Composition Analysis of Essential Oil from *Melaleuca bracteata* Leaves Using Ultrasound-assisted Extraction and its Antioxidative and Antimicrobial Activities

Yongyu Li,[a,b] Zhengmei Ye,[a] Wenting Wang,[a] Chao Yang,[a] Jingyuan Liu,[a] Liting Zhou,[a] Yuze Shen,[a] Zhiheng Wang,[a] Jianjun Chen,[b,*] Shaohua Wu,[a,*] and Liaoyuan Zhang [c,*]

To extract essential oil from *Melaleuca bracteata* leaves without thermal degradation, ultrasound-assisted extraction (UAE) was developed and optimized using a response surface method (RSM) based on the Box-Behnken design (BBD). Under the optimized extraction conditions, a higher essential oil yield of 4.55% was achieved in comparison to that of 1.02% *via* the conventional hydrodistillation extraction method, which suggested that UAE could be used as an alternative and efficient extraction method for the essential oil from *M. bracteata* leaves. Furthermore, the composition of the essential oil extract was analyzed by gas chromatography-mass spectrometry. The results showed that 42 constituents, including methyl eugenol (86.5%), methyl cinnamate (4.33%), 3,4,5-trimethoxybenzoic acid, methyl ester (1.77%), and germacrene D (1.24%) were identified in the essential oil of *M. bracteata* leaves. The essential oil showed strong 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activity, and reducing property. Additionally, remarkable bacteriostatic activity was observed against the tested pathogens, including *Chromobacterium violaceum* ATCC31532, *Pseudomonas aeruginosa* PAO1, *Serratia marcescens* MG1, and *Serratia marcescens* H30. These results indicated that the essential oil from *M. bracteata* leaves had potential applications due to its antioxidative and antimicrobial activities.

Keywords: Melaleuca bracteata; Essential oil; Component; Antioxidant activity; Antimicrobial activity; Ultrasound-assisted extraction

*Contact information: a: College of Horticulture, Fujian Agriculture and Forestry University, Fuzhou 350002, P.R. China; b: Environmental Horticulture Department and Mid-Florida Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Apopka, FL 32703, USA; c: College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, P.R. China; * Corresponding authors: jjchen@ufl.edu; wsh6677@hotmail.com; zliaoyuan@126.com*

INTRODUCTION

Melaleuca bracteata is an evergreen shrub or small tree with dense foliage belonging to the Myrtaceae family. It is aborigine throughout the eastern coast of Australia and now is widespread in many countries such as South Africa, Egypt, Thailand, China, and Indonesia (Aboutabl *et al.* 1991; Naidu 2003; Kardinan and Hidayat 2013; Osunsanmi *et al.* 2015). This plant is popularly used as an ornamental plant due to its aroma oil production. It has been reported to be rich in essential oils, betulinic acid, proline (betaine) analogues, and oleanolic acid (Naidu 2003; Wilkinson and Cavanagh 2005; Adesanwo *et al.* 2009; Osunsanmi *et al.* 2015). *Melaleuca bracteata* essential oil (BEO) has been regarded as an excellent source of biological agricultural chemical ingredients, and is

primarily used as an antiseptic due to its antibacterial, antiulcer, antimicrobial, and insecticidal properties (Aboutabl *et al.* 1991; Wilkinson and Cavanagh 2005; Kardinan and Hidayat 2013; Oyedeji *et al.* 2014). Chemical composition analysis showed that the ingredients and their contents in BEO were remarkably different among *Melaleuca* species (Yatagai *et al*. 1998). Besides the difference of *Melaleuca* species, other factors such as the BEO extraction methods and conditions also affected the analysis of the ingredients and their contents, and then resulted in different BEO bioactivity (Samaram *et al.* 2015; Ben Ahmed *et al.* 2016).

