri et al. (2017), whereby they reported chitosan-glucose complex coating and tragacanth gum incorporating *Zataria multiflora* EO coating had a positive effect on the antioxidant activity of shiitake and white button mushrooms, respectively.

It is well known fact that the antioxidant activity is usually related to the TPc (Ahmed & Tavaszi-Sarosi, 2019). Results also showed a linear correlation between the RSC and TPc. During 0-day of assay, CSNP and CjEO-CSNP treated mushrooms exhibited significantly ($p < 0.05$) higher TPc than subsequent days of assay, which is consistent with the expectations, since CjEO in the CSNP coating possesses remarkable antioxidant ability (Fig. 6A). However, CjEO-CSNP coated mushrooms showed slightly lower loss of TPc, probably as a consequence of more phenolic substances accumulation. The accumulated phenolic compounds showed higher RSC, which could be due to the delay of browning by maintaining the integrity of cell membrane (Zhang, Liu, Sun, Wang, & Li, 2020). These results coincide with those reported by Louis et al. (2021), who noted that mushrooms coated with alginate-based cinnamaldehyde EO nanoemulsion exhibited a greater retention of total polyphenols. Overall results showed that CjEO-CSNP is an outstanding free radical scavenger and act as strong antioxidant, which could be used to protect mushrooms from free radicals mediated oxidation, resulting enhancement of their shelf-life.

3.9. Determination of consumers' acceptability

The sensory analysis is a very important parameter when recommending any new bioactive coatings to appraise the acceptability of the coated product by the consumers. The average scores marked by the assessors for the undertaken attributes during storage periods are shown in Fig. 6B & C. The scores for off-odour in CSNP coated mushrooms significantly ($p < 0.05$) declined after 3 and 7-days of storage. However, CjEO-CSNP coated mushrooms showed more acceptable results throughout the storage period. Similarly, the scores for cap color and uniformity of CSNP coated mushrooms showed declining trends for both the storage periods. Contrary to this, CjEO-CSNP coated mushrooms showed better trend to the color and uniformity of the cap surface. These results showed that mushrooms coated with CjEO-CSNP could maintain the sensory attributes (negligible off-odor, cap color, and uniformity) more efficient than CS coating alone. The better scores of CjEO-CSNP coated mushrooms could be linked to the inhibition of microbial growth (possibly as a result of the controlled release of EO), preservation of their antioxidant properties, water vapour barrier property, low respiration rate, and retention of the antioxidant enzyme levels (such as superoxide dismutase and catalase) in coated fruiting bodies (Huang, Qian, Jiang, & Zheng, 2019). This result agreed with those from previous studies showing significant improvement of sensory attributes after coating of strawberries and fresh cut apples with chitosan incorporating lemon, and alginate containing lemongrass EOs, respectively (Azarakhsh, Osman, Ghazali, Tan, & Adzahan, 2014). Our results showed that the CjEO-CSNP used in this study is capable of preserving the sensory properties of the coated mushrooms and increasing its consumer willingness.

3.10. Oral acute toxicity assessment of CjEO-CSNP

In this study, the oral acute toxicity of CjEO-CSNP was performed on mice model to assess its safety and further application as food preservative. No significant signs of toxicity in the body of mice were evident after the oral administration of the CjEO-CSNP up to 1200 mg/Kg body weight. This might be endowed due to non-toxic nature of CS with reported LD_{50} value of 16 g/Kg body weight in laboratory mice that is equivalent to those of sugar and salt. Further, because of its high safety

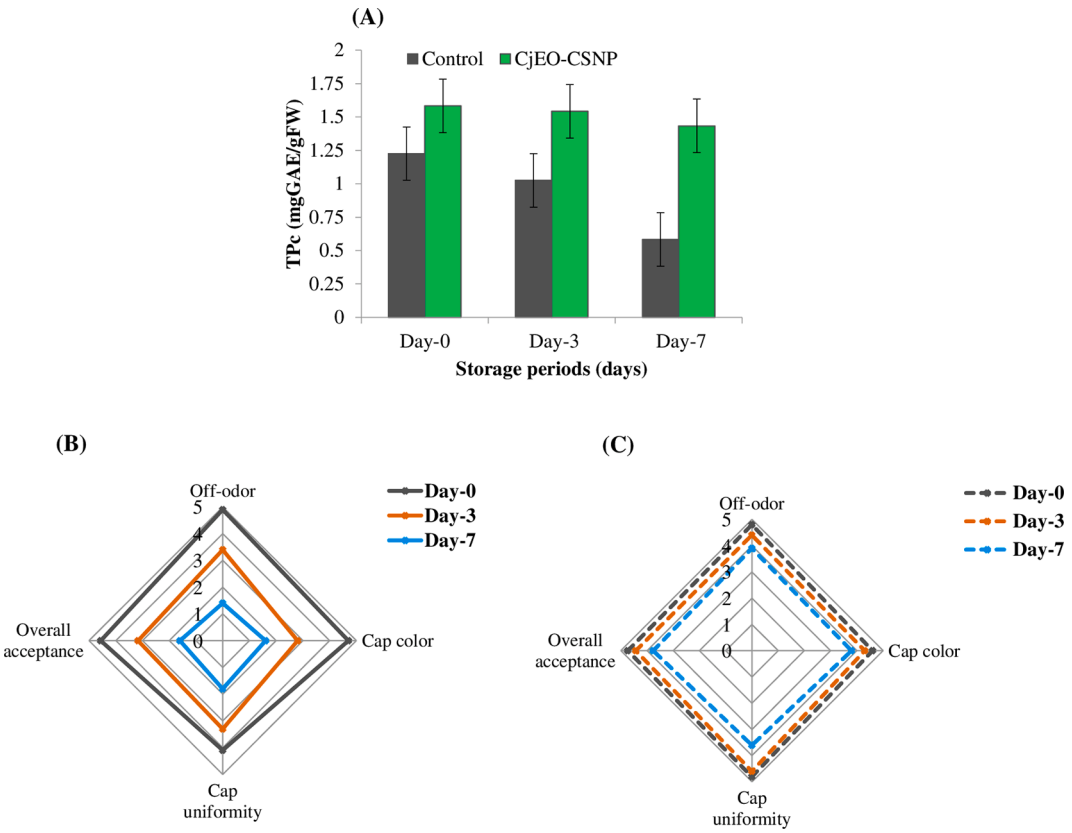

Fig. 6. Effects of CjEO-CSNP coating on (A) total phenolic contents, and (B & C) sensory characteristics of mushrooms during storage. Values are presented as mean ± standard error.

profile, CS has already been approved as food additive in different countries including Japan, Korea, Italy and Finland (Chaudhari et al., 2021). However, beyond this dose (at 1400 mg/Kg body weight), the test mice showed toxicity symptoms consisting of fast breathing, tremors, ataxia, arching, and rolling, validating its lower LD_{50} value compared with un-encapsulated EO (LD_{50} = 1600 mg/Kg body weight) previously determined (Chaudhari et al., 2022). Death was lately recorded for some mice but the survived ones were completely recovered and gradually resumed normal activity. The higher doses of CjEO-CSNP such as 1600, 1800 and 2000 mg/Kg body weight had lead to the loss of consciousness followed by death (65, 70, and 80% dead animals, respectively). This is probably due to the extremely small size of the nanoparticles, which may exhibit cytotoxic effect via modifying absorption, distribution, and metabolism of the body, resulting in adverse effects on the mice health's upon consumption (de Souza Simões et al., 2017). Research also confirmed that small sized particles are more reactive and cytotoxic than larger ones owing to its increased surface area, that likely to emphasize their intrinsic toxic properties. On the other hand, no sign of toxicity were observed at the lower doses, suggesting a significant safety profile of CjEO-CSNP.

4. Conclusion

The results of this study demonstrate that cajuputi EO was successfully encapsulated into CS nanomatrix with good nanoencapsulation efficiency and loading efficiency. Further, CjEO-CSNP was able to preserve the quality of coated mushrooms via preventing weight loss, respiration rate, and preserving firmness, color, and antioxidant metabolites. Moreover, high safety profile with satisfactory consumers acceptability was observed for CjEO-CSNP coated mushrooms. Based on overall findings, it can be inferred that CjEO-CSNP could be a novel agent for the bioactive packaging of edible mushrooms.

CRediT authorship contribution statement

Anand Kumar Chaudhari: Conceptualization, Investigation, Methodology, Software, Writing – original draft. **Somenath Das:** Data curation, Formal analysis, Methodology. **Bijendra Kumar Singh:** Formal analysis. **Nawal Kishore Dubey:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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SHORT COMMUNICATION

PHYSICOCHEMICAL PROPERTIES, CHEMICAL COMPONENTS, AND ANTIBACTERIAL ACTIVITY OF *MELALEUCA CAJUPUTI* POWELL ESSENTIAL OIL LEAVES FROM QUANG TRI PROVINCE, VIETNAM

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ABSTRACT. Essential oil (EO) was extracted from *Melaleuca cajuputi* Powell leaves using the steam distillation method. The main aim of this study is to determine several physicochemical properties of EO, including acid value, saponification value, ester value, relative density, absolute density, and freezing point. In addition, the chemical components were also analyzed using the gas chromatography–mass spectrometry (GC–MS) method. The obtained 1,8-cineol content is quite high (71.83%). In addition, these components in EO had an antioxidant capacity and antibacterial activity, including gram-positive and gram-negative bacteria (using the paper disc diffusion method for antibiotic susceptibility testing). Particularly, this EO inhibited the growth of *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076), and *Staphylococcus aureus* (ATCC 25923).

KEY WORDS: Antimicrobial activity, Antioxidant capacity, GC-MS, Essential oil, *Melaleuca cajuputi* Powell.

INTRODUCTION

Melaleuca cajuputi Powell (common name: cajuput tree or white tea tree) is a vigorous plant adapted to nutrient-poor soils, especially acid soils, cracking black clay, black peaty sand, and clay loam [1, 2]. This plant has a high economic value in Vietnam; it can be used as building materials, made into paper powder, or the EO extracted from its leaves can be applied in pharmaceutical technology [1]. This is a medium to tall tree (2-46 m), with papery bark and grey, brownish, pink-tan, or whitish tree. Leaves are long- or short-petiolate; blade glabrescent, narrowly elliptic, 40–140 mm long, and 7.5–60 mm wide. Flowering is recorded from March to November. This plant is found in Western and Northern Australia, Indonesia, East Timor, Vietnam, etc. [2].

Currently, the cajuput tree is distributed everywhere in Vietnam, especially Hue, Quang Binh, and Quang Tri province, etc. because the demands for EO in the drug, cosmetic, and flavoring field have increased significantly. The main component of this EO is 1,8-cineol (eucalyptol), and it has many uses such as treating bronchitis, sinusitis, chronic rhinitis, and asthma [3]. In addition, Atta *and* Alkofahi [4] also reported that *M. cajuputi* EO has antioxidant, anti-inflammatory, and antimicrobial activities.

Nowadays, many scientists have studied EO from *M. cajuputi* leaves. The results indicate that the chemical composition and yield of extraction of EO depend on different extraction methods and sources of the plant [5, 6]. The chemical composition significantly affects the physicochemical properties, antioxidant capacity, and antibacterial activity of EO [7, 8]. However, until now, there have been no studies on the chemical components, antibacterial activity, and physical properties of EO of *M. cajuputi* leaves from Quang Tri province (Vietnam). Therefore, the major purpose of this study is to clarify these issues mentioned above.

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EXPERIMENTAL

Material and EO extraction. Fresh leaves of *M. cajuputi* were collected and harvested from Quang Tri province (Vietnam). Materials were distilled using the steam distillation method at 100 ºC. Then, the obtained EO was finally collected after decantation and stored at 4 ºC in dark until further analyses.

Bacteria strains. Four bacteria strains were tested in this study, including one gram-positive bacteria (*Staphylococcus aureus*, ATCC 25923) and three gram-negative bacteria (*Salmonella enteritidis*, ATCC 13076; *Escherichia coli*, ATCC 25922; *Pseudomonas aeruginosa*, ATCC 27853).

Determination of the relative density (RD) and absolute density (AD) of EO. According to ISO 279:1998 [9], the RD was determined by the proportion of the mass of a given volume of the EO to the mass of an equal volume of distilled water at 20 ºC, while the AD was determined by the proportion of the mass of a given volume of the EO to the same volume.

Determination of the freezing temperature of EO. 5 mL of the obtained EO was added to the test tube. Next, it was put into a freezing container. The temperature in the freezing container was decreased slowly until the EO appeared to crystallize. The freezing temperature was recorded at this moment [10].

Determination of acid value (AV) of EO. The AV was performed according to the procedure of Quoc [8]. The obtained EO (1 g) was dissolved in 5 mL of 96% ethanol, and a few drops of 1% phenolphthalein was added to the mixture. The KOH solution (0.1 M) was used to titrate this mixture until it turned pink.

$$AV = \frac{V_{KOH} \times 0.1 \times 56.1}{Mass\ of\ essential\ oil}$$

Determination of saponification value (SV) of EO. For the determination of SV, 2 g of EO and 25 mL of the ethanol solution of KOH (0.5 M) were mixed in a glass flask (250 mL). This mixture was heated for 60 min in the condenser system. Next, 25 mL of distilled water and a few drops of 1% phenolphthalein were added to the mixture. The HCl solution (0.5 M) was used to titrate this mixture until it turned colorless [8].

$$SV = \frac{(V_{blank} - V_{sample}) \times 56.1 \times 0.5}{Mass\ of\ essential\ oil}$$

Determination of antioxidant capacity (AC) of EO. The procedure to determine the AC of the EO to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was described according to Kirby and Schmidt [11] with some minor changes. The EO from *M. cajuputi* leaves was dissolved in ethanol (96%, v/v) to obtain different concentrations (512, 256, 128, 64, 32, and 16 mg/mL). 0.3 mL of the obtained solution and 2.7 mL DPPH in ethanol solution (concentration of 40 µg/mL) were mixed together. The mixture was kept in the dark for 30 min at room temperature. The AC was recorded by observing the decrease in absorbance at 517 nm against a control sample (containing only DPPH in ethanol solution without the tested sample). The AC of EO was compared to that of ascorbic acid as a standard. Percent inhibition was plotted against EO concentrations to estimate the concentration providing 50% inhibition (IC_{50}). The AC was calculated using the following expression:

$$\%inhibition = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Determination of antibacterial activity (AA) of EO. In this study, the paper disc diffusion method for antibiotic susceptibility testing was used to test the AA of EO according to the method of Bauer *et al.* [12] with some small corrections. Firstly, 100 µL of bacteria suspension (0.5 McFarland standard, approximately 1.5×10^8 CFU/mL) were spread on MHA media (Mueller-Hinton agar) by a sterile hockey stick. Next, the sterile paper discs (6 mm diameter) were impregnated by the obtained EO (5 µL), while gentamicin (10 µg/disc) and dimethylsulfoxide (DMSO) solution (5%, v/v) were used as positive and negative controls, respectively. Then, all dishes were incubated for 24 h at 37 ºC, and the inhibition zones were expressed in mm including the disc diameter of 6 mm.

Gas chromatography-mass spectrometry (GC–MS) analysis. The chemical composition of EO was analyzed using the GC–MS method. 1 µL of EO was injected into a gas chromatograph (Agilent HP 6890N, USA) with a capillary column (HP-5ms, 30 m×0.25 mm×0.45 µm, Agilent Technologies, USA) equipped with a quadrupole mass analyzer (Agilent HP 5972, USA). Helium was used as a carrier gas at a constant flow rate of 0.5 mL/min, and a split ratio of 10:1. The injection temperature was 250 ºC and the temperature program was set as follows: initial temperature of 50 ºC, held for 2 min, increased until 300 ºC at a rate of 10 ºC/min, and held for 5 min. Mass spectra were recorded at the ionization energy of 70 eV in EI mode.

Statistical data analysis. All experiments were conducted in triplicate and the results were expressed as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) method with Fisher's least significant difference (LSD) procedure was carried out to test significant differences between the means ($p < 0.05$). The data were analyzed using Statgraphics Centurion XV software (version 15.1.02, Statgraphics Technologies, Inc., USA).

RESULTS AND DISCUSSION

Determination of the physicochemical properties of M. cajuputi leaves EO. *M. cajuputi* leaves EO is a pale-yellow liquid with a characteristic odor. Some physicochemical properties of *M. cajuputi* leaves EO are presented in Table 1. The pH value of EO reached approximately 4.46, which was significantly different than values reported from previous studies; for instance, the pH values of EOs of *Ceratonia siliqua* pulp and seeds were 4.3±0.5 and 5.2±0.3, respectively [7]. This can be explained by the fact that the pH value depends significantly on the chemical composition of EOs. It is observed that the obtained EO is a little denser than that of other materials, showing RD of 0.9102±0.0002, whereas that of *Skimmia laureola* leaves EO and *Zanthoxylum armatum* leaves EO were 0.792±0.001 and 0.816±0.001, respectively [13]. In addition, the AD for EO of *M. cajuputi* leaves (0.9086±0.0002 g/mL) was not significantly higher than the EO of *C. siliqua* seeds (0.910±0.04 g/mL) [7] and *Myrtus communis* leaves (0.894±0.001 g/mL) [14]. As for the freezing point of EO, it can be seen in Table 1 that this value is quite low (approximate -45 ºC). Considering that previous scientific reports on the freezing point of this EO are nonexistent, it is difficult to make some further comments regarding our results. However, the chemical composition of EO is the most important factor that extremely affects these EO properties.

Table 1. Some physicochemical properties of *M. cajuputi* leaves EO.

No	Physicochemical properties	Value
1	pH	4.46±0.01
2	Relative density	0.9102±0.0002
3	Absolute density (g/mL)	0.9086±0.0002
4	Freezing point (ºC)	-45
5	Acid value (mg KOH/g EO)	0.59±0.15
6	Saponification value (mg KOH/g EO)	28.05±2.55

The AV recorded in this study was 0.59±0.15, which was lower than that *S. laureola* leaves EO (1.78±0.01) and *Z. armatum* leaves EO (1.98±0.01) [13], and *M. communis* leaves EO (4.451±0.710) [14]. Besides, the SV was also quite low (28.05±2.55), and it was found to be in accordance with the conclusion of Barkatullah *et al.* [13], who indicated that the SV value of plants is usually lower than the range of 188 to 196. In general, both the AV and SV values are two basic elements to evaluate the EO quality.

Determination of the chemical compositions of M. cajuputi leaves EO. GC–MS analysis was used to determine the chemical compositions of *M. cajuputi* leaves EO. The obtained results indicate that there are 23 different components in EO that comprised approximately 98% of the total chemical constituents of the EO (Table 2). All compounds were detected under retention time (RT) ranging from 6.3 min to 21.94 min. The compounds occupying the highest content in EO included 1,8-cineol (71.83%), *p*-menth-1-en-8-ol (6.01%), *p*-cymene (2.87%), γ-terpinene (2.73%), (+)-4-carene (2.7%), linalool (2.65%), (1R)-(+)-α-pinene (2.23%), and tyranton (2.03%). These components play an important role in EO quality. These chemical compositions in *M. cajuputi* leaves EO were very different from those in EO from leaves collected in various regions. Some compounds in the obtained EO were similar to those from many previous reports. The authors also studied the same material, for instance, 1,8-cineol, γ-terpinene, α-pinene, β-pinene, β-myrcene, benzaldehyde, caryophyllene [6], linalool, and p-cymene [15]. In general, there were minor changes in the proportion of components but there were no differences in major components, especially 1,8-cineol.This is the most important compound to evaluate the quality of EO from *M. cajuputi* leaves. Many previous studies reported that 1,8-cineol usually presents in *M. cajuputi* leaves EO with a high yield such as 7.3–23.2% [6], 46.9–57.9% [15], and 41.6–59.9% [16]. In some cases, there is no 1,8-cineol in *M. cajuputi* leaves EO [17], or the EO contained a low quantity (<3%) of this compound with the material from other places [5]. The difference in the chemical composition of EO can be explained by differences in the climatic conditions, time of harvesting, extraction method, and age of the plant, etc. [8].

Table 2. Chemical composition of *M. cajuputi* leaves EO.