Ultrasound-assisted extraction (UAE) is a relatively new, facile, and cost-effective alternative to the conventional technique for recovery essential oils from a wide variety of sources (Sereshti *et al*. 2012; Samaram *et al*. 2015). Moreover, sonication can improve the extraction efficiency and rate of target compounds despite a short processing duration, low temperature, reduced solvent consumption, and less energy input (Kowalski *et al*. 2015). Hence, UAE may be designated as a green and ideal option in the edible oils industry for low processing temperatures that preserve the structural and molecular properties of bioactive compounds from thermal degradation (Tian *et al*. 2013). To study the potential industrial applications of *M. bracteata* as a raw material, a UAE method for efficiently extracting essential oils from *M. bracteata* leaves was developed, and a higher yield was achieved under the optimal extraction conditions. The essential oil obtained by UAE was analyzed to reveal the chemical composition *via* gas chromatography-mass spectrometry (GC-MS). Additionally, the essential oils from *M. bracteata* exhibited excellent antioxidant and antimicrobial activities.

EXPERIMENTAL

Materials

The pest-free and disease-free leaves of *M. bracteata* without deformity were collected from Minhou county, Fujian province of China (East of China-Fujian: north latitude 25° 47′- 26° 37′, east longitude 118° 51′- 119° 25′, altitude 29 m). These leaves were cleaned, pitted, and vacuum-dried (Four-Ring Science Instrument Co., Beijing, China) to a constant weight, followed by electric grinding (IKA-Works, Staufen, Germany). The ground powder was sieved through a 425 μm screen, collected, sealed, and preserved at 4 °C until further use.

1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), *n*-alkanes, dichloromethane, ampicillin, and kanamycin were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Petroleum ether (b.p. 60 °C to 90 °C), anhydrous sodium sulfate, absolute ethyl alcohol, methanol, sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), potassium ferricyanide, iron trichloride, trichloroacetic acid (TCA), and potassium persulfate ($K_2S_2O_8$) were purchased from China National Pharmaceutical Group Co. (Beijing, China). Only analytical grade chemicals and solvents were used.

The tested microorganisms included *S. marcescens* H30, *S. marcescens* MG1, *S. aureus* ATCC25933, *C. violaceum* ATCC31532, and *P. aeruginosa* PAO1 purchased from the China Center of Industrial Culture Collection (Beijing, China) and the American Type Culture Collection (Manassas, VA, USA), respectively. The bacteria were cultured on a nutrient agar (NA) medium that was sterilized at 121 °C for 20 min in an autoclave.

Methods

UAE of essential oils

In this study, an ultrasonic extraction crasher Scientz-IID (25 kHz, maximum to 950 W, Scientz, Ningbo, China) was used for the recovery of essential oils from *M. bracteata* leaves. Dried ground leaves samples were mixed into petroleum ether solution (300 mL) to prepare the UAE-based system. Different experimental parameters, including ultrasonic power (X_1, 190 W to 570 W), ultrasonic time (X_2, 5 min to 25 min), solvent-to-solid ratio (X_3, 5 mL/g to 25 mL/g), and extraction temperature (X_4, 20 °C to 40 °C), were used to conduct the extraction process as described in a previous study (Samaram *et al.* 2015). The effect of each parameter was analyzed independently to determine the appropriate range of each variable using single-factor experiments, and subsequently their optimal levels for high extraction yield were obtained using response surface methodology (RSM). After UAE, the slurry was centrifuged at 8000 × g for 10 min to collect the supernatant in a volumetric flask. Each trial was conducted five times. The extract was preserved in dark vials at 4 °C until further use.

Box-Behnken design and optimization

The RSM based on the Box-Behnken design (BBD; Design-Expert, version 8.0.6, Stat-Ease Inc., Minneapolis, MN, USA) with three coded levels was employed to determine the optimal essential oil extraction conditions of the ultrasound power (X_1), ultrasound time (X_2), and solvent-to-solid ratio (X_3). The level of each factor was designed in Table 2 according to the results of single-factor experiments. The low, middle, and high levels of each independent variable were designated as −1, 0, and +1, respectively, and the dependent variables were the extraction yields of the essential oil.