No	Compound	RT (min.)	(%)
1	Tyranton	6.3	2.03
2	4-Methyl-1-(1-methylethyl)bicyclo[3.1.0]hexane didehydro deriv.	8.38	0.47
3	(1R)-(+)-α-Pinene	8.58	2.23
4	Benzaldehyde	9.2	0.17
5	β-Pinene	9.68	1.5
6	β-Myrcene	9.93	1.5
7	α-Phellandrene	10.34	0.36
8	δ-Terpinene	10.66	0.53
9	*p*-Cymene	10.88	2.87
10	1,8-Cineol	11.12	71.83
11	γ-Terpinene	11.73	2.73
12	(+)-4-Carene	12.5	2.7
13	Linalool	12.71	2.65
14	Methylenecyclooctane	14.5	0.11
15	4-Terpinenol	14.77	0.66
16	*p*-Cymen-8-ol	14.9	0.11
17	*p*-Menth-1-en-8-ol	15.1	6.01
18	β-Sesquiphellandrene	19.39	0.09
19	Caryophyllene	20.51	0.5
20	d,l-trans-4-methyl-5-methoxy-1-(1-methoxy-1-isopropyl)-cyclohex-3-ene	21.07	0.11
21	α-Caryophyllene	21.24	0.3
22	2,5,9,9-Tetramethyl-3,4,4a,5,8,9a-hexahydrobenzo [7] annulene	21.72	0.2
23	1H-Cyclopropa[a]naphthalene, decahydro-1,1,3a-trimethyl-7-methylene-, [1aS-(1aα,3aα,7aβ,7bα)]-	21.94	0.37

Determination of the antioxidant capacity (AC) of M. cajuputi leaves EO. By the DPPH technique, with an increase in EO concentration from 16 to 512 mg/mL, a significant increase in the AC of *M. cajuputi* leaves EO was detected (Figure 1). The AC peaks at 38% for an EO concentration of 512 mg/mL, while the AC of the original EO was approximately 40.88±0.85%. This revealed that the IC_{50} was not found in the EO concentration ranges tested, while the AC of ascorbic acid (IC_{50} of 16 µg/mL) was more efficient than that of the EO concentrations obtained (Figure 2). In general, the AC depends significantly on the chemical components, especially 1,8-cineol, γ-terpinen, and 4-carene. They are classified as terpene groups and can be found in large amounts in *M. cajuputi* leaves EO. Many previous studies reported that these compounds have a strong AC [18-20]. Essentially, there are no published studies on the AC of *M. cajuputi* leaves EO in the literature, it was not possible to compare our results.

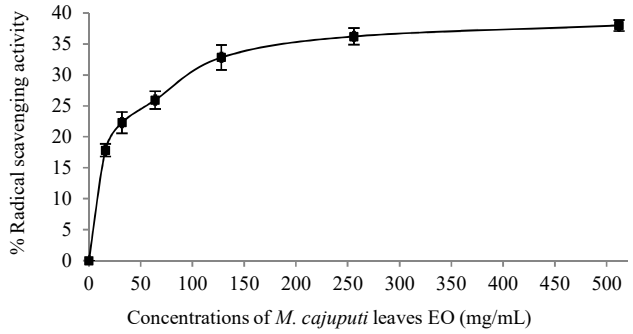

Figure 1. Radical scavenging activity of *M. cajuputi* leaves EO

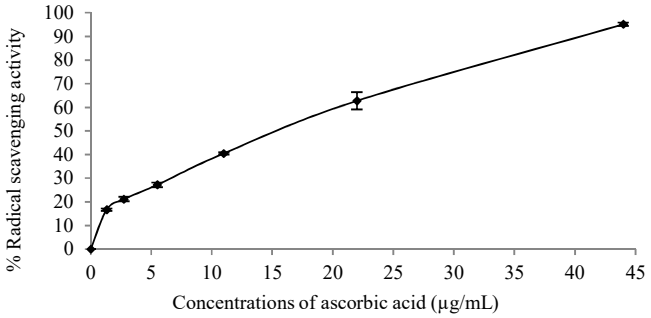

Figure 2. Radical scavenging activity of ascorbic acid

Determination of the antibacterial activity (AA) of M. cajuputi leaves EO. *M. cajuputi* leaves EO is considered a non-toxic agent. Beyond this, it has been noticed that cajuput EO is a disinfectant against bacteria [21]. In this study, the AA of cajuput EO is arranged in descending order: *S. aureus* > *S. enteritidis* > *E. coli* > *P. aeruginosa* (Table 3). However, cajuput EO did not inhibit the growth of *P. aeruginosa*, whereas it had a strong AA for the other three bacteria. In particular, the AA of EO for *S. aureus* and *E. coli*is significantly stronger than that of the positive controls. The same tendency of the AA of cajuput EO for *S. aureus*, *E. coli*, and *P. aeruginosa* is also observed in the study of Bharat *and* Praveen [22].

Although there is wide variation in the chemical composition of cajuput EO, the most important bioactive compound still is 1,8-cineol. The obtained EO in this study contains substantial amounts of 1,8-cineol (71.83%), and it was the main active component responsible for

its AA [23]. In addition, there are many minor compounds that exist in EO that also inhibit the growth of bacteria, for instance, linalool, γ-terpinene [21], myrcene [24], etc. This also proved that the AA can be a result of the synergistic action of major and minor components of the EO.

Table 3. The AA of *M. cajuputi* leaves EO.

No	Microorganisms	Diameter of the inhibitory zones of EO (mm)	Diameter of the inhibitory zones of positive control (gentamicin, mm)
1	*S. enteritidis*	23.67 ± 0.58^{Ab}	23 ± 0^{Ac}
2	*E. coli*	21.33 ± 1.15^{Ba}	15 ± 0^{Aa}
3	*P. aeruginosa*	-	17.67 ± 0.58^{b}
4	*S. aureus*	27.33 ± 0.58^{Bc}	25.67 ± 0.58^{Ad}

Different lowercase letters in the same column indicate significant differences between microorganisms at the $p < 0.05$ level. Different capital letters in the same row indicate significant differences between samples at the $p < 0.05$ level.

CONCLUSION

In general, the *M. cajuputi* leaves EO collected in Quang Tri province revealed that its physicochemical properties are very different compared to those of other EOs. It had high AC and AA. By the GC-MS method, 23 major components were identified in cajuput EO. This is a natural, rich source of 1,8-cineol that could be widely used in the cosmetic, food, and pharmaceutical industry.

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ARTICLES FOR FACULTY MEMBERS

CULTURAL HERITAGE

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Phytoconstituents and biological activities of *Melaleuca cajuputi* Powell: A scoping review

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Biological activities, medicinal plant, *Melaleuca cajuputi* Powell, phytoconstituents.

ABSTRACT

Melaleuca cajuputi Powell is a medicinal plant of the Myrtaceae family that is widely distributed in Asia and Southern Australia. The Major bioactive compounds reported in *M. cajuputi* powell include quinones, flavonoids, phenols, alkaloids, glycosdes, 18-cineol, α-pinene, linalool, β- caryophyllene, nerolidol, and terpenoids. Reports from several studies have shown an excellent antimicrobial effect of *M. cajuputi* Powell extracts against bacteria, viruses, protozoa, and fungal species. The plant also has remarkable insecticidal and antioxidant properties. Various toxicity experiments on rats and brine shrimps, on the other hand, have revealed a mild toxicity effect. With the potent biological activities and mild toxicity reported in *M. cajuputi* Powell, the plant could be exploited to develop potential plant-based novel drugs.

INTRODUCTION

Herbal formulations are not uncommon in treating diseases as they have been utilized to treat different diseases in humans and animals for millennia. Despite screening conducted on several other plant species, research studies on plant-based bioactive compounds remain paramount as better and safer agents with a broad spectrum of bioactivity are greatly needed (Arifullah *et al*., 2014). There is a long history of the medicinal uses of the plant in Southeast Asian countries, with some being sold in different forms for health remedies (Liew *et al*., 2020). It is an appropriate time to develop novel bioactive compounds from natural sources such as higher plants (Manga *et al*., 2018). This is due to the antimicrobial, insecticidal, and antioxidant properties these plants possess; therefore, they are accepted for everyday use in both traditional and modern medicine (Arifullah *et al*, 2014). Herbs are essential in dealing with different health conditions; this could be attributed to their enormous bioactive compounds, thus attracting the interest of researchers to the field of drug discovery (Oliveira *et al*., 2020).

Melaleuca cajuputi Powell plant is a member of the Myrtaceae family, popularly called "Gelam," "kayu putih," "paperbark," "Cajuput tree," "*Melaleuca leucadendron,*" "Cajuput oil," or "tea tree" (Sharif *et al*., 2019). The leaves are greyish-green and fragrant with a relaxing aroma. Its height ranges from 4 to 9 cm (Fig. 1). The flowers are whitish with a unique stamen with 16 cm long spikes, which are said to resemble bottlebrushes, and the encircled seeds are essential in folk medicine (Sharif *et al*., 2019). *Melaleuca* essential oil has a yellow-green color with a solid herbal aroma that is similar to eucalyptus essential oil (Wińska *et al*., 2019). The tea tree can be found naturally in tropical areas including Malaysia, Indonesia, Thailand, and Australia. *Melaleuca* forests grow well in estuaries and coastal swamps in the hot and humid tropics with a temperature range

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Figure 1. *Melaleuca cajuputi* Powell in its natural habitat (source: author).

of 17°C–33°C. The *Melaleuca*-rich region receives an average of 1.3–1.7 mm of precipitation per year, with typical monsoon conditions (Lim and Midon, 2001).

Several studies on the different parts of *M. cajuputi* Powell extracts have been reported previously (Al-Abd *et al*., 2015; Noor *et al*., 2020). However, the information obtained from these studies is extremely fragmented. Due to this, an in-depth literature review on *M. cajuputi* Powell will undoubtedly provide the necessary information which is crucial in understanding the knowledge gaps of this plant and stimulating future research opportunities. This scoping review focuses on the types of *M. cajuputi* Powell extracts examined and their phytoconstituents as well as the research findings on their biological activities.

METHODOLOGY

This review followed the methodological framework established by Arksey and O'Malley (2005). In addition, this study was processed according to the procedure of systematic database search, selection, and inclusion strategy as described by (Moher *et al*., 2009). This scoping review consisted of defining objectives, searching for relevant data, proper selection, gathering relevant information based on the study objectives, and summarizing the findings.

Relevant studies on the phytoconstituents and biological activities of *M. cajuputi* Powell published in the last two decades (2001 to 2022) were retrieved from Google, Science Direct, Google Scholar, Scopus, and PubMed. However, only research papers on *M. cajuputi* Powell written in English from January 2001 to early 2022 were reviewed. Theses, dissertations, and conference proceedings were included as well. Several key search terms were included such as "*M. cajuputi* Powell," "Gelam extracts," "tea tree," "cajuput tree," "*M. leucadendron*," "antibacterial," "antifungal," "antimicrobial," "antioxidant activities," "insecticidal," "toxicity effect," "bioactive," and "phytochemical compounds" by using the Boolean operators of AND/OR (Table 1).

Figure 2 illustrates the record selection criteria. Research articles from the various databases were first harmonized and sorted based on the titles to exclude all duplicates. Titles and abstracts were reviewed to exclude all records with inaccurate subjects or outcomes related to this review. Lastly, the full-text articles were screened further to exclude other irrelevant data.

Data recording

Relevant data regarding studies of *M. cajuputi* Powell were recorded. The information obtained consisted of various parts of the plant, such as leaves, flowers, stems, and essential oils (Fig. 3). The extraction procedures, bioactive compounds, type of biological activity, methods employed/outcomes, organisms tested, and references are shown in Table 2.

Collection, reporting, and summary of the review outcomes

All findings were recorded and summarized in Table 2. The research gaps were identified to establish a roadmap for future research to provide knowledge on novel drug discoveries from *M. cajuputi* Powell.

RESULT AND DISCUSSION

Medicinal plants are essential sources of unique bioactive compounds in creating new, effective, and safe medicines (3,311) research articles on the bioactivity of different parts of *M. cajuputi* Powell were retrieved via an electronic survey in interdisciplinary databases, with 325 duplicates removed from the list. As shown in Figure 2, The remaining 2,986 articles were then checked for title and abstract eligibility. In all, 2,892 irrelevant articles were excluded, leaving 94 possible relevant articles for full-text analysis, out of which only 34 papers were selected based on the review's selection criteria and objectives.

Ethnomedicinal uses of *M. cajuputi* Powell

Medicinal plants have been reported and investigated for natural bioproducts to control diseases and lessen reliance on conventional antibiotics worldwide (Isah *et al*., 2020). According to historical records, herbal therapy and Ayurvedic medicine have been utilized in China for over 5,000 years (Idris *et al*., 2019). Moreover, humans also have been relying heavily on natural products such as plant-based food and treatment for thousands of years (Idris *et al*., 2019). Herbal decoctions can be used in oral administration, topical application, or steaming for disease treatment, depending on the formulation and plant parts. Tablespoons, cups, and bottles are used to measure the dosage of these herbal formulations (Bunalema *et al*., 2014).

The leaves of Gelam are edible and are used to treat digestive problems, cough, common cold, and stomach pain (Daud *et al*., 2015). Meanwhile, herbal preparation made from *M. cajuputi* Powell leaves and stems is used to treat joint soreness, abdominal discomfort, and muscle ache (Wolter *et al*., 2002). Furthermore, tea tree leaves exhibit antimicrobial, antioxidant, anodyne, insect repellent, and anti-inflammatory properties and are utilized in traditional medicine to treat dyspepsia, burns, pain, and influenza (Ko *et al*., 2009).

Similarly, *M. cajuputi* Powell is an excellent producer of therapeutic essential oil that contains many phytochemicals (Hai *et al*., 2019). Steam or hydrodistillation extracts the Cajuput oil from the plant's leaves or other parts. It is utilized to heal wounds, body itch, coughs, stomach pains, asthma, and rashes in folk medicine, particularly in Southeast Asia (Toan *et al*., 2020). Additionally, the oil has a synergistic effect when combined with other plants in

Table 1. Keywords and search phrases used for literature search.

Keywords	Search phrases
#1 Biological activities	"Antimicrobial activity" OR "Antibacterial activity" OR "Antifungal agents" OR "Antioxidant activity" OR "Insecticidal effect" OR "Toxicity effects" AND "Bioactive compounds" OR "Phytochemical compounds."
#2 *M. cajuputi* Powell	"*Melaleuca* cajuputi Powell" OR "Gelam extract" OR "Tea tree" OR "Cajuput tree" OR "*M. Leucadendron*" OR "Cajeput oil".
#3	"Ethnomedicinal uses"
#4	#1, #2, AND #3

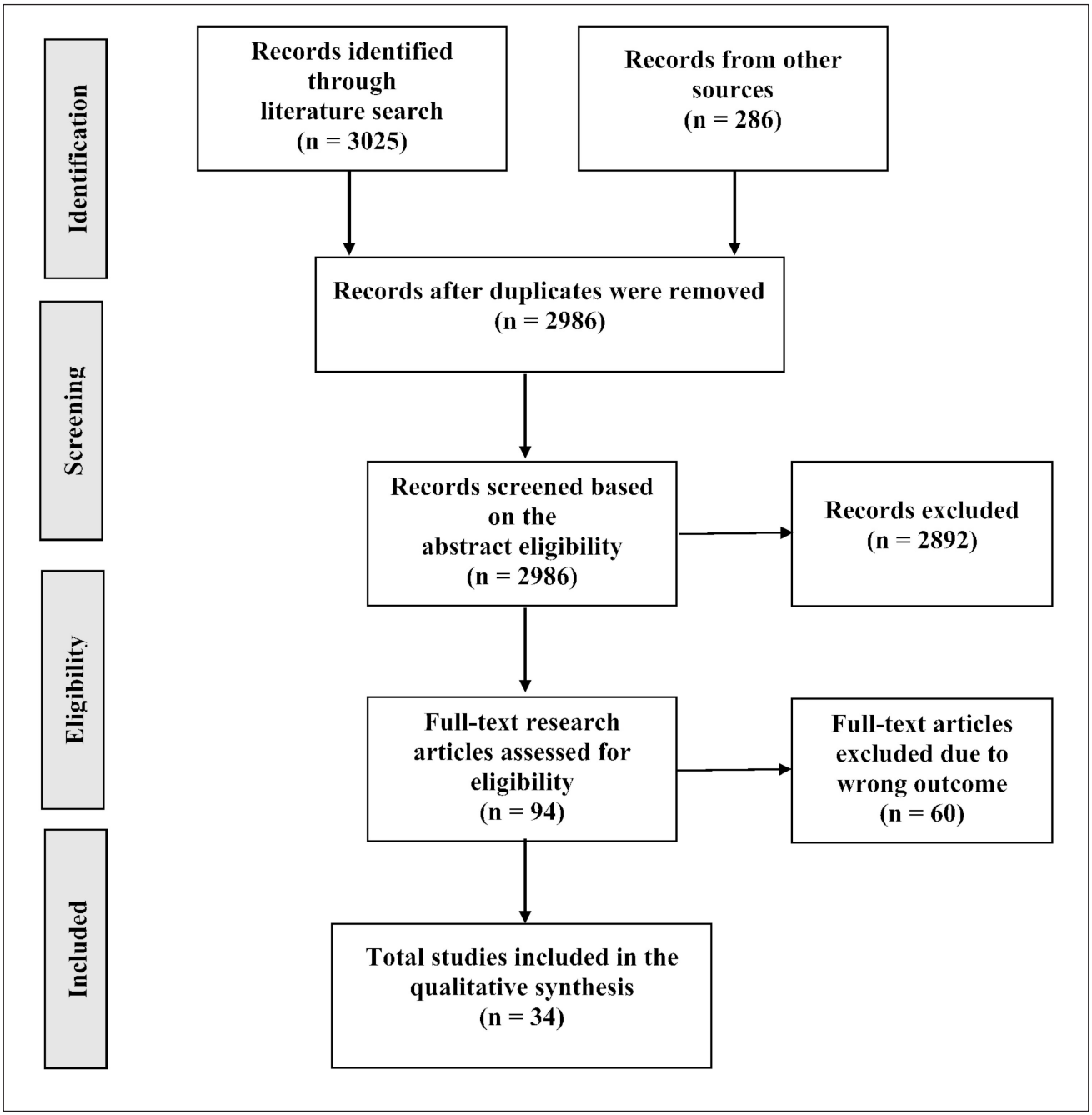

Figure 2. Record identification and selection protocol based on PRISMA guidelines (PRISMA, 2009).

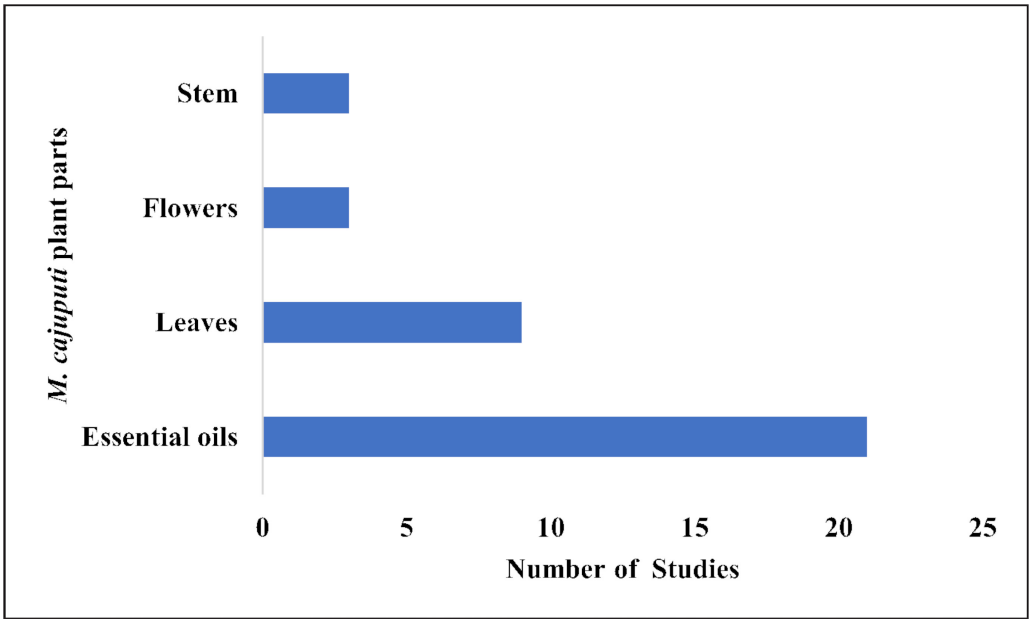

Figure 3. Bioactivity studies on the different parts of *M. cajuputi* Powell.