The correlation between the coded and real values, for statistical analysis, was established by Eq. 1,

$$X_i = \frac{X_i - X_o}{\Delta X_i} \quad (1)$$

where X_i indicates the coded value of the variable, X_o indicates the true value of X_i at the centre point, and ΔX_i is the step change in the variable.

Thus, herein, a three-level-three-factor BBD was employed that required 17 experiments, including 12 factorials and 5 replicates at the center point, for the optimization of the extraction parameters. The experimental yields were fitted to the second-order polynomial equation (quadratic model) for the prediction of the optimized parameters of the extraction process as follows (Eq. 2),

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (2)$$

where Y represents the response function (in this case the extraction yield of essential oils), β_o indicates a constant coefficient, β_i, β_{ii}, and β_{ij} indicate the regression coefficients of linear, quadratic, and interactive terms, respectively; and X_i and X_j represent the coded independent variables (ultrasound power, ultrasound time, solvent-to-solid ratio). A statistical analysis was conducted using Design-Expert 8.0.6 software. The results were fitted to a second-order polynomial regression model comprised of the coefficient of individual linear, quadratic, and interactive parameters. An analysis of variance (ANOVA)

with a 95% confidence level for each response variable examined the significance and suitability of the model.

Conventional hydrodistillation extraction (CHE) of essential oils

Essential oils in *M. bracteata* leaves were extracted using a traditional hydrodistillation method following the methods of Sereshti *et al*. (2012). Briefly, 50 g of each leaf powder were immersed in 500 mL water and hydrodistilled in a full glass Clevenger-type apparatus to extract for 2 h (until no more essential oil was obtained). Then, the system was cooled down and the condensed essential oil was decanted. To improve the recovery, the essential oils were immersed in n-pentane, dried under anhydrous sulphate, and stored in a dark glass bottle at 4 °C until their use.

Determination of extraction yield

The extraction yield was computed as the amount of the extracted essential oils divided by the initial amount of leaf powder. For an accurate measurement, a 0.0001 g analytical balance (Mettler-Toledo International, Greinfensee, Switzerland) was used. The final percentage of the extraction yield was obtained as follows (Samaram *et al.* 2015) (Eq. 3):

$$\text{Extraction yield (\%)} = [\text{Essential oil amount} / \text{Initial sample amount}] \times 100 \quad (3)$$

GC-MS analysis

The essential oil extracted from the *M. bracteata* leaf was subjected to a GC-MS analysis using an Agilent 6890N GC (Agilent Technologies Co. Ltd., Palo Alto, USA) equipped with an Agilent DB-5MS quartz capillary column (30 m × 0.25 mm, 0.25-μm film thickness) and an Agilent 5973I mass selective detector in the electronic ionization (EI) mode (Mothana *et al.* 2013; Chen *et al.* 2014a) with slight modification. Helium served as the carrier gas at a flow rate of 1 mL/min, ionization energy 70 eV with a scan time of 1 s, mass range 45 m/z to 550 m/z, and solvent delay of 3.5 min. The temperature of the injector and detector was 250 °C and that of the transport line was 300 °C. The ion source and quadrupole temperatures were set as 230 °C and 150 °C, respectively. The column temperature was initially set at 50 °C (maintained for 5 min), and then increased to 100 °C at a rate of 3 °C/min (maintained for 5 min), followed by a rate of 5 °C/min up to 250 °C, which was maintained for 2 min. A 1 μL sample using a split mode with 20:1 was loaded into the GC column. The volatiles were extracted and analyzed six times. The chromatographic peaks were considered as signals when simultaneously they were different from the blank control and the signal-to-noise ratio was higher than 3:1. The data obtained were validated by comparison of the mass spectrum either to those of the reliable compounds or to the published date for the identification of the volatile leaf compounds. The relative concentrations of the components were obtained by peak area normalization.

Determination of antioxidant activity

The antioxidative properties of BEO were evaluated by the scavenging activity against DPPH and ABTS radicals and the determination of reducing power according to the methods described by Chen *et al*. (2014b), Dahmoune *et al*. (2015), and Huang *et al*. (2009), respectively.

Determination of antimicrobial ability

The inhibitory zone (IZ) assay of the essential oil on tested microorganisms was performed using the disc diffusion method (Al-Abd *et al.* 2015) with slight modifications. In brief, the essential oil was diluted to between 10 mg/mL and 40 mg/mL using dimethyl sulfoxide (DMSO) and filter-sterilized through 0.22-mm Millipore filters. All of the tested strains, adjusted to a microbial suspension of 10^6 CFU/mL, were cultured in nutrient medium (per liter: peptone 10 g, beef powder 3 g, NaCl 5.0 g, and a pH of 7.3) at 35 °C for 24 h with 150 rpm agitation.

A 30 μL filter-sterilized essential oil sample was spotted on a sterile paper disc (6-mm diameter), which was then placed on the surface of the agar plate (NA) preinoculated with the tested strains. Similar discs were prepared for ampicillin and methyl eugenol (10 mg/mL to 40 mg/mL) that served as positive controls, whereas DMSO was used as the negative control. These samples diffused into the agar plates for 1 h, and then the plates were inverted and incubated at 35 °C for 24 h. The diameter of the inhibition zone (mm) was measured to evaluate the antimicrobial activity of essential oil from *M. bracteata* leaves. Each assay was performed in triplicate and repeated at least twice to confirm the results as average values.

The minimum inhibition concentration (MIC) was determined *via* a broth dilution method as previously described (Al-Abd *et al.* 2015) with slight modifications. Each microorganism was evaluated with the essential oil sample in the concentration range of 0.08 mg/mL to 40 mg/mL and diluted by using the nutrient medium solution. A 180 μL mixture of nutrient medium and essential oil DMSO solution sterilized with a 0.22-mm Millipore filter was loaded into a 96-well plate. Then, 20 μL microorganism suspension (10^6 CFU/mL) was inoculated and cultured at 30 °C for 24 h with 150 rpm in a rotary shaker.

The culture concentration was determined using a microplate reader (iMark; Bio-Rad Laboratories Inc., Hercules, USA) after incubation. Culture medium without bacterial inoculation was used as the negative control. The MIC value was estimated as the minimum concentration of the sample in the 96-well where there was no visible bacterial growth after incubation.

According to the MIC values, 5 μL of the culture medium that showed no increase in turbidity was transferred from each well and streaked on a solid NA culture medium, followed by incubation at 35 °C for 24 h. The lowest concentration in the medium without bacterial growth was deemed as the minimum bactericide concentration (MBC) (Al-Abd *et al.* 2015).

Statistical analysis

All of the experiments were performed in triplicate, and the data were recorded as mean ± SE (standard error). The statistical analysis was used to evaluate the significance of differences between groups using the Statistical Product and Service Solutions version 19.0 software (IBM Corporation, Armonk, NY, USA). The comparisons between the groups were determined by Fisher's Least Significant Difference (LSD) at $P < 0.05$ or $P < 0.01$. The IC_{50} (the concentration of antioxidant at which 50% of the reaction was inhibited) was determined using SPSS for Windows, version 19.0 (IBM Corporation, Armonk, NY, USA).

RESULTS AND DISCUSSION

Optimizing BEO Yield Through Ultrasound-assisted Extraction using RSM

The designs and results of the single-factor experiments are shown in Table 1 and Fig. 1. The ultrasound power, extraction time, and liquid-to-solid ratio showed obvious effects on the BEO yield, while only slight influence on the BEO yield was observed when the extraction temperature was tested in the range from 20 °C to 40 °C.