Table 2. Biological activities and phytochemical constituents of *M. cajuputi* Powell.

Plant part	Extraction method (solvent)	Analysis of phytoconstituents	Major phytoconstituents	Biological activities	Method/ outcomes	Organism tested (reaction)	References
Essential oil	Commercial	GC-MS/O	Active compounds reported: caryophyllene, 1,8-cineole, ylangene, α-pinene, nerolidol, and linalool	Flavoring ingredient	Nasal impact frequency	Not tested on organisms	(Septiana, Yuliana, and Wijaya *et al*., 2020)
Essential oil	Commercial	GC-MS	1,8-Cineole, β-caryophyllene, α-terpineol, α-pinene, and γ-terpinene	Flavoring agent	Metabolomics approach	Not tested on organisms	(Septiana, Yuliana, and Bachtiar *et al*., 2020)
Essential oil	Commercial	NR	NR	Antimicrobial	Broth microdilutions MIC 800–3200 µg/mL and 3,200–6,400 µg/ml against *S. aureus* and *E. coli,* respectively	*Staphylococcus aureus* DSM 1104 (S) and *E. coli* DSM 1103 (S)	(Thielmann *et al*., 2019)
Essential oil	Commercial	According to Pranarôm	Cineole, a monoterpene ether	Antibacterial	Disc diffusion (10.0 mm) at conc. of 6 µl/ paper disc	*Streptococcus pyogenes* CIP 104226 (S)	(Sfeir *et al*., 2013)
Essential oil	Commercial	NR	NR	Antifungal	Agar diffusion optimum growth inhibition at 1.5625% (*v*/*v*)	*Aspergillus flavus* IMI 242684 (S)	(Thanaboripat *et al*., 2007)

Continued

Plant part	Extraction method (solvent)	Analysis of phytoconstituents	Major phytoconstituents	Biological activities	Method/ outcomes	Organism tested (reaction)	References
Flowers	Maceration (methanol)	NR	NR	Antifilarial (targeting bacterial endosymbiont "*Wolbachia"*)	Cell culture (Aa23 cells)	Filarial worm (*B. pahangi*) and bacterial endosymbiont "*Wolbachia*" (S)	(Hnawia *et al*., 2012)
Flowers and fruit	Maceration (methanol)	Qualitative	Alkaloids, flavonoids, terpenoids, and saponins; others are quinones, steroids, tannins, and phenols	Antibacterial	MIC and MBC for flower extract were 1.67 and 2.083 mg/ml, respectively. For fruit, both MIC and MBC values were 3.334 mg/ml	*E. coli* ATCC 25922 (S)	(Isnaini *et al*., 2021)
Flowers and fruit	Maceration (methanol)	Qualitative	Alkaloids, flavonoids, terpenoids, and saponins; others are quinones, steroids, tannins, and phenols	Antifungal	Flower extract MIC and MBC were 3.125 and 25 mg/ml, respectively. Fruit extract, 3.125 and 12.5 mg/ml were the MIC and MBC, respectively	*Candida albicans* ATCC 10231 (S)	(Isnaini *et al*., 2021)
Leaves	Maceration (methanol)	NR	NR	Antimicrobial	Disc diffusion (10–20 mm) at conc. 1,000 µg/ml	*Staphylococcus aureus* (S), *Escherichia coli* (R), *C. albicans* (S), and *Aspergillus niger* (S)	(Khalaf *et al*., 2021)
Leaves	Maceration (methanol)	NR	NR	Antioxidant	IC_{50} values in the DPPH= 2,2-diphenyl-1-picrylhydrazyl assay were 34.60–60.97 µg/ml		(Khalaf *et al*., 2021)
Leaves	Maceration (methanol)	NR	NR	Antibacterial	Disc diffusion. The diameter zone of inhibition was 1.00 to 13.87 mm at 10 µl	*Shigella dysentriae* (S), *Bacillus* species (S), *Pseudomonas aeruginosa* (R), *E. coli* (R), *S. aureus* (S), *Vibrio cholerae* (S), and *Enterococcus faecalis* (S).	(Ukit *et al*., 2019)

Continued

Plant part	Extraction method (solvent)	Analysis of phytoconstituents	Major phytoconstituents	Biological activities	Method/ outcomes	Organism tested (reaction)	References
						Enterococcus faecalis ATCC 29212 (S),	
					Broth microdilutions	*P. aeruginosa* ATCC 10145 (S),	
Leaves	Maceration (ethanol)	NR	NR	Antibacterial	MIC and MBC for AgNPs were 7.81–31.25 µg/ml and 62.20–125 µg/ml, respectively	*E. coli* ATCC 25922 (S), *S. aureus* ATCC 29213 (S), *K. pneumoniae* ATCC 700603 (S), *E. coli* O157:H7 (S), *A. baumannii* ATCC 19606 (S)	(Paosen *et al*., 2017)
Leaves	Soxhlet and superficial fluid extraction (SFE) (hexane)	GC and GC/MS	Isoeugenitine, β-elemene, β-caryophyllene, α-humulene, viridiflorol, platyphyllol, β-eudesmol, bulnesol, (Z, Z)-farnesol, (E,E)-farnesal, 9-epi-β-caryophyllene, and δδ-elemene	NR	NR	Not tested on organisms	(Jajaei *et al*., 2010)
Leaves	Maceration (methanol, hexane, ethyl acetate, and dichloromethane)	NR	NR	Insecticidal	Hexane extract against *Ae. aegypti* LC_{50} 0.015 mg/cm^2 and *Aedes albopictus* 0.022 mg/cm^2 while dichloromethane extracts *Aedes aegypti* and *Ae. Albopictus,* the LC_{50} was 104.8 mg/l and 106 mg/l, respectively	*Aedes aegypti* and *Aedes albopictus*	(Bakar, 2020)
Leaves (essential oil)	Hydrodistillation	GCMS	1,8-Cineole (23.59%), (-)-α-pinene (9.12%), (-)-1S-β-pinene (5.87%), α-terpineol (4.91%), α-terpinene (4.74%), (β)-limonene (4.42%), β-caryophyllene (5.32%) and α-humulene (4.76%)	Antimycobacterial	Broth microdilution **(**resazurin microtiter assay**)** MIC values ranged between ≤0.5 and 16% (*v*/*v*)	*Mycobacterium tuberculosis* (S)	(Bua *et al*., 2020)

Continued

Plant part	Extraction method (solvent)	Analysis of phytoconstituents	Major phytoconstituents	Biological activities	Method/ outcomes	Organism tested (reaction)	References
Leaves (essential oil)	Commercial	NR	NR	Antitrichomonal	Broth microdilution MIC 0.08 ± 0.05% and 0.06 ± 0.05%	*Trichomonas vaginalis* (S)	(Thi Ha Trinh *et al*., 2021)
Leaves (essential oil)	Steam distillation (EO) organic solvent **(**methanol, aqueous, and decoction**)**	GCMS	The most dominant compounds are terpenoids (α-pinene (20.353%), β-caryophyllene (4.943%), and α-terpinene (3.556%)), α-pinene (2.026), α-phellandrene (2.974), and 4-terpineol (2.227)	Antioxidant	Folin-Ciocalteu and aluminum chloride colorimetric (4.90–19.29 mg GAE/g) TPC (0.14–13.29 mg QE/g) and TFC	Not tested on organisms	(Noor *et al*., 2020)
Leaves (essential oil)	Hydrodistillation	GCMS	Eucalyptol (27.512%), terpinolene (9.047%), γ-terpinene (8.59%), α-terpineol (4.108%), α-selinene (3.889%), α-caryophyllene (3.522%), β-eudesmene (3.359%), and 1R-α-pinene (2.158%)	Antibacterial	Disc diffusion. Activity ranged from 5 to 7 mm at 0.1 ml	*Salmonella typhimurium* NRRL-B-2354 (S), *Bacillus cereus* NRRL-B-354 (S), *S. aureus* NRRL-B-313 (S), *E. coli* NRRL-B-409 (S), and *P. aeruginosa* NRRL-B-14781 (S)	(Toan *et al*., 2020)
Leaves (essential oil)	Steam distillation	GCMS	Cineol (31.6%), terpineol (10.7%), β-selinenol (6.8%), α-eudesmol (6.7 5%), guaiol (6.5%), γ-eudesmol (4.3%), bulnesol (1.9%), β-myrcene (0.9%), terpinen-4-ol (0.9%), and linalool (0.6%)	Anticoronavirus	Docking simulation	Coronavirus (S)	(My *et al*., 2020)
Leaves (essential oil)	Steam distillation	GCMS	B-Caryophyllene (20.16%), α-terpinolene (17.0%), and α-humulene (11.91%), also β-elemene (7.62%) and γ-terpinene (5.62%)	NR	NR	Not tested on organisms	(Sharif *et al*., 2019)

Continued

Plant part	Extraction method (solvent)	Analysis of phytoconstituents	Major phytoconstituents	Biological activities	Method/ outcomes	Organism tested (reaction)	References
Leaves (essential oil)	Steam distillation	GCMS	1,8-Cineole (42–60%), α-terpineol (4–18%), caryophyllene (0.6–11%), α-selinene (6.73 %), α-pinene (3-12%), α-gurjunene (17.31%), and 2,4-pentanediol (11.44%)	NR	NR	Not tested on organisms	(Sutrisno *et al*., 2018)
Leaves (essential oil)	Steam distillation	Qualitative analysis (using wet reactions)	Saponins, reducing sugar, cardiac glycoside, coumarins and lactones, and steroids	Antimicrobial	Agar well diffusion at conc. Oil: methanol *v/v* 50 µl. The zone of inhibition ranged between 5 ± 0.085–27 ± 0.05 mm and 2 ± 0.12–19.5 ± 0.12 for bacteria and fungi, respectively	*Staphylococcus aureus* (S), *P. aeruginosa* (S), *E. coli* (S), *Klebsiella* species (S), *Acinetobacter* species (S), *Salmonella typhi* (R), *Salmonella paratyphi* (R), *Rhizopus nigricans* (R), *Candida albicans* (R), and *A. niger* (S)	(Dahiya *et al*., 2016)
Leaves (essential oil)	Hydrodistillation	GC and GCMS	Major component; 1,8-cineole (43.7 ± 0.5%)	NR	NR	Not tested on organisms	(Silva *et al*., 2007)
Leaves (essential oil)	Steam hydrodistillation	GCMS	Major chemical compounds, 2-propenoic acid (29.55%) and caryophyllene (20.04%)	Insecticidal	LC_{50} 120.99 mg/l and 222.58 mg/l for *A. aegypti* and *A. albopictus,* respectively	*Aedes aegypti* and *Aedes albopictus*	(Bakar *et al*., 2019)
Leaves (essential oil)	Steam distillation	GCMS	1,8-Cineole (53.90%), α-terpineol (9.53%), D-(+)-limonene (6.52%), and β- caryophyllene (4.11%)	Antifungal	Agar well diffusion. IC_{50} ranged from 0.44 to 7.71 mg/ml	*Fusarium oxysporum* (NBRC 31213) (S), *Thanatephorus cucumeris* (NBRC 30937) (S), and *Rhizopus oryzae* (NBRC 31005) (S)	(Pujiarti *et al*., 2011)
Leaves (essential oil)	Steam distillation	GCMS	1,8-Cineole (53.90%), α-terpineol (9.53%), D-(+)-limonene (6.52%), and β-caryophyllene (4.11%)	Antioxidant	1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (IC_{50} 4.24 mg/ml)		(Pujiarti *et al*., 2011)

Continued

Plant part	Extraction method (solvent)	Analysis of phytoconstituents	Major phytoconstituents	Biological activities	Method/ outcomes	Organism tested (reaction)	References
Leaves (essential oil)	Commercial	NR	NR	Antifungal	Vapor-phase-mediated susceptibility assay (using 96-well microtiter plate) at 20 µl of essential oil	*Candida albicans* SC5314 (S) and *Candida glabrata* ATCC 2001 (S)	(Feyaerts *et al*., 2018)
Leaves (essential oil)	Hydrodistillation	NR	NR	Antimicrobial	Broth microdilutions at conc. of 60 µl EO sample	*Streptococcus mutans* Xc (S) and *C. albicans* (S)	(Septiana *et al*., 2019)
Leaves (essential oil)	Hydrodistillation	GCMS	Alpha-pinene (4.15%)	Enzyme inhibition (acetylcholinesterase)	Ellman's method (21.18 ± 0.54%) inhibitory activity. IC_{50} not evaluated		(Sareedenchai *et al*., 2019)
Leaves and flowers	Maceration (methanol)	LCMS and GCMS	The major compounds include Terpenoids Aromatics Phenolics Fatty acids Flavonoids	Antimicrobial	Disc diffusion (100 mg/ml) and broth dilution. The MIC was 12.5 mg/ml and MBC 25.0 mg/ml for leaves extract. For flower 12.5–25 mg/ml MIC and 25–50 mg/ml MBC	*Staphylococcus epidermidis* MTCC 3615 (S), *S. aureus* RF 122 (S), *B. cereus* ATCC 11778 (S), *Streptococcus pneumoniae* ATCC 10015 (R), *E. coli* UT181 (R), *S. typhimurium* ATCC 14028 (R), *Klebsiella pneumonia* ATCC 13883 (R), and *Pasteurella multocida* (R)	(Al-Abd *et al*., 2015)
Leaves and flowers	Maceration (methanol)	LCMS and GCMS	The major compounds include Terpenoids Aromatics Phenolics Fatty acids Flavonoids	Antioxidant	DPPH radical scavenging activity. IC_{50} value 25 µg/ml for flower extract and IC_{50} value 10 µg/ml for leaves extract		(Al-Abd *et al*., 2015)
Leaves and twigs	Reflux (ethanol)	Preparative HPLC	β-Triketone flavanone hybrid (cajuputones A, B, and C)	NR	NR	Not tested on organisms	(Xu *et al*., 2020)
Leaves and twigs (essential oil)	Hydrodistillation	GCMS	β-Elemene (5.09%). α-pyrone (10.11%), γ-terpinene (8.00%), terpinolene (9.26%), β-caryophyllene (6.36%), and 1,8-naphthyridine derivatives (10.46%)	Antifungal	Broth microdilution. 0.31–1.25 µl/ml and 0.63–1.25 µl/ml were the MICs and MFCs, respectively	*Candida albicans* ATCC 90028 (S)	(Keereedach *et al*., 2020)

Continued

Plant part	Extraction method (solvent)	Analysis of phytoconstituents	Major phytoconstituents	Biological activities	Method/ outcomes	Organism tested (reaction)	References
Stem	Maceration(methanol and ethanol)	NR	NR	Antibacterial	Broth microdilution (MIC and MBC. No activity	*Propionibacterium acnes* ATCC 6919 (R)	(Batubara *et al*., 2009)
Stem	Maceration (methanol and ethanol)	NR	NR	Lipase inhibition	Using 2,3-dimercapto-1-propanol tributyrate. No inhibition	Lipase enzyme	(Batubara *et al*., 2009)
Stem	Maceration (methanol and ethanol)	NR	NR	Antioxidant	Using DPPH assay at IC_{50} 58.41 ± 1.32 and 5.79 ± 1.13 µg/ml % inhibition		(Batubara *et al*., 2009)

NR = not reported, (R) = resistant, and (S) = susceptible.

decoction to treat rheumatoid arthritis (Toan *et al*., 2020). Externally, the utilization of *M. cajuputi* oil includes relieving neuralgia and rheumatism (typically in the form of ointments and salves), as well as toothache, cancer, worms (particularly roundworms), and infections of the genitourinary system (Silva *et al*., 2007).

Solvent selection and methods for extracting phytochemicals from *M. cajuputi* Powell extracts

Plant extraction can be performed with different solvent systems ranging from nonpolar, intermediate, and highly polar to separate and purify bioactive compounds (Ukit *et al*., 2019). The resulting filtrate is tested for bioactivity screening at different concentrations. The most common solvent systems used to extract the bioactive compounds from *M. cajuputi* Powell were methanol, ethanol, hexane, ethyl acetate, dichloromethane, and water (Table 3). Methanol, in particular, was highlighted several times in previous studies due to its excellent properties in dissolving polar and nonpolar bioactive compounds from plant material (Ukit *et al*., 2019). Bioactive compounds can be separated and isolated by using different chromatographic techniques. Subsequently, various spectrometric approaches can be employed to identify the plant's chemical structure and functional groups of extracted bioactive components (Xu *et al*., 2020).

Table 3. Solvent's system used in *M. cajuputi* Powell extraction process from 2001 to 2021.

Extraction solvent	Number of studies
Methanol	10
Ethanol	3
Hexane	2
Ethyl acetate	1
Dichloromethane	1
Aqueous	1

Phytochemical composition of *M. cajuputi* Powell

The bioactive compounds reported in *M. cajuputi* Powell extracts include monoterpenes, sesquiterpenes, flavonoids, and phenolic compounds (Table 2). Figure 4 depicts the chemical structures of the main bioactive molecules. Monoterpenes are the most common chemical compounds, with structures comprised two isoprene units and organic functional groups involving hydrocarbons (pinene, terpinene, p-cimene, and terpinene), alcohols (linalool, cineol, and 4-terpineol), and ethers like 1,8-cineol (Jajaei *et al*., 2010). Sesquiterpenes are unsaturated chemicals that are comprised of hydrocarbons (caryophyllene), oxygenated sesquiterpenes (nerolidol), and alcohols (bisabolol and nerolidol). Other organic compounds such as steroids, quinones, saponins, tannins, and alkaloids were also reported in *M. cajuputi* Powell extracts (Dahiya *et al*., 2016; Isnaini *et al*., 2021).

Plants usually produce primary metabolites such as simple sugars, amino acids, polypeptides, and lactic acid, which are found virtually in all plants as part of their regular metabolic activity. They also produce secondary metabolites such as antibiotics, poisons, organic acids, and pigments for defense purposes (Angelo, 2015). As most of the products are used for self-defense, these metabolites and pigments could contain bioactive molecules, including inulin, quinine, morphine, and codeine (Angelo, 2015). Some of these bioactive compounds reported in *M. cajuputi* Powell extracts could interfere with the cell membrane integrity and distort cell wall structure, leading to cell death. For example, terpenoids could divide lipid membranes and cause irreversible leakage of cellular components, leading to cell destruction (Sharif *et al*., 2019). Another bioactive compound is 4-terpineol, an isomer of terpineol that blocks respiration and interferes with cellular metabolism (Sharif *et al*., 2019).

Antimicrobial activity of *M. cajuputi* Powell

The antimicrobial efficacy of *M. cajuputi* Powell extracts from leaves, flowers, stem bark, and essential oil against bacteria, fungi, protozoa, and viruses was evaluated *in vitro* using agar well diffusion, disc diffusion, and broth microdilution. *Melaleuca cajuputi* Powell crude extracts showed antimicrobial activity at

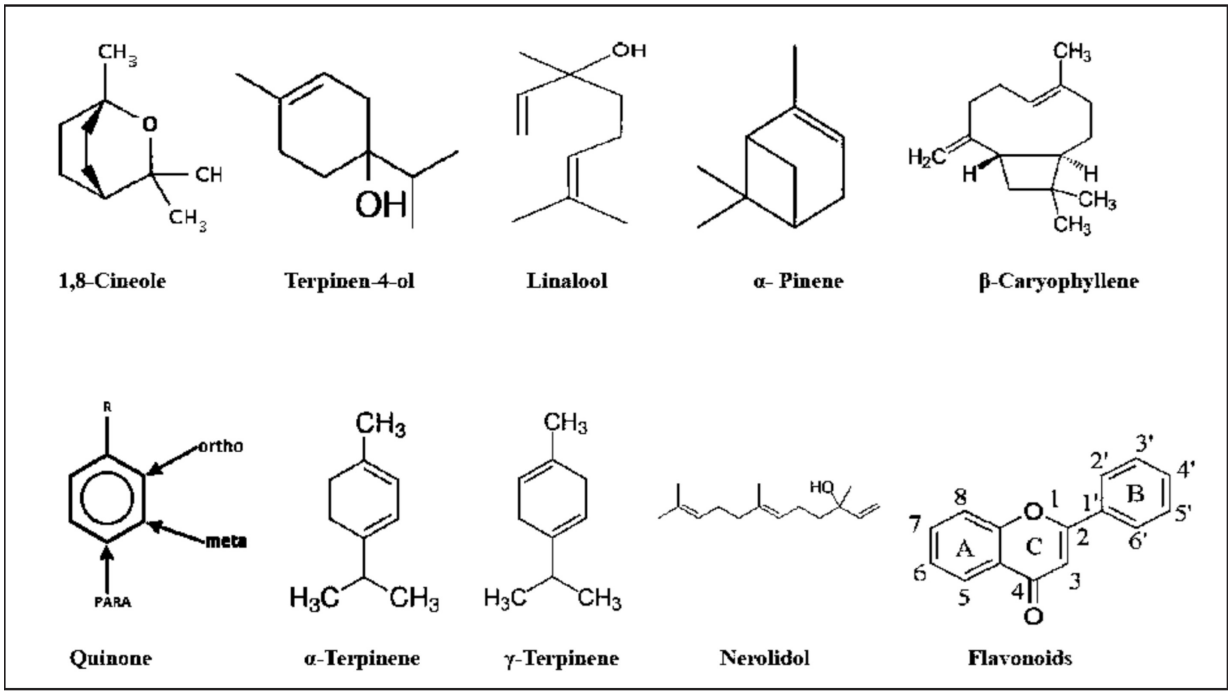

Figure 4. Major bioactive compounds reported in *M. cajuputi* Powell extracts and their chemical structures.