Table 1. Single-factor Experimental Design

Experiment Name	Ultrasound Power (W)	Extraction Time (min)	Liquid-to-solid Ratio (mL/g)	Extraction Temperature (°C)
Ultrasound Power Optimization	190-570	20	20	25
Extraction Time Optimization	380	5-25	20	25
Liquid-to-solid Ratio Optimization	380	20	5-25	25
Extraction Temperature Optimization	380	20	20	20-40

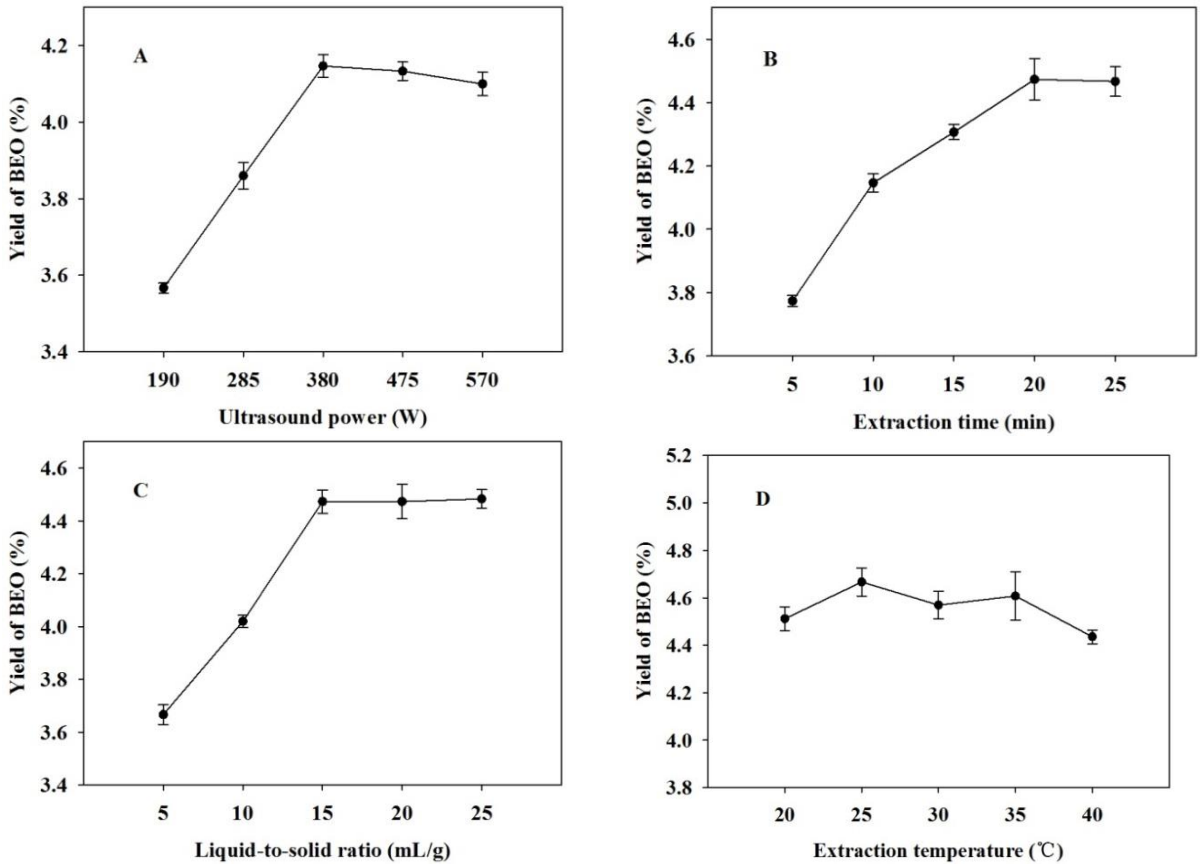

Fig. 1. The effect of ultrasound power (A), extraction time (B), liquid-to-solid ratio (C), and extraction temperature (D) on the yield of essential oil extracted from *M. bracteata* leaves (n = 3)

To achieve the high BEO yield, three factors, including ultrasound power, extraction time, and liquid-to-solid ratio, were further optimized to confirm the optimal levels by using RSM based on the Box-Behnken design. The experimental design and corresponding response data for BEO are shown in Table 2. Out of the 17 experiments that also included 5 replicates, experiment 9 (ultrasound power 380 W, extraction time 25 min, and liquid-to-solid ratio 20 mL/g) produced the highest BEO yield at 4.51%, while the lowest yield of 4.20% was observed in experiment 4 (ultrasound power 285 W, extraction time 15 s, and liquid-to-solid ratio 15 mL/g).