0.007–25.00 mg/ml and 0.062–50.0 mg/ml as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)values, respectively. The diameter zone of inhibition ranged from 1.00 to 27.00 mm (Table 2).

The report on the antimicrobial properties showed that *M. cajuputi* Powell extracts were effective against fungal species, bacteria, viruses, and protozoa at varying concentrations (Bua *et al*., 2020; Shivappa *et al*., 2015; Thanaboripat *et al*., 2007; Trinh *et al*., 2021).

The essential oil at a concentration of 0.714% (w/w) was reported to show antibacterial activity against pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) (Wahab *et al*., 2022). As determined *in silico* and verified by flow cytometry and transmission electronic microscopy, *M. leucadendra* essential oil enhances cell membrane permeability and disruption as well as cell wall lysis of bacteria by inhibiting enzymes (peptidoglycan glycosyltransferase) (Bautista-Silva *et al*., 2020). According to Chaudhari *et al*. (2022), *M. cajuputi* essential oil interferes with the integrity of the plasma membrane of *Aspergillus flavus* cells by interfering with the biosynthesis of ergosterol.

When compared to other parts of the plant, Cajuput oil was far more effective against all the microorganisms tested, including the virulent coronavirus (My *et al*., 2020). Furthermore, the plant was reported to have an antifilarial effect against *Brugia pahangi* by inhibiting the bacterial endosymbiont "*Wolbachia*" (Al-Abd *et al*., 2016). Figure 5 depicts the number of microorganisms used in the previous studies.

Antioxidant properties of *M. cajuputi* Powell

Melaleuca cajuputi Powell possesses significant antioxidant activity. Different parts of the plant have several antioxidant compounds, such as α-terpineol, D-(+)-limonene, and β-caryophyllene, which possess excellent antioxidant activities (Table 2).

Several physiological processes in the human and animal body produce free radicals, and they are involved in cellular biochemical functions such as gene regulation, transcription, translation, and signaling (Pérez-Rosés *et al*., 2016). However, it is important to highlight that there must be a balance between free radicals and antioxidants for normal physiological functioning. Oxidative stress can arise when free radicals outstrip the body's ability to manage them, leading to diabetes, inflammation, cancer, and cardiovascular disease (Abubakar and Loh, 2016). Therefore, natural antioxidant compounds from a natural source, such as high plants, are essential for the body's normal physiological function (Dandashire *et al*., 2019).

Toxicological studies

There are ongoing debates on the safety of herbal medicine in modern medicine and disease treatment, but scientists have continued to be fascinated with herbal medicine. Therefore, it is essential to provide knowledge of previous reports regarding the toxicity profile of *M. cajuputi* Powell to support its usage in disease treatment.

*Melaleuca cajuput*i Powell essential oil causes termite mortality at LC_{50} = 4.60% (Roszaini *et al*., 2013). Furthermore, *M. cajuputi* Powell essential oil caused contact toxicity on *Sitophilus*

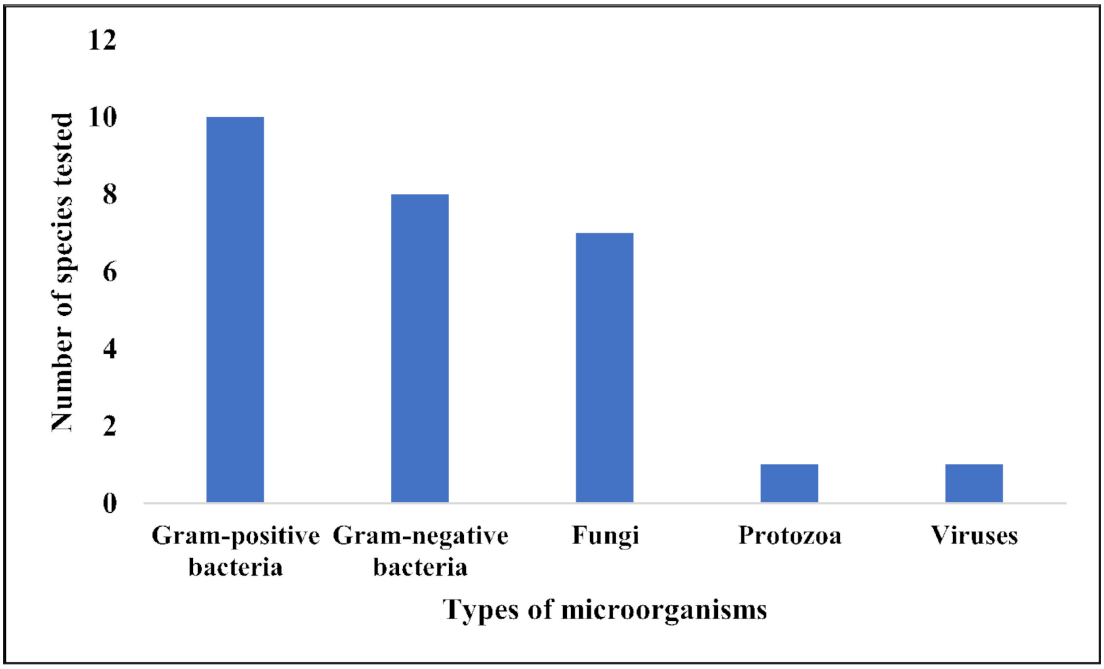

Figure 5. Types of microorganisms susceptible to *M. cajuputi* Powell extracts as reported in the various literatures (n = 27).

zeamais and *Tribolium castaneum* at LC_{50} values of 178.23 and 213.17 µl L^{-1}, respectively (Ko *et al*., 2009). Similarly, the methanolic extract of *M. cajuputi* Powell stems induced a toxicity effect on *Camponotus* sp. at LT_{50} = 84.3% (Visheentha *et al*., 2018). On the other hand, the methanolic leaves extract at doses between 50 and 200 mg/kg showed no toxic effect on Sprague-Dawley rats (Daud *et al*., 2018). However, in a brine shrimp lethality test, the extract induced a mild toxicity effect at LC_{50} 427 µg/ml (Noor *et al*., 2020). The aqueous and decoction extracts of *M. cajuputi* Powell had no toxicity effect at LC_{50} 1062 and 2477 µg/ml, respectively (Noor *et al*., 2020). According to the Meyer toxicity index, the plant extract is considered toxic when the LC_{50} is less than 1,000 µg/ml, and it is nontoxic when the LC_{50} is greater than 1,000 µg/ml (Noor *et al*., 2020). Clarkson on the other hand defined the toxicity as nontoxic if the LC_{50} is greater than 1,000 µg/ml, lowly toxic if the LC_{50} is between 500 and 1,000 µg/ml, highly toxic if the LC_{50} is between 100 and 500 µg/ml, and extremely toxic if the LC_{50} is between 0 and 100 µg/ml (Hamidi *et al*., 2014).

CONCLUSION

The extracts from *M. cajuputi* Powell showed a wide range of *in vitro* and *in vivo* biological effects. The reported phytoconstituents such as phenolic, aromatic, flavonoid, and alcohol groups could be responsible for the biological activities in the plant. The antimicrobial, antioxidant, and toxicity profiles of *M. cajuputi* Powell signified that the plant could be an excellent source of particular pharmacological active compounds. Further studies on bioassay-guided fractionation to obtain the pure compounds of *M. cajuputi* Powell extracts and the investigation of their antimicrobial mechanisms are necessary to unveil the plant's full potential as a good source of novel pharmaceutical compounds for disease remedy.

AUTHOR'S CONTRIBUTIONS

Assoc. Prof. Dr. Mohd Dasuki Sul'ain, Dr. Wan-Nor-Amilah Wan Abdul Wahab, and Isah Musa contributed to the concept and design. Dr. Wan-Nor-Amilah Wan Abdul Wahab and Isah Musa contributed to data acquisition and interpretation. Isah Musa drafted the manuscript. Prof. Dr. Wan Rosli Wan Ishak, Assoc. Prof. Dr. Hasmah Abdullahi, Dr. Wan-Nor-Amilah Wan Abdul Wahab, and Rasmaizatul Akma Rosdi critically reviewed the manuscript.

CONFLICT OF INTEREST

The authors have no known conflicts of interest.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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Original Article

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Screening of acetylcholinesterase inhibitory activity in essential oil from Myrtaceae

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ABSTRACT

Objective: This research was to investigate acetylcholinesterase inhibitory (AChEI) activity of the essential oil from Myrtaceae and its components. **Materials and Methods:** The essential oils were extracted from a fresh leave of Myrtaceae plants: *Eucalyptus globulus* Labill (Eucalyptus), *Melaleuca cajuputi* Powell (Samed-Khao), *Melaleuca citrina* (Curtis) Dum. Cours (Bottlebrush tree), *Psidium guajava* Linn. (Gauva), *Syzygium cumini* (L.) Skeel (Wha), and *Syzygium samarangense* (Blume) Merr. and L. M. Perry (Chompoo Nam Dok Mai) by hydrodistillation and analyzed chemical constituent by gas chromatography. The AChE inhibition was determined based on Ellman's method. **Results:** The essential oil from *M. citrina* presented the greatest inhibitory activity (71.77 ± 2.11%) and followed by *E. globulus* (47.65 ± 2.26%), *P. guajava* (24.96 ± 2.38%), *M. cajuputi* (21.18 ± 0.54%), *S. cumini* (19.97 ± 1.10%), and *S. samarangense* (13.78 ± 1.52%), respectively. Alpha-pinene was found in the essential oil of 6 species. 1,8-Cineole was the main compound of the essential oil from *M. citrina* and acts as an active constituent on AChEI. The essential oil from *M. cajuputi*, *S. cumini,* and *S. samarangense* contained a small amount of α-pinene, and without 1,8-cineole, then they were less potency on AChEI. **Conclusion:** The essential oil containing higher amount of 1,8-cineole presented strong activity to inhibit the AChE. Moreover, there are also other constituents in the essential oils that may affect the AChEI activity and also present synergistic effect for AChEI.

Keywords: 1,8-Cineole, acetylcholinesterase inhibitor, essential oil, myrtaceae

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease of cholinergic neurons, accompanied by loss of the neurotransmitter acetylcholine (ACh). Acetylcholine is involved in the regulation of cognitive function. The causes of AD are still unknown, but it was found in clinical studies that by giving acetylcholinesterase inhibitors (AChEI) to patients resulted in the increase of ACh in the central nervous system. The used of AChEI is one of the methods to prevent ACh from breaking down the process, consequently delaying disease progression.[1,2]

Several herbs has been evaluated for their biological activity on AChEI. There have been a lot of research, searching a new compounds for management the AD.[3-5] Some of Amaryllidaceae alkaloids were reported for AChEI activity. Galantamine, alkaloids, from the bulb of *Galanthus caucasicus,* was licensed as a medicine for treating AD. Furthermore, numerous essential oils and their monoterpene constituents were tested for AChEI. The essential oil of *Salvia lavandulaefolia* and its terpenoinds, composing of 1,8-cineole, camphor, α-pinene, α-pinene, borneol, caryophyllene oxide, linalool, and bornyl acetate, was investigated an activity of AChE inhibition. The studies indicated that the major compounds of essential oils, 1,8-cineole, α-pinene, and α-pinene, presented a potency on AChEI.[6-8] Thymoquinone constituent from the essential oil of *Thymus vulgaris* L. exhibited the strongest AChEI.[9] The commercial essential oils, *Artemisia dracunculus* L., *Inula graveolens* L., *Lavandula officinalis* Chaix, and *Ocimum sanctum* L. inhibited AChE activity. The terpenoids from the essential oil were investigated. 1,8-cineole, α-pinene, eugenol, α-terpineol, and terpinene-4-ol were showed AChEI.[10] The extracted and essential oil from *Satureja thymbra* L. showed an activity on AChEI. Carvacrol was a major component of essential oil but less potency on AChEI than thymol.[11] The 5 essential oils from the commercial, Eucalyptus, Cajuput, Sweet Marjoram, Camphor, and Rosemary, composing 1,8-cineole, showed a potency on AChEI.[12] Moreover, the essential oil from *Salvia*

lavandulaefolia inhibits the acetylcholinesterase in vivo. The essential oil of *S. lavandulaefolia* was administered orally in rat for 5 days. The cortex, hippocampus and striatum were dissected and evaluated AChE activity. Acetylcholinesterase activity was decrease in the striatum and hippocampus but no change in the cortex of the rat brain. That was supported, the constituents of essential oil reach the brain and inhibit AChE in specific areas.[13] Many studies on essential oil indicated that 1,8-cineole and α-pinene are strong inhibitors of AChE. 1,8-cineole and α-pinene can be found in the various genus of Myrtaceae; Eucalypts, Melaleucas, or Leptospermums.[14]

Myrtaceae originate from the central and south of America. They distribute in tropical and subtropical regions. Myrtaceae plant, shrub or tree, is the accumulation of essential oils in schizogenic glands. Myrtaceae is an important economic in the product from timber, fruits, spices, and essential oil. The essential oil from the member of Myrtaceae was enriched in terpene; 1, 8-cineole, α-pinene, limonene, linalool, and terpinen-4-ol, etc. For this reasons, the leaves, flowers, and fruits, provided essential oils, used for several purposes as a food, medicine, spice, or fragrance.[15,16] The objective of this study was to investigate AChEI activity form the essential oil of Myrtaceae species which was grown in Thailand and its components using gas chromatography (GC).

MATERIALS AND METHODS

Plant Materials and Distillation of Essential Oils

Fresh plant of 5 species, *Eucalyptus globulus* Labill (Eucalyptus), *Melaleuca cajuputi* Powell (Samed-Khao), *Melaleuca citrina* (Curtis) Dum. Cours (Bottle brush tree), *Psidium guajava* Linn. (Gauva), *Syzygium cumini* (L.) Skeel (Wha), and *Syzygium samarangense* (Blume) Merr and L.M. Perry, were collected from Nakhonnayok. Only *M. citrina* (Curtis) Dum. Cours (Bottle brush tree) was collected from Nakhon Si Thammarat. The voucher specimen of *M. cajuputi* was kept at Faculty of Pharmacy, Srinakharinwirot University. Fresh leaves of 6 species were cleaned and cut into small piece. The essential oils were extracted from the fresh leave by hydrodistillation with Clevenger apparatus for 4 h. Anhydrous sodium sulfate was added in the essential oils for eliminate water. They were kept in an amber bottle and stored at 4°C for analyzed by gas chromatography–mass spectrometry (GC-MS).

GC-MS

The essential oil was analyzed the component using GC on a Finnigan Trace GC-MS ultra (Thermo Electron Corporation, USA). The detector was Finnigan DSQ Quadrupole MS. The column was BPX5 fused silica column (30 m × 0.25 mm, 0.25 uM film thickness). The carrier gas was Helium, flown in 1 ml/min. The injector temperature was 180°C. 1 μl of sample was injected by splitter (1:100). The oven temperature programming was 60°C for 1 min. then ramp to 240°C with the rate of 3°C/min. MS was performed by EI positive mode at 70 eV ionization voltages. The compounds were identified by comparing by matching their mass spectra and retention indices with Adams EO Mass Spectral library and NIST05 Mass Spectral library.

AChE Inhibition Assay

The AChEI was determined in a 96-well plate assay based on the colorimetric Ellman's method (1961)[17] and modified method from Salah and Jäger.[18] In briefly, 25 μl of 1.5 mM acetylcholine iodide, 25 μl of sample, and 125 μl of 3mM of 5,5'-dinitrobis-2-nitrobezoic acid in 50 μl of 50 mMTris/HCl buffer pH8 were added in 96 well plates. Each sample was dissolve with methanol to 10 mg/ml for stock solution, diluted with buffer to 1 mg/ml. 1,8-Cineol and essential oil from *M. citrina* were prepared in various concentration (1.0–0.25 mg/ml) for investigated IC_{50}. The absorbance was measured in kinetics mode (OD/min), every 13 s for five cycles, by Anthos Zenyth, model 200 RT at 405 nm. Then, 25 μl of 0.20 U/ml AChE was added in the reaction. The hydrolysis of acetylcholine iodide was monitor by the formation of the 5-thio-2-nitrobenzoate which was measured again every 13 s for eight cycles. The reactions were performed in triplicate. 1,8-Cineole was used as positive control. The inhibitory activity was calculated from this formula.

$$\% \text{ Inhibition} = [1 - \frac{\text{OD Sample}}{\text{OD Control}}] \times 100$$

RESULTS AND DISCUSSION

The essential oil was obtained by hydrodistillation of the fresh leave of plant. The yield of essential oils was shown in Table 1.

Table 1: The inhibition activity on AChE of the essential oils *E. globulus*, *M. cacajuputi, M. citrina, P. guajava, S. cumini, S. samarangense*, and 1,8-cineol, final concentration at 0.10 mg/ml (n=3)

Sample	% yield v/w	% Inhibition±SD at 0.10 mg/ml	IC_{50} (mg/ml)
Eucalyptus globulus	0.40	47.65±2.26	NE
Melaleuca cajuputi	0.31	21.18±0.54	NE
Melaleuca citrina	0.18	71.77±2.11	0.037
Psidium guajava	0.19	24.96±2.38	NE
Syzygium cumini	0.24	19.97±1.10	NE
Syzygium samarangense	0.08	13.78±1.52	NE
1,8-cineol	-	66.18±0.53	0.052

NE: Non evaluated. *E. globulus: Eucalyptus globulus, M. cajuputi: Melaleuca cajuputi, M. citrine: Melaleuca citrine, P. guajava: Psidium guajava, S. cumini: Syzygium cumini, S. samarangense: Syzygium samarangense.* AChE: Acetylcholinesterase

E. globulus provided the highest yield of essential oil at 0.40% v/w, followed by essential oil from *M. cajuputi* (0.31%), *S. cumini* (0.24%), *P. guajava* (0.19%), and *M. citrina* (0.18%). The lowest yield was found in *S. samarangense* (0.08%). The chemical composition of essential oil was analyzed by GC-MS. The essential oil composed various constituents [Table 2] and the GC chromatograms of essential oil were shown in Figure 1. The essential oil was consisted of a mixture of a monoterpene, sesquiterpene, and phenolic compounds. They were in a various functional groups such as acid, alcohol, aldehydes, ester, ketones, or lactones. The major compounds (>10%) of each essential oil were found as the following: *E. globulus* were γ-terpinene and *p*-cymene; *M. cajuputi* were terpinolene, γ-terpinene and *E*-caryophyllene; *M. citrina* were 1,8-cineole, and α-pinene; *P. guajava* was limonene and α-pinene; *S. cumini* were terpinolene, γ-terpinene and *E*-caryophyllene; and *S. samarangense* was *o*-cymene. All major components were monoterpene; only *E*-caryophyllene was bicyclic sesquiterpene. Alpha-pinene was found all 6 species and had been report AChEI, but it was less potency than

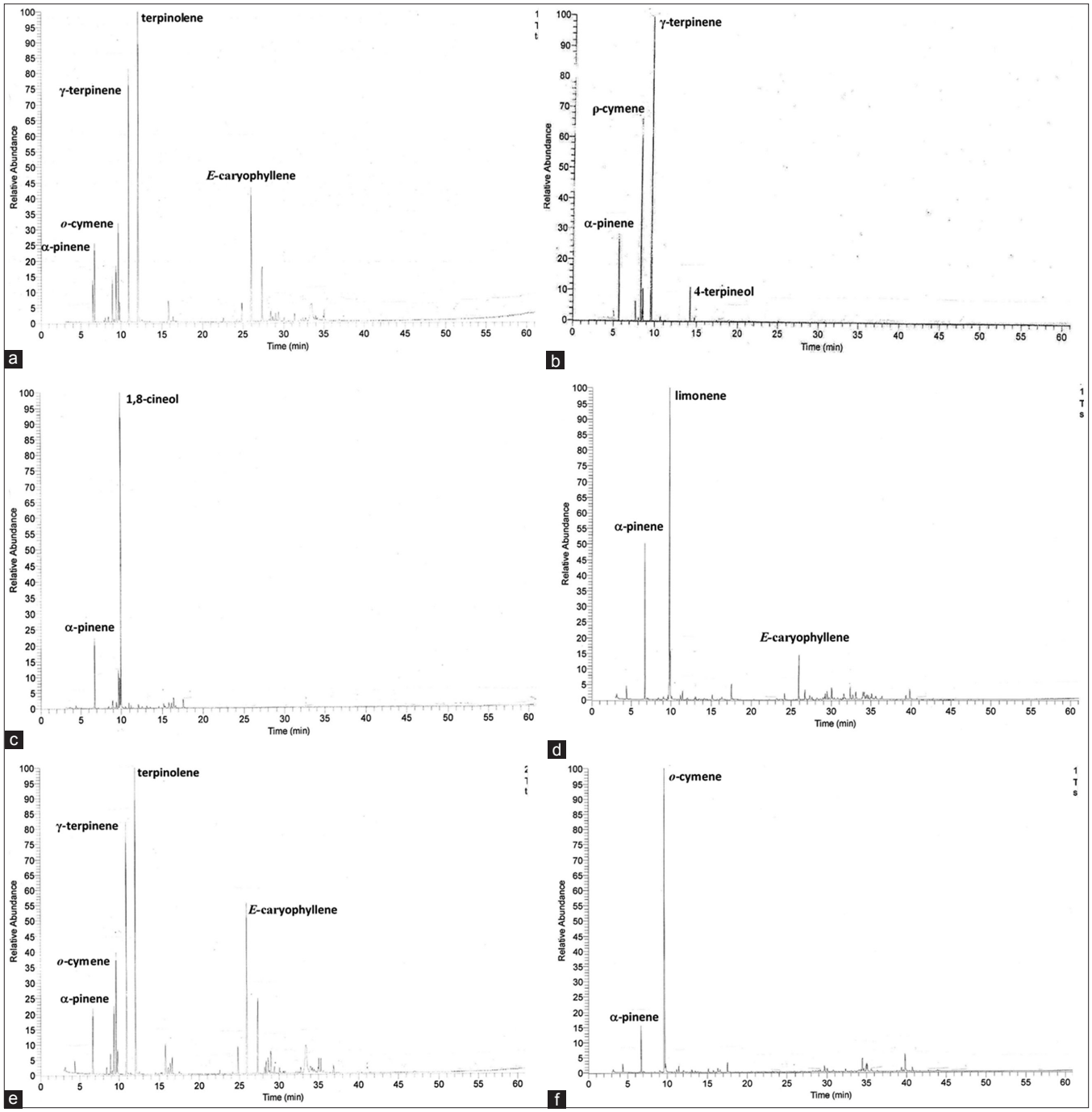

Figure 1: Gas chromatography of essential oil from (a) *Eucalyptus globulus*, (b) *Melaleuca cajuputi*, (c) *Melaleuca citrina*, (d) *Psidium guajava*, (e) *Syzygium cumini,* and (f) *Syzygium samarangense*

Table 2: The chemical constituent of the essential oils from Myrtaceae plant by GC-MS.

Compound	Area percentage (%)					
	E. globulus	***M. cajuputi***	***M. citrina***	***P. guajava***	***S. cumini***	***S. samarangense***
α-Amorphene		1.16			0.85	
Aromadendrene				1.88		
β-Bisabolene				1.46		
α-Bisabolene				1.02		
Bornyl Chloride			1.23	0.77		1.11
γ-Cadinene						1.64
epi-α-Cadinol						4.30
Camphene			0.11			
1,8-Cineole	4.48		58.06	7.37		
E-Caryophyllene		12.22		7.22	12.25	
Trans-Calamenene				2.70		1.03
o-Cymene		7.34	7.56	0.60	7.34	54.33
p-Cymene	28.75					
β-Elemene		1.65			2.02	
Globulol				1.46		
α-Humulene		4.92		0.77	5.58	
Limonene		1.85	6.23	36.55	1.69	2.79
epi-α-Muurolol						2.07
E-Nerolidol				1.48		
α-Phellandrene	2.78	2.66	1.68		1.14	
α- Pinene	8.57	4.15	10.98	14.70	3.41	7.51
β- Pinene	0.09	8.03	0.66			
Piperitone	0.31					
Platyphyllos		7.50			9.69	
D-sylvestrene	2.00					
α-Terpinene	0.41	3.48	1.14		3.82	
γ-Terpinene	44.60	17.81			16.63	
4-Terpineol	5.42	2.02	1.45		2.47	0.57
α-Terpineol	0.55	0.75	2.69	0.65	1.19	
α-Terpineol-acetate			2.03		0.34	
Terpinolene	0.82	21.61			19.08	0.49
α-Thujene		2.46				
Thymol methyl ether						1.19

E. globulus: Eucalyptus globulus, M. cajuputi: Melaleuca cajuputi, M. citrine: Melaleuca citrine, P. guajava: Psidium guajava, S. cumini: Syzygium cumini, S. samarangense: Syzygium samarangense, GC-MS: Gas chromatography–mass spectrometry

1,8-cineole.[8,10] *Eucalyptus* spp. and *Melaleuca* spp. are enrich in 1,8-cineole[10]. This resulted, 1,8-cineole was found only from *M. citrina*, *E. globulus,* and *P. guajava*. *E*-caryophyllene had been report as a main component of Thai *P. guajava*.[20] This contrasting pattern of the result, *E*-caryophyllene was found a small amount in the essential oil of *P. guajava*.

The essential oils from six species were tested on various concentration in AChEI assay which was limited the final concentration at 0.1 mg/ml. The essential oil was not possible to test in the higher concentrations than 0.1 mg/ml cause the appearance of turbidity in the test solution. All essential oils from this studying inhibited AChE [Table 1]. At 0.1 mg/ml, the essential oil of *M. citrina* showed a high potency on AChEI activity (71.77 ± 2.11%), followed with *E. globulus* (47.65 ± 2.26%), *P. guajava* (24.96 ± 2.38%), *M. cajuputi* (21.18 ± 0.54%), *S. cumini* (19.97 ± 1.10%), and *S. samarangense* (13.78 ± 1.52%). They compared with 1,8-cineole [Figure 2]. The essential oil from *Syzygium* spp. was less activity to inhibit AChE. Furthermore, the essential oil of *M. citrina* and 1,8-cineole was investigated IC_{50} values and showed 0.037 mg/ml and 0.052 mg/ml, respectively [Table 1]. Phrompittiyarat *et al*. (2014) investigated AChEI

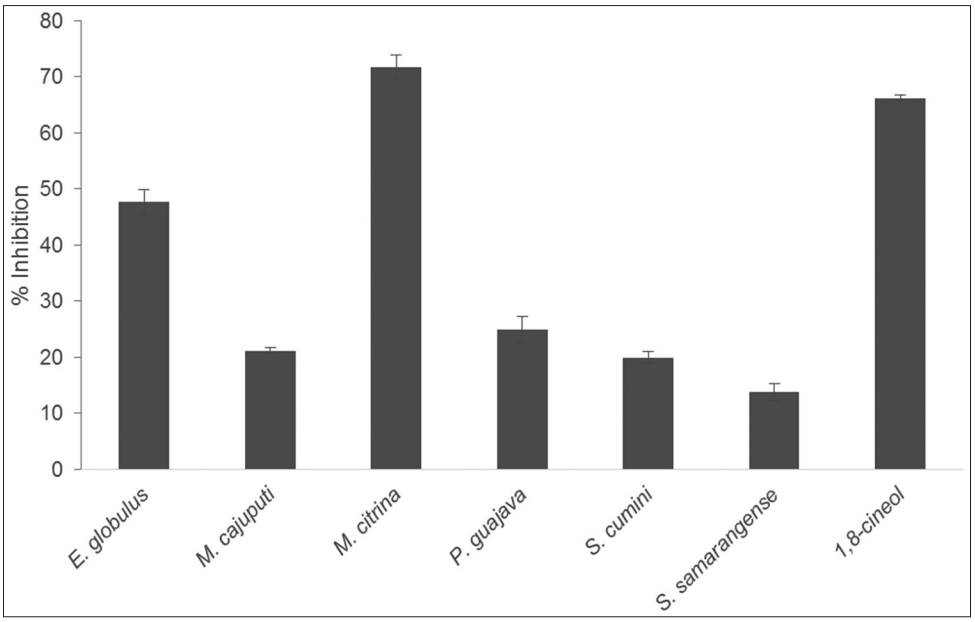

Figure 2: The inhibition acetylcholinesterase activity of essential oils from *Eucalyptus globulus*, *Melaleuca cacajuputi*, *Melaleuca citrina, Psidium guajava*, *Syzygium cumini*, *Syzygium samarangense,* and 1, 8 cineole at 0.10 mg/ml (n=3)

activity from the commercial essential oil. Eucalyptus oil, cajuput oil, and sweet majoram oil showed the potency on AChEI at 68.49%, 68.68%, and 63.51%, respectively, and they performed a high content of 1,8-cineole at 83.30%, 70.16%, and 63.51%, respectively, while the current study, eucalyptus oil and cajuput oil from distillation presented an AChEI activity at 47.65±2.26% and 21.18%. The essential oil from *E. globulus* contained 1,8-cineole only 4.48% and *M. cajuputi* do not show any 1,8-cineole.[12] This has been confirm that the essential oil containing higher amount of 1,8-cineole showed potency on AChEI. The essential oil from *M. citrina* had 1,8-cineole and α-pinene as a main constituent and presented higher inhibition activity on AChE. The essential oils of *M. cajuputi*, *S. samarangense,* and *S. cumini* showed weak activity in AChEI, due to absent of 1,8-cineole. Then they presented less potency to inhibit on AChE. The essential oil from *E. globulus* had γ-terpinene and *p*-cymene as the main constituent but composed small amounts of 1,8-cineole. Even though the essential oil from *E. globulus* contained 1,8-cineole (4.48%), small amount than the essential oil from *P. guajava* (7.37%) but it presented moderate AChEI activity (47.65%). Gamma-terpinene had been report AChEI activity[11] and showed a competitive inhibitor on AChE,[16] but *p*-cymene did not present any inhibition on AChE[11]. Gamma-terpinene, α-pinene, and 1,8-cineole responded for AChEI in the essential oil of *E. globulus*. The essential oil from *P. guajava* had limonene and α-pinene and contained a small amount of 1,8-cineole (7.37%). Miyazawa *et al*. (1997) studied AChEI activity on monoterpene. At 1.2 mM, (−)-limonene and (+)-limonene inhibited AChE at 25% and 22%, respectively, they showed less potency on AChEI[19]. The AChEI activity of the essential oil from *P. guajava* should be from α-pinene, 1,8-cineole, and limonene. *E*-caryophyllene, *o*-cymene, and terpinolene had not been report any AChEI activity; then they should be further study on AChEI activity. The essential oil contained 1,8-cineole which present a potency on the inhibition of AChE. Savelev *et al*. had been investigate the AChEI activity by combinations of terpenoids. The synergy was apparent in 1,8-cineole/α-pinene and 1,8-cineole/caryophyllene oxide. Antagonism was found in 1,8-cineole/camphor. The AChEI activity of essential oil resulted from the complex interaction between its constituents cause synergistic and antagonistic activity.[8] The components on essential oil are important on the biological activity of the plant. Not only major compound but also minor chemical constituent in essential oil influence on their activity. They could presented a synergist or an antagonist to AChEI activity. In addition, the synergism or antagonism of the substance from essential oil should be further investigated.

CONCLUSION

The essential oil from Myrtaceae contained higher constituent of 1,8-cineole that presents potency on AChEI. Moreover, there are also other constituents in the essential oils that may affect the AChEI activity by a synergistic and antagonistic activity.

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Unveiling the cellular and molecular mode of action of *Melaleuca cajuputi* Powell. essential oil against aflatoxigenic strains of *Aspergillus flavus* isolated from stored maize samples

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ABSTRACT

This study aimed to reveal the bio-efficacy of *Melaleuca cajuputi* essential oil (McEO) against aflatoxigenic fungi and lipid peroxidation causing deterioration of stored maize samples. Three different toxigenic strains of *Aspergillus flavus*, namely AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8 were investigated. Gas chromatography-mass spectrometry (GC-MS) analysis of EO revealed the presence of α-pinene (49.24%) as major compound. Investigation on efficacy showed that McEO exhibited remarkable inhibitory activity against growth and AFB_1 production by AF-LHP-M2 (2.0 and 1.4 μL mL^{-1}, respectively), AF-LHP-SP2 (1.2 and 1.0 μL mL^{-1}, respectively), and AF-LHP-VS8 (0.8 μL mL^{-1}) ($p < 0.05$). The McEO inhibited fungal growth via inhibition of ergosterol biosynthesis, cellular constituents' leakage, and damage of mitochondrial membrane potential, while AFB_1 production by inhibition of intracellular methylglyoxal. Further, molecular docking study was carried out to unveil the binding affinities of major compounds with the target protein Nor-1 (primarily catalyze an important step in AFB_1 biosynthesis), and the results revealed good correlation with the experimental findings. In addition, McEO showed significantly ($p < 0.05$) higher $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity with IC_{50} values 3.16 and 4.29 μL mL^{-1}, respectively. Interestingly, McEO inhibited AFB_1 production, and malondialdehyde content in fumigated maize samples without significantly ($p < 0.05$) changing their sensory attributes, ascertaining its efficacy in food system with high safety profile ($LD_{50} = 1800$ mg kg^{-1} body weight) on mice model. The overall results proved McEO's potential as natural food preservative of stored food products.

1. Introduction

The majority of the maize (*Zea mays* L.) produced worldwide are susceptible to qualitative deterioration caused by fungal attack, mycotoxins production, and lipid peroxidation during storage, which do not only lessen their shelf-life but also lead to unpleasant taste (Chaudhari et al., 2021). Amongst different food borne contaminants, *Aspergillus flavus*, which is associated with the production of aflatoxin B_1 (AFB_1) is of alarming concern, owing to its hepatotoxic, mutagenic, teratogenic, immunosuppressive, neurotoxic, nephrotoxic, and estrogenic properties (Manso et al., 2014; Yogendrarajah et al., 2016). In addition, AFB_1 has been reported to induce toxicity via epigenetic modifications, including DNA methylation, histone protein modification, and non-coding RNAs regulations (Dai et al., 2017). Because of its extreme toxicities, many countries have set very strict guidelines to restrict or keep AFB_1 and total AFs concentration very low in the stored food commodities.

Generally, the elimination and degradation of this ubiquitous contaminant is achieved by application of chemical preservatives; however, in the past few years, their excessive use has begun to be questioned because many of them have deleterious effects, including residual toxicity to non-target organisms (aquatic and terrestrial organisms), emergence of resistant fungal strains, and ecotoxicity due to non-biodegradable nature, hence there is a growing interest for some novel alternatives (Ali et al., 2017; Chaudhari et al., 2019). In recent years, essential oils (EOs) have been explored as promising substitute of the chemical preservatives, owing to their biodegradable, non-residual, and non-toxic nature with strong antimicrobial, antimycotoxigenic, and antioxidant properties as well as high consumers safety (not directly

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edible, but shows high median lethal dose in mammalian system) (Chaudhari et al., 2021; Majeed et al., 2015). These EOs have an added effect of maintaining the overall quality (including organoleptic, nutritional, and functional) of the treated food items, since they are highly volatile and do not persist in the foods after application (Pérez-Alfonso et al., 2012).

Melaleuca cajuputi Powell. (Family: Myrtaceae), commonly called as 'cajeput' is a perennial aromatic tree, well known for the production of EO, which hold quite a good potential for the treatment of influenza, cough, abdominal flatulence, internal disorder, intestinal problems, and insect bite effects (Noor et al., 2020; Septiana, Yuliana, Bachtiar, Putri, et al., 2020). In addition, the EO has been reported to exhibit promising antibacterial, antifungal, and antioxidant activities (Siddique et al., 2020). United States Food and Drug Administration (US-FDA) has also approved it for use in foods (Septiana, Yuliana, Bachtiar, & Wijaya, 2020). However, to the best of our knowledge, there are no comprehensive studies in the literature showing the food preservative potential of this EO against qualitative losses caused by aflatoxigenic fungi and lipid peroxidation.

Thus, the main objective of this research was to analyze the chemical compositions of *M. cajuputi* EO (McEO) and to investigate its *in vitro* as well as *in situ* antifungal efficacies against three different toxigenic strains of *A. flavus* (AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8) isolated from different varieties of stored maize, with special emphasis on cellular, biochemical, and molecular (binding interactions of the EO's major components with the putative target protein) levels. The outcome of this study may provide significant inferences for the development and commercialization of new antifungal food preservatives based on plant EOs.

2. Materials and methods

2.1. Materials

The major chemicals viz., dimethyl sulfoxide (DMSO), methylglyoxal (MG), 1,2-diaminobenzene, perchloric acid, Tween 20, Tween 80, silica gel-G, ethanol, methanol, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and ascorbic acid were supplied by Hi-Media laboratories, Mumbai, India. The culture media viz., potato, dextrose, and agar (PDA), and sucrose, $MgSO_4{\cdot}7H_2O$, KNO_3, and yeast extract (SMKY) were purchased from Sisco Research Laboratories (SRL), Mumbai, India.

2.2. Fungal strains

For this study, three different toxigenic strains of the *A. flavus*, namely AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8, respectively isolated from Maukyrwat, Sohru Pnah II, and Varun suma variety of maize were selected as test fungi. The cultures were maintained in Tween 80 (0.1%) in the form of spore suspension (density = 10^3 spores mL^{-1}) at 4 °C.

2.3. Extraction and characterization of McEO

The EO was extracted from the mature leaves of *M. cajuputi* via hydro-distillation using a Clevenger's apparatus (Council of Europe, 1997). The voucher specimen (Melaleuca. 2021/1) was deposited in the herbarium of the Botany Department (Banaras Hindu University, Varanasi). For extraction, the mature leaves (500 g) of *M. cajuputi* were suspended in distilled water and subjected to a 5-L round bottom flask connected to the Clevenger's hydro-distillation apparatus. Temperature, time, and power were controlled by the operating system (power regulator and thermocouple) of the hydro-distillation apparatus. Next, the samples were heated to a fixed temperature of 75 °C for 3 h. The obtained oil was dried over anhydrous sodium sulfate, and stored in a dark amber glass vial at 4 °C until analysis.

Quantification of main components in the McEO was performed by means of gas chromatography-mass spectrometer (GC-MS) coupled with flame-ionization detector (FID). GC was performed on a TRACE 1300 series GC (Agilent Technologies) equipped with a TG-5 column (30 m length × 0.25 mm diameter × 0.25 μm thickness). During analysis, initial oven temperature was maintained at 60 °C for 2 min and then increased to 250 °C with a heating rate of 5 °C/min. Injector and detector temperatures were 220 °C and 250 °C, respectively. The EO was diluted with hexane (10 μL in 1 mL of hexane). Injection mode was pulsed with a split ratio equivalent to 1/50 with an injection volume 2 μL. Helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. The column effluents were directly transferred into MS recorded with ionization energy of 70 eV. The components were identified by comparing their retention indices (RI) and mass fragmentation pattern with those available in the literature (Adams, 2007). The RI values of different components were calculated by using the retention times (RT) of a homologous series of n-alkanes (C_9–C_{33}) running in parallel with McEO under similar conditions.