The multiple regression analysis on the experimental data demonstrated that the response variable and the independent variables correlated by the following second-order polynomial model as follows (Eq. 4),

$$Y(BEO) = 4.44 + 0.021X_1 + 0.00575X_2 + 0.089X_3 - 0.028X_1X_2 + 0.057X_1X_3 + 0.038X_2X_3 - 0.12X_1^2 - 0.079X_2^2 + 0.00575X_3^2 \quad (4)$$

where Y is the predicted BEO yield and X_1, X_2, and X_3 are the coded values for ultrasound power, extraction time, and liquid-to-solid ratio, respectively.

The model was further substantiated by the ANOVA. The regression coefficient and ANOVA of the second-order polynomial model for BEO yield are presented in Table 3. Only two linear parameters, ultrasound power (X_1) and the liquid-to-solid ratio (X_3), were significant ($P < 0.05$). Two quadratic parameters, ultrasound power (X_1) and extraction time (X_2), were highly significant ($P < 0.01$). The interactions X_1X_2, X_1X_3, and X_2X_3 were also significant ($P < 0.05$ or $P < 0.01$).

Table 2. Experimental Design and the Observed Responses Value with Different Combinations of Factors for the Trials of Box-Behnken

Run	Ultrasound Power (W), X_1	Extraction Time (min), X_2	Liquid-to-solid Ratio (mL/g), X_3	BEO Yield (%)
1	380.00 (0)	25.00 (+1)	10.00 (-1)	4.25
2	475.00 (+1)	20.00 (0)	20.00 (+1)	4.50
3	285.00 (-1)	20.00 (0)	10.00 (-1)	4.27
4	285.00 (-1)	15.00 (-1)	15.00 (0)	4.20
5	285.00 (-1)	25.00 (+1)	15.00 (0)	4.25
6	380.00 (0)	20.00 (0)	15.00 (0)	4.47
7	380.00 (0)	20.00 (0)	15.00 (0)	4.41
8	380.00 (0)	20.00 (0)	15.00 (0)	4.43
9	380.00 (0)	25.00 (+1)	20.00 (+1)	4.51
10	380.00 (0)	15.00 (-1)	20.00 (+1)	4.40
11	380.00 (0)	20.00 (0)	15.00 (0)	4.42
12	380.00 (0)	15.00 (-1)	10.00 (-1)	4.30
13	475.00 (+1)	25.00 (+1)	15.00 (0)	4.23
14	475.00 (+1)	15.00 (-1)	15.00 (0)	4.29
15	475.00 (+1)	20.00 (0)	10.00 (-1)	4.21
16	380.00 (0)	20.00 (0)	15.00 (0)	4.47
17	285.00 (-1)	20.00 (0)	20.00 (+1)	4.34

Table 3. Estimated Regression Coefficients of the Quadratic Polynomial Model and ANOVA for the Experimental Results of Essential Oil Extracted from *M. bracteata* Leaves Using UAE Method