2.4. Effect of McEO on growth and AFB_1 secretion by toxigenic strains of *A. flavus*

The effect of McEO on growth of toxigenic strains of *A. flavus* was recorded in term of minimum inhibitory concentration (MIC) according to the well known poisoned food technique (Singh et al., 2008). MIC was considered as the lowest concentration of EO that inhibited the visible growth of fungus on the PDA plate. The MIC was determined by the *in vitro* experiment measuring the growth of toxigenic strains of *A. flavus* in treatment sets against control. For this, briefly, requisite amounts of McEO (dissolved separately in 5% Tween 20) were separately added to the Petri plates containing 9.5 mL PDA medium to reach the final concentrations of 0.2–2 μL mL^{-1} for AF-LHP-M2, 0.2–1.2 μL mL^{-1} for AF-LHP-SP2, and 0.2–0.8 μL mL^{-1} for AF-LHP-VS8. Each plate was inoculated with 10 μL spore suspension of respective *A. flavus* strains (density = 10^3 spores mL^{-1}) along with controls (containing PDA without EO), sealed with parafilm to prevent EO volatilization, followed by incubation at 27 ± 2 °C. Parafilm sealing completely prevented hypoxia, hence, did not compromise the growth of *A. flavus* (Briard et al., 2016). After 7 days, the antifungal activity was evaluated by measuring the growth of fungus in treatments against control and inhibition rate (IR) was calculated using the following formula:

$$IR\ (\%) = (C_{FG} - T_{FG}/\ C_{FG}) \times 100$$

where,

C_{FG} = Fungal growth in control sets (cm)
T_{FG} = Fungal growth in treatment sets (cm)

The inhibitory effect of McEO on AFB_1 production was assessed in SMKY medium using our previously reported method (Chaudhari, Singh, Dwivedy, et al., 2020). Briefly, requisite amounts of McEO were separately added to the conical flasks containing SMKY medium to obtain the above-mentioned concentrations against respective *A. flavus* strains. Controls were prepared using SMKY without test EO. Then, each flask was inoculated with 25 μL spore suspension of test fungus (density = 10^3 spores mL^{-1}) and incubated in biological oxygen demand (BOD) incubator at 27 ± 2 °C for 10 days. Following incubation, the mycelia developed in the medium were filtered and dry weights were determined. During incubation, the conical flasks were sealed with cotton plugs, permitting the air to diffuse, resulting in normal growth of the test fungus. AFB_1 in the medium was extracted with 20 mL chloroform in a separating funnel and allowed to evaporate on water bath (70 °C). The residues left were re-suspended in 1 mL of methanol and 50 μL of each sample was spotted onto the silica gel-G thin layer chromatography (TLC) plates and developed in the mobile phase consisting of toluene:

isoamyl-alcohol: methanol (90:32:2 v/v/v). The amount of AFB_1 in the sample was calculated from the following equation:

$$AFB_1 \text{ amount } (\mu g\ mL^{-1}) = (\text{Absorbance of sample} \times \text{Molecular mass of } AFB_1) / (\text{Molar extinction coefficient} \times \text{Path length}) \times 1000$$

2.5. Antifungal mechanism of McEO

2.5.1. Effect on fungal plasma membrane integrity

The integrity of fungal plasma membrane was examined by determining the inhibition of ergosterol (Chaudhari, Singh, Singh, et al., 2020). Briefly, 25 μL spore suspension of each fungus (density = 10^3 spores mL^{-1}) was separately inoculated into the conical flasks containing SMKY medium amended with 0.2–2 μL mL^{-1} concentrations of McEO for AF-LHP-M2, 0.2–1.2 μL mL^{-1} for AF-LHP-SP2, and 0.2–0.8 μL mL^{-1} for AF-LHP-VS8. After 4-days of incubation at 27 ± 2 °C, the mycelia were extracted, mixed with 5 mL of 25% alcoholic KOH, vortexed, and incubated on water bath at 85 °C. The ergosterol was extracted from the sample by adding 2 mL of sterile distilled water and 5 mL of *n*-heptane. Following 2 min of vortexing, *n*-heptane layer was collected and analyzed by scanning between 230 and 300 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan).

2.5.2. Effect on permeability of plasma membrane

The permeability of fungal plasma membrane was analyzed by measuring the efflux of cellular cations (Ca^{2+}, Mg^{2+} and K^+) and release of 260 (nucleic acids) and 280 nm (proteins) absorbing materials into the suspensions of McEO treated *A. flavus* cells as described previously by Das et al. (2019) with slight modifications. For ion leakage, 5-days old mycelium mass of each *A. flavus* strain was harvested, washed with distilled water, and suspended in 0.85% NaCl solution containing different concentrations of McEO (1/2MIC, MIC, and 2MIC). The fungal cultures without McEO were served as controls. The suspensions were then incubated at 27 ± 2 °C for overnight. The samples were centrifuged (13,000×*g* for 10 min) and respective ions were measured using Atomic Absorption Spectrophotometer (AAnalyst 800, PerkinElmer, USA).

For 260 and 280 nm absorbing materials, 5-days old cultured mycelial biomass of each test fungus was harvested by centrifugation (5000×*g*) for 10 min, washed thrice with phosphate buffer saline (PBS), and re-suspended in the same solution bearing 1/2MIC, MIC, and 2MIC concentration of McEO. After overnight incubation at 27 ± 2 °C, the mycelia were centrifuged and supernatants were subjected to UV–visible spectrophotometry (Hitachi-2900, Shimadzu, Japan) at 260 and 280 nm, respectively.

2.5.3. Effect on fungal mitochondrial membrane potential (MMp)

The effect of McEO on MMp was measured using fluorescent dye rhodamine (Rho123) according to Tian et al. (2012) with slight modifications. First, spore suspension of each fungus was adjusted to 10^6 cells mL^{-1} in PBS and then treated with different concentrations of McEO (1/2MIC, MIC, and 2MIC) for overnight. Samples without McEO were served as controls. Each sample was then centrifuged (5000×*g*), washed, dissolved in PBS, and stained with Rho123 at a final concentration of 1 μg mL^{-1}. After dark incubation for 30 min, the cells were centrifuged and fluorescence intensity of supernatants were measured at the excitation and emission wavelengths of 488 and 525 nm, respectively, using fluorescence spectrophotometer.

2.6. Antiaflatoxigenic mechanism of McEO

2.6.1. Effect on cellular methylglyoxal (MG) content

The antiaflatoxigenic mode of action of McEO was unravelled by determining the level of intracellular MG, which is one of the main precursors for up-regulating the biosynthesis of AFB_1 in the culture) according to our previously reported method (Upadhyay et al., 2018). Initially, an aliquot containing 25 μL spore suspension of AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8 (density = 10^3 spores mL^{-1}) were inoculated into the conical flasks containing SMKY medium and different concentrations of McEO (0.2–2 μL mL^{-1}, 0.2–1.2 μL mL^{-1}, 0.2–0.8 μL mL^{-1}, respectively). The control sets were prepared without EO. After 7-days of incubation at 27 ± 2 °C, the tissue was extracted by addition of 3 mL of 0.5 M perchloric acid and incubated on ice bath for 15 min followed by centrifugation at 13000×*g* for 10 min. The supernatant was neutralized by drop-wise addition of saturated K_2CO_3 and centrifuged at 13000×*g* for 10 min. The supernatants were used for the estimation of MG in a total volume of 1 mL reaction mixture containing 250 μL 7.2 mM 1,2-diaminobenzene, 100 μL 5 M perchloric acid and 650 μL of resulting supernatant. Thereafter, absorbance of the sample was recorded at 341 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan).

2.6.2. Homology modeling of Nor-1 gene product and its interaction with the major components of EO: Molecular docking

The molecular docking study was performed to interpret the molecular interaction of major bioactive components of McEO (viz., α-pinene, bornyl acetate, and camphor) with Nor-1 gene product. The docking procedure began with the preparation of the amino acid sequences of Nor-1 protein, which were obtained from UniProtKB database (http://www.uniprot.org) and submitted to the SWISS-MODEL (https://swissmodel.expasy.org). The quality of modeled protein was assessed through QMEAN Z-Scores and molprobity scores obtained from the results. The ligand structure of test compounds was downloaded directly from PubChem.

The structures were then prepared for molecular docking between the test compounds and Nor-1 protein using Molegro Virtual Docker 6.0.1. Before executing the docking process, protein and ligand structures were refined using protein preparation wizard and ligand preparation wizard, respectively. Ligand binding sites in protein was detected using auto detection mode and grid resolution was set to at 0.30 Å and number of runs at 30. Rest parameters were set to the default. The highest affinity-bound-ligands were predicted using MolDock Score.

2.7. Antioxidant activity of McEO: In vitro

2.7.1. DPPH radical scavenging assay

The $DPPH^{\bullet}$ scavenging activity of McEO was performed following the procedure performed previously (Tomi et al., 2011) with minor modifications. Briefly, 0.004% solution of DPPH in methanol was prepared and different concentrations (2–12 μL mL^{-1}) of McEO were added to 2 mL DPPH solution. After 30 min of reaction under dark (color changed from purple to straw), absorbance of the sample was measured at 517 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan). BHT, BHA, and ascorbic acid were used as positive control. The free radical scavenging (FRS) activity was calculated according to the following equation:

FRS activity (%) = [A $_{(blank)}$ − A $_{(sample)}$ / A $_{(blank)}$] × 100

where,

A $_{(blank)}$ = Absorbance of DPPH solution
A $_{(sample)}$ = Absorbance of DPPH solution containing McEO

2.7.2. ABTS radical scavenging assay

The FRS activity of the McEO against $ABTS^{\bullet+}$ was determined according to the standard protocol suggested by Re et al. (1999) with some modifications. Initially, $ABTS^{\bullet+}$ was produced by reacting 7 mM of ABTS solution with 2.45 mM potassium persulphate, and the mixture was placed in the dark at room temperature for 16 h. The solution was diluted with absolute ethanol to obtain the absorbance of 0.70 ± 0.02 at 734 nm. Then, different concentrations (2–12 μL mL^{-1}) of McEO were added to 2 mL of $ABTS^{\bullet+}$ solution and mixed thoroughly. BHT, BHA, and ascorbic acid were used as reference control. After 6 min of reaction, the absorbance of the sample was recorded at 734 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan). The FRS activity was calculated similar to DPPH.

2.7.3. Determination of total phenolic content

The total phenolic content of McEO was assessed using Folin-Ciocalteu's (FC) reagent (Dwivedy et al., 2017). In brief, 100 μL McEO (mixed in DMSO) was added to a conical flask containing 23 mL of distilled water. To this sample, 0.5 mL FC reagent was added and incubated for 3 min. After thorough mixing, 2 mL of 2% Na_2CO_3 was added and allowed to stand for 1 h under dark condition. The absorbance of the sample was read at 760 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan). The contents were presented as μg gallic acid equivalent per gram of EO (μg GAE g^{-1} EO).

2.8. Application of McEO in food system: In situ study

2.8.1. Experimental design

Herein, three different varieties of maize (Maukyrwat, Sohru pnah II, and Varun suma) were selected as model food commodity to demonstrate the *in situ* efficacy of McEO. These varieties were preferred because *A. flavus* strains namely AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8 were isolated from these respective varieties. Samples were obtained from local market of Varanasi and Meghalaya, and then immediately transferred to the closed plastic containers (volume 0.5 L). It is crucial to understand the impact of interacting environmental factors such as water activity (a_w), temperature, pH, and moisture contents on growth and aflatoxins production by *A. flavus* in maize in order to predict the possible risk of AFB_1 contamination during storage (Garcia et al., 2012). However, among them, a_w is regarded as the main controlling factor, that influence both the rate of fungal growth and aflatoxin production (Magan & Aldred, 2007). The calculated a_w of the Maukyrwat, Sohru pnah II, and Varun suma were found to be 0.96, 0.97, and 0.90, respectively. Maize samples were divided into three different groups. In one group (inoculated treatment at MIC), maize samples (Maukyrwat, Sohru pnah II, and Varun suma) were fumigated with 2.0, 1.2, and 0.8 μL mL^{-1} concentration of McEO (impregnated in cotton swabs) and inoculated with 500 μL spore suspension (density = 10^3 spores mL^{-1}) of AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8, respectively. In second group (inoculated treatment at 2MIC), maize samples were fumigated with 4.0, 2.4, and 1.6 μL mL^{-1} concentration of McEO and inoculated with 500 μL spore suspension (density = 10^3 spores mL^{-1}) of respective fungal strains. In third group, the samples were kept without McEO. All samples were then stored at 25 °C and observation for the presence of AFB_1 was made after three months of storage.

2.8.2. AFB_1 extraction and analysis

The AFB_1 extraction was performance according to the method of Upadhyay et al. (2021) with slight modifications. For this, approximately 10 g of the well milled representative samples were separately added to the conical flasks and mixed with 20 mL of methanol: double distilled water (8:10, v/v) with stirring on a mechanical shaker (300×*g*) for 30 min. Thereafter, the samples were centrifuged at 5000×*g* for 5 min and supernatants obtained were mixed with 300 μL of chloroform and 6 mL double distilled water containing 3% KBr. After 10 min of centrifugation at 5000×*g*, the settled phase were collected and evaporated on hot water bath. The residues obtained were suspended with 50 μL of HPLC grade methanol and injected to the high performance liquid chromatography (HPLC) system.

The AFB_1 in the sample was quantified using a Waters 515 HPLC instrument coupled with fluorescent detector set at the excitation and emission of 365 and 435 nm, respectively. The chromatographic separation was performed on a Nova Pack C18 column (4.6 mm × 25 cm × 5 μm) under isocratic condition at a flow rate of 1.2 mL min^{-1} using a mixture of methanol, acetonitrile, and Millipore water (17:19:64, v/v/v) and delivered at a flow rate of 1 mL min^{-1}. The limits of detection (LOD) and limit of quantification (LOQ) of AFB_1 were 0.5 and 1.5 ng mL^{-1}, respectively.

2.9. Efficacy of McEO against lipid peroxidation

The extent of lipid oxidation inhibition during storage was validated by measuring the level of thiobarbituric acid reactive substances (TBARS) as previously described with modifications (Mancini et al., 2017). This method is based on the spectrophotometric quantification of the pink coloured complex formed due to the reaction of malondialdehyde (MDA) with TBA. Briefly, 1 g powdered maize samples from the above control and treatments were separately homogenized for 5 min in 10 mL distilled water containing 4 mL 0.375% (w/v) TBA, 15% (w/v) trichloroacetic acid (TCA), and 2.5 mL 0.2 N of HCl. The solution was mixed and heated on a water bath at 80 °C for 30 min. Finally, after cooling at room temperature, the samples were centrifuged at 10,000×*g* for 10 min, and the absorbance was measured at 532 against a blank (consisting of the mixture of distilled water and TBA). Results were expressed as μg of MDA per gram of sample fresh weight (μg MDA $g^{-1}FW^{-1}$).

2.10. Oral acute toxicity assessment of McEO

In this study, a population of Swiss albino male mice (*Mus musculus* L., 3 months olds) were divided into 10 groups, each composed of 10 individuals. After being marked and weighed, the mice were transferred to polypropylene cages under controlled laboratory conditions and fasted overnight prior to the testing. The oral acute toxicity study was performed in accordance with Organization for Economic Cooperation and Development (OECD) guidelines (OECD, 2002 Test no. 423) class method at serial doses of 200–2000 mg kg^{-1} body weight diluted in Tween 80 (1%, v/v). The control groups consisted of 0.5% Tween 80 and distilled water. The assay was performed by the oral administration. Animals were monitored for 24 h before proceeding to the next dose. In addition, the animals were monitored for a week for possible long term lethal outcome. Finally, the numbers of dead mice were counted and median lethal dose (LD_{50} value, concentration required to kill 50% population of mice) was calculated by the probit analysis (Finney, 1971).

2.11. Organoleptic attributes analysis

The sensory acceptance of fumigated maize samples was performed by a panel of ten untrained assessors having experience in evaluating food quality following Stojanović-Radić et al. (2018). Maize samples from the *in situ* experiment were taken and prepared by roasting in a preheated oven (2 min at 100 °C). Tests were run on a 7-point hedonic scale (7 = like extremely, 1 = dislike extremely). Sensory evaluation were done for: color, texture, aroma, taste, and overall acceptance. Samples were served at room temperature, and marked with 3-digit

arbitrary codes.

2.12. Statistical analysis

Each experiment was conducted a minimum of three times, and each analysis was carried out in triplicate. The experimental data were subjected to one way analysis of variance (ANOVA), and significant differences between means were evaluated by Tukey's B multiple-range test (SPSS 16.0.). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Extraction and characterization of McEO

The yield of EO obtained from *M. cajuputi* leaves via hydrodistillation was 4.5 mL kg^{-1} fresh weight. The EO was light yellow in color and exhibited strong aroma. The GC-MS analysis of EO revealed the identification of 10 principal components, making 85.83% composition of the oil (Table 1). The most frequent compounds were α-pinene (49.24%) followed by bornyl acetate (21.07%), and camphor (11.70). In addition, McEO also contained considerable amount of minor constituents such as 1,8-cineole (0.83%), p-cymene (0.77%), limonene (0.66%), fenchone (0.58%), camphene (0.41%), p-menthenol (0.37%), and α-terpineol (0.20%).

3.2. Effect of McEO on growth and AFB_1 secretion by toxigenic strains of *A. flavus*

The efficacy of McEO on growth and AFB_1 biosynthesis by toxigenic stains of *A. flavus* are presented in Fig. 1A–C. As can be seen in the figures, there were significant ($p < 0.05$) efficacy against fungal strains at tested concentrations comparable to the controls. The McEO caused complete inhibition of mycelial growth of AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8 with MIC value of 2.0, 1.2, and 0.8 μL mL^{-1}, respectively (Fig. 1A–C). Additionally, McEO achieved complete suppression of AFB_1 secretion by respective fungal strains at 1.4, 1.0, and 0.8 μL mL^{-1} concentrations, respectively (Fig. 1A–C).

3.3. Antifungal mechanism of McEO

The results of ergosterol inhibition in plasma membrane of toxigenic strains of *A. flavus* is shown in Fig. 1D. Results revealed a significant ($p < 0.05$) decline in ergosterol contents with the increasing concentrations of McEO. The reduction percentage were 7.06–100% for AF-LHP-M2, 25.34–100% for AF-LHP-SP2, and 24.49–100% for AF-LHP-VS8 when the concentrations of McEO increased from 0.2 to 1.6 μL mL^{-1}, 0.2–1.0 μL mL^{-1}, and 0.2–0.8 μL mL^{-1}, respectively (Fig. 1D). Further, a significant enhancement ($p < 0.05$) in the leakage of Ca^{2+}, Mg^{2+}, and K^{+} as well as 260 and 280 nm absorbing materials was observed (Fig. 2A–F). The fungal strains treated with 2MIC concentrations showed greater leakage of cellular materials than treated at MIC doses. The results of disruption of MMp in *A. flavus* cells exposed to 1/2MIC, MIC and 2MIC concentrations of McEO is presented in Fig. 3A. A dose-dependent degradation of fluorescence intensity of Rho123 dye was noted with the increasing concentrations of McEO.

Table 1
Chemical composition of *M. cajuputi* EO analyzed through GC-MS analysis.

Peak. no.	Identified compounds	Area (%)	RT	RI calculated	KI (Adams, 2007)
1	**α-pinene**	**49.24**	**8.25**	**964**	939
2	Camphene	0.41	9.78	1005	954
3	p-cymene	0.77	12.49	1068	1024
4	Limonene	0.66	12.69	1073	1029
5	1,8-cineole	0.83	12.78	1082	1031
6	Fenchone	0.58	15.56	1137	1086
7	**Camphor**	**11.70**	**18.51**	**1202**	1146
8	α-terpineol	0.20	20.99	1257	1188
9	p-menthenol	0.37	23.28	1308	1177
10	**Bornyl acetate**	**21.07**	**25.13**	**1351**	1285
	Total	85.83%			

Compounds are listed in order of their elution from a MS column.
Compounds in bolds represent the major components.
RT = Retention times; RI = Retention indices; KI = Kovats indices; Area (%) = Percentage of compounds.