Source[a]	Estimated Coefficients	Standard Error	Sum of Squares	DF[b]	Mean Square	*F*-value	Prob > *F*
Model			1.77×10^{-1}	9	1.96×10^{-2}	37.50	< 0.0001
Intercept	4.44	1.02×10^{-2}		1			
X_1	2.08×10^{-2}	8.09×10^{-3}	3.44×10^{-3}	1	3.44×10^{-3}	6.59	0.0372
X_2	5.75×10^{-3}	8.09×10^{-3}	2.65×10^{-4}	1	2.65×10^{-4}	0.51	0.5000
X_3	8.93×10^{-2}	8.09×10^{-3}	6.37×10^{-2}	1	6.37×10^{-2}	121.83	< 0.0001
X_1X_2	-2.80×10^{-2}	1.14×10^{-2}	3.14×10^{-3}	1	3.14×10^{-3}	6.00	0.0442
X_1X_3	5.65×10^{-2}	1.14×10^{-2}	1.28×10^{-2}	1	1.28×10^{-2}	24.41	0.0017
X_2X_3	3.85×10^{-2}	1.14×10^{-2}	5.93×10^{-3}	1	5.93×10^{-3}	11.33	0.0120
X_1^2	-1.16×10^{-1}	1.11×10^{-2}	5.64×10^{-2}	1	5.64×10^{-2}	107.85	< 0.0001
X_2^2	-7.93×10^{-2}	1.11×10^{-2}	2.64×10^{-2}	1	2.64×10^{-2}	50.56	0.0002
X_3^2	5.75×10^{-3}	1.11×10^{-2}	1.39×10^{-4}	1	1.39×10^{-4}	0.27	0.6218
Residual			3.66×10^{-3}	7	5.23×10^{-4}		
Lack of Fit			5.45×10^{-4}	3	1.82×10^{-4}	0.23	0.8691
Pure Error			3.12×10^{-3}	4	7.79×10^{-4}		
Corrected Total			1.80×10^{-1}	16			
$R^2 = 0.98$ $R^2_{adj} = 0.95$ CV = 0.53							

[a] Coefficients refer to the general model; [b] Degree of freedom

The ANOVA analysis of the experimental results summarized in Table 3 implied that the quadratic polynomial model was highly significant ($P < 0.0001$) for representing the actual relationship between the response and parameters. Furthermore, the determination coefficient (R^2) of 0.98 and the adjusted determination coefficient (R^2_{Adj}) of 0.95 were obtained for the response of BEO, which further validated the adequacy of the model. However, a large R^2 value does not necessarily indicate the reliability of the regression model; however, the R^2_{Adj} should be statistically comparable to R^2 (Dahmoune *et al*. 2015). As shown in Table 3, the R^2 and R^2_{Adj} values for the model did not differ greatly. The model also showed a statistically insignificant lack of fit at 95% confidence, which thereby indicated the adequacy of the fitted models. The value of pure error was low, which suggested the reliability and reproducibility of the model was in agreement with the previous data obtained by ANOVA. These results indicated the suitability of the model for the prediction of BEO extract from *M. bracteata* leaves.

The levels of the variables for BEO yield from *M. bracteata* leaves were determined using two-dimensional and three-dimensional surface plots of multiple non-linear regression models, which are displayed in Fig. 2. The response surface plots were constructed in Table 3 to assess the significant ($P < 0.05$) effect of the ultrasound extraction variables' interaction on the BEO yield. The UAE process at the high ultrasound power and time with a high liquid-to-solid ratio resulted in a highly efficient extraction of essential oils from *M. bracteata* leaves.