3.4. Antiaflatoxigenic mechanism of McEO

The antiaflatoxigenic mode of action of test EO was determined by measuring the level of cellular MG. There were a good linear relationship between the inhibition of MG contents with the values 695.55–8.56 μM g^{-1} FW^{-1}, 550.02–29.97 μM g^{-1} FW^{-1}, and 698.80–50.02 μM g^{-1} FW^{-1} exposed to 0.2–2.0 μL mL^{-1} concentrations against AF-LHP-M2, 0.2–1.2 μL mL^{-1} against AF-LHP-SP2, and 0.2–0.8 μL mL^{-1} concentrations of McEO against AF-LHP-VS8 were observed (Fig. 3B).

To reveal the molecular mechanism behind the inhibitory role of McEO on AFB_1 biosynthesis, molecular docking between the test compounds and Nor-1 protein was performed. The 3D structure of Nor-1 protein obtained through homology modeling was found suitable for the molecular docking and revealed 88.82% of amino acid residues in the favored region. The results revealed that the test compounds strongly interacted with the amino acid residues of the target protein Nor-1. The binding affinity was calculated in terms of MolDock score, which was found to be −37.414, −58.178, and −38.628 for α-pinene, bornyl acetate, and camphor, respectively (Fig. 4).

3.5. Antioxidant activity of McEO: In vitro

The antioxidant activity of the McEO at different concentrations against DPPH and ABTS radicals are presented in Fig. 5A and B, with BHT, BHA, and ascorbic acid as positive control. McEO showed very strong FRS activity with IC_{50} value 4.29 and 3.16 μL mL^{-1} against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, respectively. These values were much higher than that of the BHT (IC_{50} = 6.6 and 5.13 μL mL^{-1}, respectively), BHA (IC_{50} = 5.12 and 3.91 μL mL^{-1}, respectively), and ascorbic acid (IC_{50} = 4.31 and 3.52 μL mL^{-1}, respectively). The total phenolic content of McEO was found to be 11.23 μg GAE g^{-1} EO.

3.6. Application of McEO in food system: In situ study

From HPLC results, it was found that the maize samples fumigated with McEO had significant differences ($p < 0.05$) in the AFB_1 contamination rate. Control sample presented the highest level of AFB_1 contamination (131.58 μg kg^{-1}). However, the samples (Maukyrwat, Sohru pnah II, and Varun suma) fumigated with McEO at MIC concentrations showed relatively lower AFB_1 content (4.021, 7.967, and 3.754 μg kg^{-1}, respectively), which was non-detectable in 2MIC fumigated maize samples.

3.7. Efficacy of McEO against lipid peroxidation

The effect of different doses of McEO (MIC and 2MIC) on TBARS values is shown in Fig. 6A. The MDA contents significantly decreased ($p < 0.05$) in all McEO fumigated maize samples as compared to controls. MDA content in control set was 917.2 μM g^{-1} FW^{-1}. After fumigation with MIC and 2MIC doses, MDA contents were decreased in all tested varieties of the maize (Maukyrwat, Sohru pnah II, and Varun suma). Highest MDA inhibition occurred in the maize treated with 2MIC doses.

3.8. Oral acute toxicity assessment of McEO

There were no significant changes in the behaviour of the treated

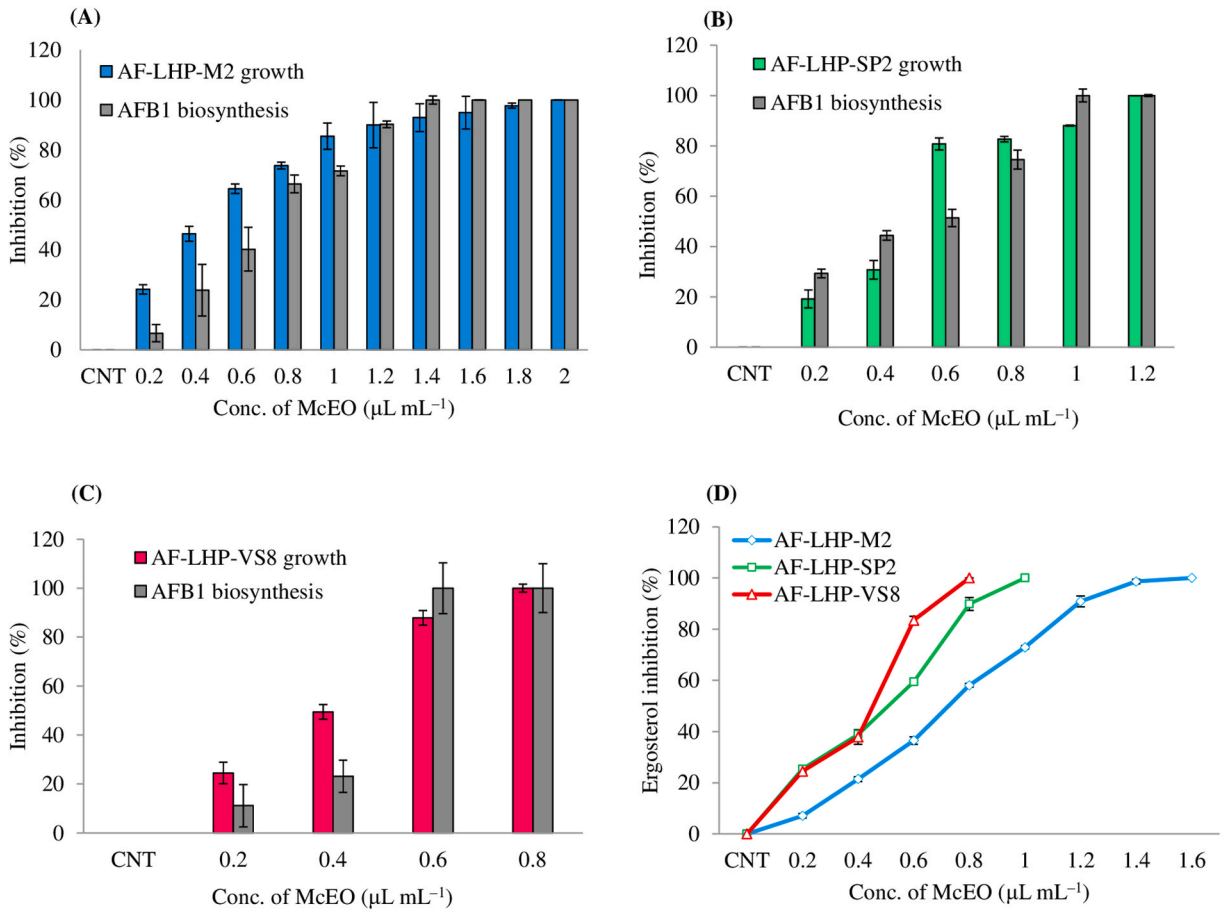

Fig. 1. Effect of varying concentrations of McEO on growth and AFB_1 production of toxigenic strains of *A. flavus*: (A) AF-LHP-M2, (B) AF-LHP-SP2, (C) AF-LHP-VS8, and (D) plasma membrane integrity (ergosterol contents). Error bars indicate the mean ± standard error.

animal groups and no evident signs of toxicity as well as mortality were noted following administration of McEO at the dose levels of 200–1600 mg kg^{-1} body weight. The symptoms of toxicity was observed at higher doses, mice having taken McEO >1600 mg kg^{-1} body weight showing symptoms such as torpor, nose and eyelid bleeding within 24 h of administration. All animals died at 2000 mg kg^{-1} body weight. The LD_{50} in mice was found to be 1800 mg kg^{-1} body weight. These results demonstrate that the mice appeared to well tolerate the McEO at doses between 200 and 1600 mg kg^{-1} body weight. The toxicity was sub-acute when the dose exceeds from 1600 to 1800 mg kg^{-1} body weight, and acute at the dose of up to 2000 mg kg^{-1} body weight, indicating its dose-dependent effects. As far as the current study is concerned, the no observed adverse effect level (NOAEL) of the McEO can be established at the dose of 1600 mg kg^{-1} per day, since at this dose, no sign of toxicity as well as mortality of any mice was observed.

3.9. Organoleptic attributes analysis

The results of the organoleptic evaluation of maize samples fumigated with MIC and 2MIC concentrations of McEO are plotted in Fig. 6B–D, where the sensory scores of different maize samples were shown. The results obtained indicated that McEO fumigated maize samples had significantly higher ($p < 0.05$) scores as compared to control samples. In contrast, maize fumigated with MIC and 2MIC concentrations of McEO showed significant improvement ($p < 0.05$) in the sensory scores for almost all the tested parameters except aroma, which achieved lower scores for all maize varieties at 2MIC.

4. Discussion

It is generally evident that the biological activity of EO is depending upon their chemical composition and the percentage of these compounds may be influenced by environmental factors (Kedia, Prakash, Mishra, & Dubey, 2014). Therefore, the identification of actual chemical composition of EO is crucial before recommending its use as food preservative and to assure consumers safety. The chemical composition of McEO recorded in the present investigation showed slight variation from the previously recorded chemical profiles of the same EO obtained from Indonesia by Septiana, Yuliana, Bachtiar, Putri, et al. (2020). The authors reported 1,8-cineole, α-terpineol, caryophyllene, α-pinene, and γ-terpinene as the major components. However, there were some differences in the per cent composition. This might have resulted from the differences in climatic condition, geographical location, plant part used, genetic variations, and methods of extraction (Chaudhari, Singh, Das, et al., 2020; Teerarak & Laosinwattana, 2019).

The present results on antifungal activity showed that McEO exhibited potent inhibitory activity against tested toxigenic strains of *A. flavus*, the main deteriorating strains found in stored maize samples. These results are in consistent with those reported by Boukaew et al. (2017), who noted the effect of capsicum, cinnamon, clove, and vatica EO treatment on growth inhibition of ten isolates of *A. flavus*. In an attempt to investigate the efficacy of EO as natural fungicides, Hu et al. (2021) also examined the inhibitory effect of *Perilla frutescens* EO on toxigenic *A. flavus* strain. The authors reported that test EO had a pronounced antifungal activity at MIC value of 0.4 µL mL^{-1}. More importantly, in the present investigation, MIC values of 2.0, 1.2, and 0.8 µL mL^{-1} were reported because complete inhibition of visual growth of

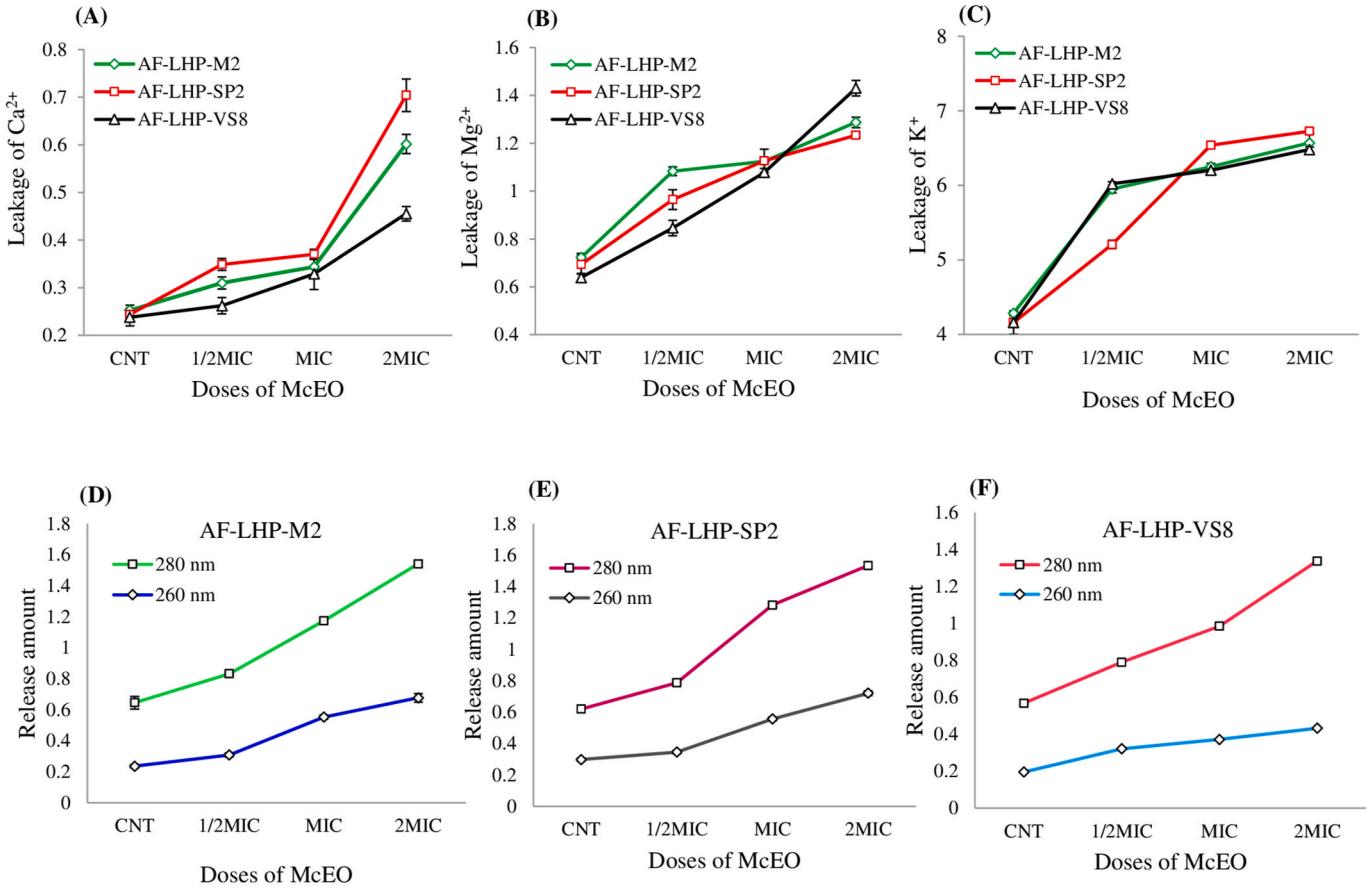

Fig. 2. Effect of varying concentrations (1/2MIC, MIC, and 2MIC) of McEO on plasma membrane permeability of toxigenic strains of *A. flavus*: (A–C) Leakage of cellular ions, and (D–F) Release of 260 (nucleic acids) and 280 nm (proteins) absorbing materials. Error bars indicate the mean ± standard error.

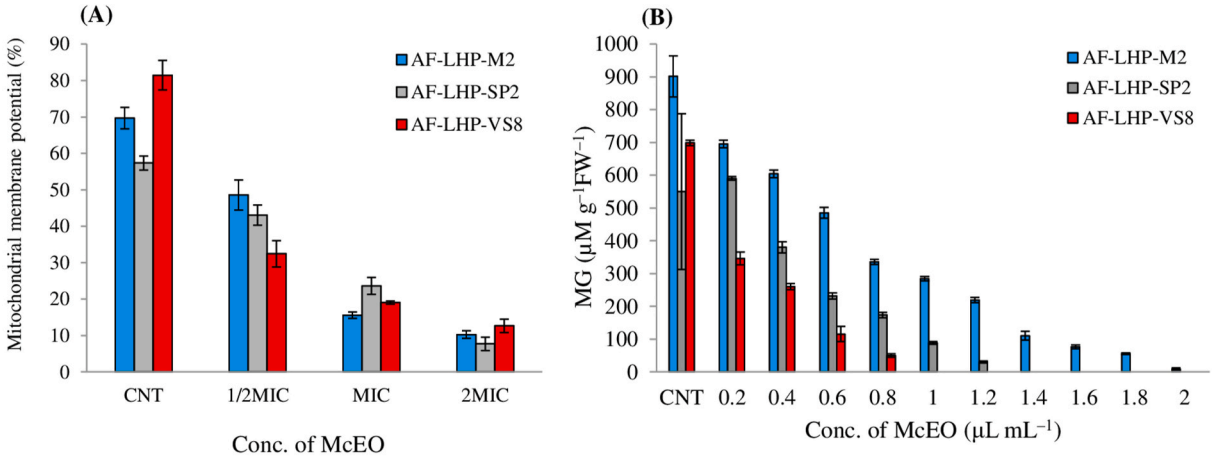

Fig. 3. Effect of different concentrations of McEO on (A) Mitochondrial membrane potential, and (B) Methylglyoxal biosynthesis of toxigenic strains of *A. flavus*. Error bars indicate the mean ± standard error.

toxigenic strains of *A. flavus* by McEO has been achieved at these concentrations. Reported inhibitory activity of McEO might be attributed to the presence of high contents of monoterpene compounds, including α-pinene, bornyl acetate, camphor etc (Deba et al., 2008; Kordali et al., 2005; Yong et al., 2021). While differences in the MICs relative to controls were attributed to the sensitivity of different fungal strains toward test EO or due to the variations in its chemical composition having different mode of action (Avanço et al., 2017). However, it has been also documented that the efficacy of EO is not restricted to a single or dominant compounds but is rather a synergism of both major and minor compounds present in the oil (Farzaneh et al., 2015). Further, the presence of monoterpenes in the McEO may lead to oxidative stress, damage the cell integrity, inhibition of the respiration process in the mitochondrial membrane, and decrease the virulence as well as growth of the fungi (Abdel-Aziz et al., 2019; Hua et al., 2014). To date, the capacity of the McEO to inhibit growth and AFB_1 biosynthesis by tested *A. flavus* strains remained unexplored. Nevertheless, the antifungal effect against other fungal and bacterial pathogens has been investigated

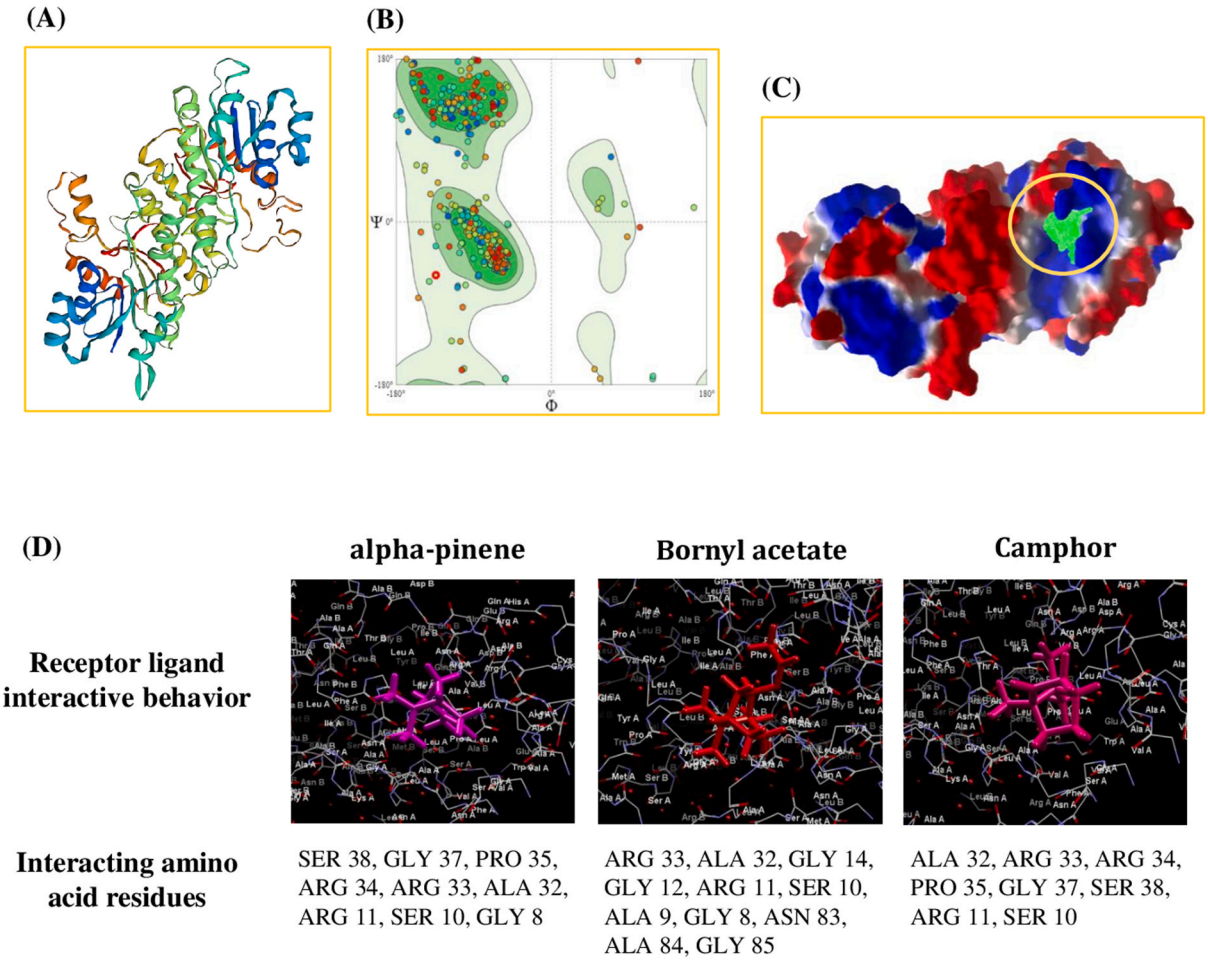

Fig. 4. Homology modeling of Nor-1 gene product and its interaction with the major components of McEO: (A) 3D model of Nor-1 protein obtained through homology modeling, (B) Ramachandran plot of the assessment of homology model, (C) Protein showing the catalytic cavity for docking, and (D) Interaction of different ligands with Nor-1 protein and interacting amino acid residues.

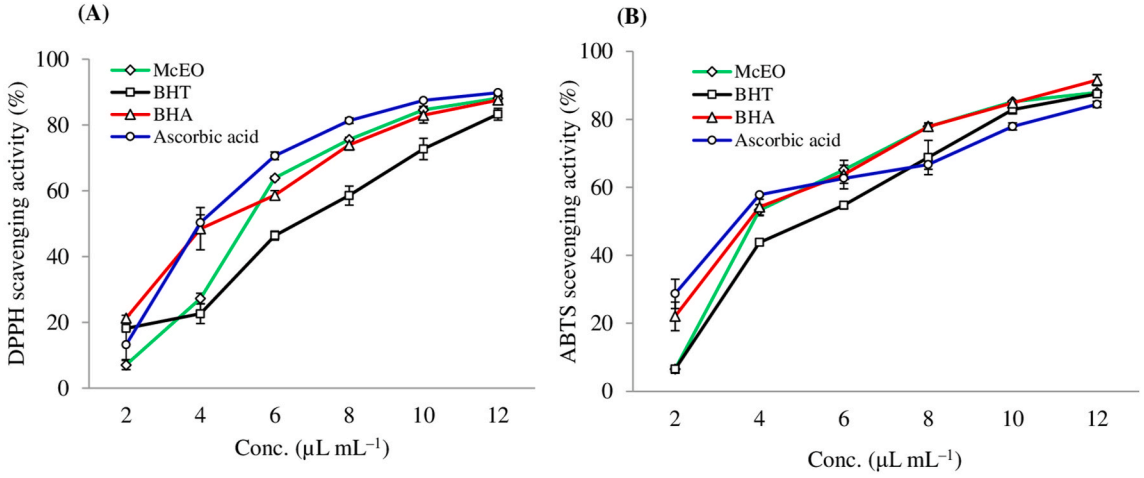

Fig. 5. Free radical scavenging activities of McEO determined through (A) DPPH, and (B) ABTS assay. Error bars indicate the mean ± standard error.

(Harkenthal et al., 1999; Keereedach et al., 2020).

The use of EOs has always been of great interest to the researchers owing to their great potential to inhibit fungal growth and mycotoxin production. Even though, numerous records are available on the efficacy of EOs to control fungal and mycotoxin production (Chaudhari et al., 2021). Kedia, Prakash, Mishra, Chanotiya, and Dubey (2014) found *Mentha spicata* EO as one of the strongest inhibitor of *A. flavus* and AFB_1 production, better than the synthetic fungicides nystatin and wettasul-80. Likewise, Kiran et al. (2016) observed complete inhibition of *A. flavus* growth and AFB_1 production after treatment with *Cinnamomum zeylanicum* EO at 0.6 and 0.3 μL mL^{-1}, respectively. Based on findings of the present study along with supportive results of others, it

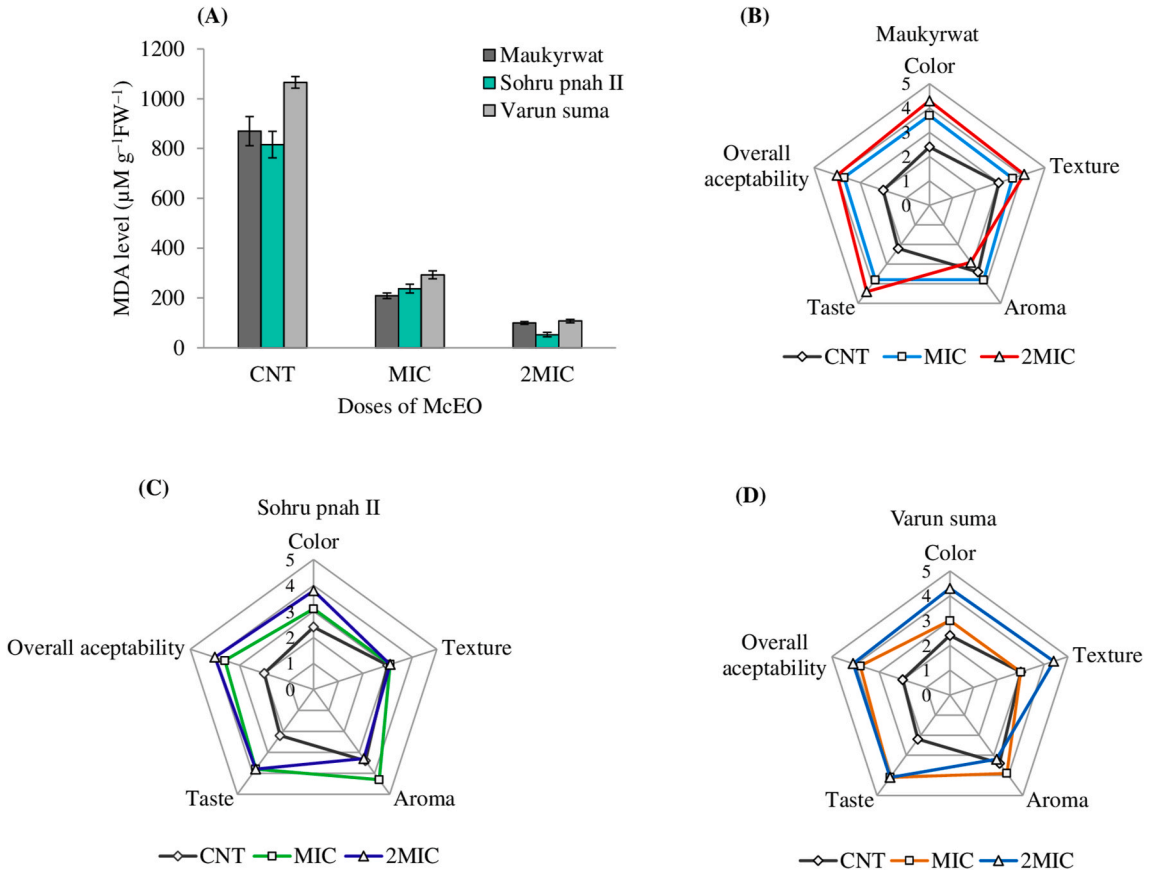

Fig. 6. Effect of varying concentrations of McEO on (A) Lipid peroxidation (TBARS value), and (B–D) Organoleptic qualities of fumigated maize samples.

may be concluded that McEO could be used for the development of effective substitute to the harmful synthetic preservatives to overcome fungal and mycotoxin contamination.

The plasma membrane provides a permeability barrier to the passage of cellular constituents, which are vital for maintaining the normal activity and metabolism of the fungal cells. Previous studies have demonstrated that plasma membrane of fungus is one of main antifungal targets of EOs. To investigate plasma membrane integrity, the ergosterol contents in McEO treated *A. flavus* strains were determined. Ergosterol is a unique and major sterol component of the fungal cell membrane, aid in regulating the structure, integrity, fluidity, and permeability of the membrane, and has also been regarded as important target site for most of the commercially available antifungal drugs such as azoles and polyenes (Siahmoshteh et al., 2018). Earlier studies suggest that even relatively minor changes in the cell membrane structure can lead to harmful effects on cell metabolism and cause cell death. A very significant inhibition of ergosterol biosynthesis was noted even at lower MIC values of McEO, indicating that this EO was effective in interdicting the plasma membrane integrity. The impairment of ergosterol biosynthesis by tested EO may be associated with the inhibition of lanosterol 14-α demethylase, a key enzyme involved in the conversion of lanosterol to ergosterol (Das et al., 2021). Similar trend for diminution of ergosterol biosynthesis in *A. flavus* by cuminaldehyde has been reported by Xu et al. (2021). The authors found 90% reduction in *Erg 25* gene expression, resulting in down-regulation of differentially expressed genes (DEGs) associated with impaired ergosterol biosynthesis. The inhibition of ergosterol biosynthesis may thus negatively affect the integrity and functions of some membrane associated proteins, resulting in permeability disorder, ultimately causing the leakage of important cellular constituents and cell death.

Cellular ions homoeostasis is an important factor for maintaining the energy status, membrane coupled soluble transport, and cellular turgor pressure (Cai et al., 2019). The release of cellular constituents could reflect changes in membrane permeability. The plasma membrane permeability was confirmed on the basis of leakage of the cellular ions and absorbance of 260 (nucleic acids) and 280 nm (proteins) absorbing materials from *A. flavus* cells exposed to McEO at 1/2MIC, MIC, and 2MIC concentrations. Our result showed that McEO caused dose-dependent enhancement of cellular ions and 260 and 280 nm absorbing materials leakage. This result is in corroboration with our previous findings, reporting enhancement of cellular cations and 260 and 280 nm absorbing leakage from *A. flavus* cells following exposure of nanoencapsulated allspice EO (Chaudhari et al., 2022). Excessive loss of these vital cellular constituents may hamper the fungal respiratory reactions, causing detrimental effect on hyphal growth and cellular metabolism. Therefore, even minor changes in permeability caused by EO fumigation could led to cytoplasmic accumulations of ions and irreversible damage to gross cellular metabolic activity (Tao et al., 2014). The results in this study suggests that the relative permeability of the fungal cell membrane increased with the increasing concentrations of McEO, causing leakage of important cellular constituents and leading to cell death.

MMp is a very sensitive indicator of the energy-coupling status of mitochondria. Rho123 is a cationic cell-permeant dye and extensively used to determine the MMp. In normal cells, Rho123 can selectively enter the mitochondrial matrix depending on membrane potential across inner membrane. The fluorescence intensity of the dye is enhanced when cell death occurs and the integrity of mitochondrial membrane is destroyed (Jouan et al., 2014; Pinto et al., 2013). Hence, changes in MMp can be determined by the change of fluorescence level. The fluorescence was increased when *A. flavus* strains were treated with 1/2MIC and MIC concentrations of McEO. When McEO concentration

reached 2MIC, the fluorescence reached maximum, indicating loss of MMp. Similar result was obtained for the EO of *Foeniculum vulgare*, suggesting antifungal mode of action through destruction of the mitochondrial membrane potential (Kumar et al., 2020).

To further understand the antiaflatoxigenic mode of action of McEO that led to AFB_1 inhibition, we investigated MG, which have been reported to induce AFB_1 biosynthesis in *A. flavus* cells. According to Chen et al. (2004), MG biosynthesis in *A. flavus* is essential for up-regulating the expression of AFB_1 biosynthesis gene (*Afl*R). The disruption or down-regulation of this gene resulted in loss of the ability to convert aflatoxin intermediates to AFB_1. Thus, it seems possible that McEO may interfere with the genes involved in AFB_1 biosynthesis, which subsequently resulted in the inhibition of AFB_1. Similar finding for the inhibition of MG biosynthesis by *Cistus ladanifer* EO in *A. flavus* cells has been reported by Upadhyay et al. (2018), who suggested that the inhibition of MG level in *A. flavus* culture might be the possible reason for down-regulation of *AflR* gene expression, and subsequently the AFB_1 biosynthesis. However, further research, especially on gene and protein expressions is needed to shed light on the possible mechanism concerned with the AFB_1 inhibition by McEO.

Molecular docking have been used to decipher the interactions that occur between the ligands with the active site residues of important proteins involved in aflatoxin biosynthesis (Singh et al., 2021). In the present investigation, molecular docking simulations were used to evaluate the complementary interactions between α-pinene, bornyl acetate, and camphor with the Nor-1 binding site. Nor-1 is a 29-kDa protein containing short chain alcohol dehydrogenase motif in its amino acid chain and primarily involved in conversion of norsolorinic acid (NOR) into averantin (AVN), which is an important step in AFB_1 biosynthesis. More importantly, Nor-1 is synthesized in the cytoplasm and transported to the vacuoles of fungal hyphae, where it carried out the regulatory step of AFB_1 biosynthesis (Hong & Linz, 2009). Hence, Nor-1 was selected as the receptor protein for molecular docking.

The result clearly confirmed that the test compounds strongly interacted with the amino acid residues of the Nor-1 protein. This result is consistent with the previous study of Kumar et al. (2020), reporting molecular interaction of anethole with Nor-1 gene products during investigating the antiaflatoxigenic mode of action of *Foeniculum vulgare* EO. Das et al. (2020) also demonstrated strong interaction of thujanol, elemicin, and myristicin (components of *Myristica fragrans* EO) with other proteins (Ver-1 and Omt-A) involved in AFB_1 biosynthesis. Regarding this interactive structure dependent binding mechanism, we concluded that the antiaflatoxigenic mechanism of action of test EO could be attributed to the bindings of its major components (ligands) with amino acids present in the protein binding pocket of Nor-1.

Owing to the complex reactive facets of plant bioactives, the antioxidant activity of plant EO cannot be measured solely by a single method, but at least by two to ascertain authenticity (Chaudhari, Singh, Singh, et al., 2020; Ye et al., 2013). For this reason the FRS activity of McEO was determined by two spectrophotometric methods, ABTS and DPPH assays. Our results showed that the McEO exhibited moderately higher FRS capacity than that of synthetic antioxidants like BHT, BHA, and ascorbic acid. This fact might be explained by the dominance of monoterpene compounds viz., α-pinene, bornyl acetate, and camphor, whose antioxidant capacity has already been reported by others (Kim et al., 2013; Kordali et al., 2005; Shahriari et al., 2018; Yang et al., 2010).

Many studies have reported that the antioxidant activity of EO largely depends on the total phenolic contents. Therefore, besides measuring the antioxidant activity, the total phenolic content of McEO was also recorded. The results showed a strong linear correlation between the FRS activity and total phenolic content. More importantly, most of the reactions involved in AFB_1 biosynthesis are mediated by oxygenases enzymes (Manso et al., 2014). Hence, blocking the enzymatic activity of a biosynthetic enzyme, probably due to the high phenolic content (high antioxidant activity) of McEO may constitutes one of the possible mechanisms for the inhibition of AFB_1 biosynthesis. In addition, the high phenolic content in McEO may also lead to its higher antifungal activity (Bagamboula et al., 2004). According to these authors, the EO that contains more phenolic compounds could interact more rapidly with fungal cell membrane structures and functions, and hence, exhibited strongest antifungal properties. The results confirmed that the evaluated McEO could exhibit great potential for being applied as a good shelf-life enhancer of the stored food items.

Maize is one of the world's highest yielding crops and rich source of carbohydrates, protein, starch, and other micro/macronutrients, and serves as an excellent substrate for fungal proliferation, especially for aflatoxigenic fungi. Therefore, in order to reveal the preservative efficacy of McEO against AFB_1 contamination in food system, *in situ* study was performed on maize (var. Maukyrwat, Sohru pnah II, and Varun suma). The results of *in situ* investigation on maize in storage containers during three months of storage confirmed the strong efficacy of EO against AFB_1 contamination. Hu et al. (2017) similarly confirmed our results as they found that *Curcuma longa* EO at 4 μL mL^{-1} to had the remarkable effect on extending the shelf-life of treated maize. Our previous research also indicated that the fumigation of maize with *Origanum majorana* EO could significantly inhibit AFB_1 accumulation during storage (Chaudhari, Singh, Das, et al., 2020). Although, it is difficult to compare the efficacy of different treatments employed to control AFB_1 contamination; however, the level of control of AFB_1 achieved in this study is relevant, and might be possible to achieve complete protection of stored maize using McEO as a fumigant.

Lipid peroxidation is one of the major factors contributing to post-harvest losses of food commodities during storage, resulting in serious loss of flavor and nutritional value. In this study, the extent of lipid peroxidation was recorded by measuring the level of TBARS in maize samples treated with McEO. TBARS test is one of the most prevalent methods used to measure the second stage oxidation products, especially MDA (Papastergiadis et al., 2012). From the obtained results, it can be concluded that the test EO retarded the rate of lipid peroxidation in fumigated maize samples during storage. Inhibition of MDA content by McEO in stored maize samples might be associated with the presence of different bioactive components having prominent free radical scavenging activities (Youdim et al., 2002). Our result is in agreement with previous study of Amiri et al. (2019), who observed significant inhibition of lipid peroxidation in ground beef patties after treatment with *Zataria multiflora* EO fortified with cinnamaldehyde. Our results in this study suggested that adding McEO might prevent the production of MDA as a secondary lipid oxidation product in stored maize and other agricultural commodities during storage.

The calculated LD_{50} value of McEO was found more higher than some of the commonly used preservatives like pyrethrum (300–500 mg kg^{-1}), bavistin (1500 mg kg^{-1}), and formic acid (700 mg kg^{-1}) (Prakash et al., 2012). Moreover, the higher LD_{50} value of McEO as compared to different EOs and bioactive components such as *Artemisia dracunculus* EO (1250 mg kg^{-1}) thujone (870 mg kg^{-1}), pulegone (150 mg kg^{-1}), and carvacrol (910 mg kg^{-1}), suggesting mammalian non-toxicity and satisfying the acceptability of consumers for large scale application in food system (Maham et al., 2014; Moazeni et al., 2019). In light of these results, fumigating model food (maize) with the McEO may be advised to consumers to prevent from fungal and AFB_1 contamination during storage, as consumption of maize treated at this recommended dose of tested EO is likely to be safe for human.

The results of sensorial properties of treated maize samples showed that the maize samples without any treatment (controls) had the lowest scores for all the tested parameters viz., color, texture, aroma, taste, and overall acceptability. This is probably due to the oxidation of essential fatty acids (palmitic, linoleic, oleic and linolenic acid) during storage, and thus, decreased scores by the panelists. However, McEO treated maize samples showed significant improvement ($p < 0.05$) in the sensory scores for almost all the tested parameters except aroma, which achieved lower scores for all maize varieties at 2MIC. This may be due to

the interactions of EO with food matrix components such as carbohydrates, fats, and proteins, resulting in decrease of their organoleptic properties, and subsequently the consumer acceptance. This result is supported by the earlier results of Castro-Rosas et al. (2017) and Sharma et al. (2017), they also reported that the application of higher doses of EOs induced negative impact on sensory characteristics of treated food items. Nevertheless, nanoencapsulation of McEO in polymeric matrix could be an effective strategy to overcome this issue, since the application of encapsulated EO in food did not negatively alter its sensory attributes due to the controlled release properties when compared to the control samples over the assessed storage period (Hasheminejad & Khodaiyan, 2020).

5. Conclusion

The outcome of this study clearly indicated that the tested McEO exhibited potential antifungal and antiaflatoxigenic activity against three toxigenic strains of *A. flavus* in culture media and in maize grains without significantly altering their sensory acceptability. The antifungal modes of action of McEO was attributed to the disruption of plasma membrane integrity, permeability, and mitochondrial membrane potential, while antiaflatoxigenic action was ascribed to the inhibition of methylglyoxal biosynthesis and molecular functioning of the Nor-1 protein in fungal cell. The non-toxic nature of McEO on mice, ascertained its high safety profile on mammalian system. The findings of the study could support the utilization of this EO as a novel substitute of synthetic chemical preservatives for the protection of stored maize grains from fungal attack, AFB_1 contamination, and lipid peroxidation.

Compliance with ethical standards

The experiment related to animal study was in accordance with the ethical standards of the institution at which the study was conducted.

CRediT authorship contribution statement

Anand Kumar Chaudhari: Conceptualization, Writing – original draft, Methodology, Investigation, Funding acquisition. **Vipin Kumar Singh:** Methodology, Investigation. **Somenath Das:** Validation, Data curation. **Anupam Kujur:** Methodology, Software. **Deepika:** Formal analysis. **Nawal Kishore Dubey:** Writing – review & editing, Supervision, All authors have reviewed and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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