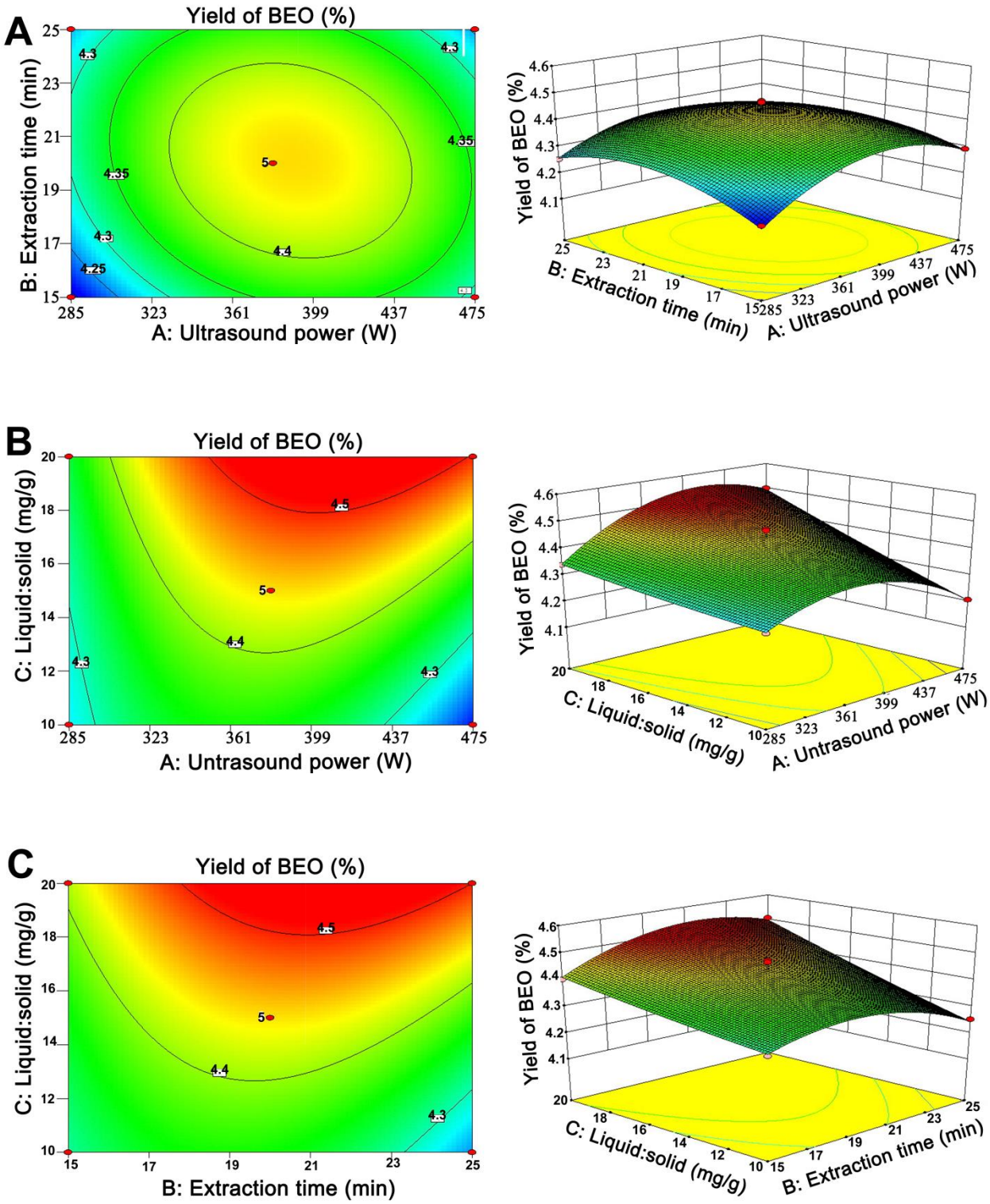

Fig. 2. Response surface plots (3D, right) and contour plots (2D, left) of the essential oil yield extracted from *M. bracteata* leaves using UAE as a function of significant interactions between factors: (A) ultrasound power and extraction time; (B) ultrasound power and liquid-to-solid ratio; and (C) extraction time and liquid-to-solid ratio

Using the Design Expert 8.0.6 software, the authors identified the optimum values of ultrasound power (408.99 W), extraction time (21.08 min), and the liquid-to-solid ratio (20 mL/g), which were the three key conditions for BEO extraction from *M. bracteata* leaves. Additionally, these parameters predicted a maximum extraction yield of 4.55% BEO. The reliability of the model was validated by five verification experiments under optimum conditions. The mean value of BEO extraction from this experiment was 4.55 ± 0.01% (w/w, $N = 5$), which coincided with the predicted value and was not significant ($P > 0.05$) by a paired t-test. This experiment showed that the RSM model was accurate and reliable. The BEO yield from this study was considerably higher than that obtained using a conventional hydrodistillation method (1.02 ± 0.01%). As a result, the regression model was considered adequate for predicting the BEO yield extracted from *M. bracteata* leaves using the UAE process. Furthermore, this extraction method was straightforward and rapid.

Chemical Composition of the *M. bracteata* Essential Oil

The essential oil extracted from *M. bracteata* leaf under the optimized UAE conditions was colorless and possessed an aromatic and minty odor. The GC-MS chromatogram of the BEO is presented in Fig. 3. Table 4 illustrates the chemical components and their peak area ratios of BEO. A total of 42 volatile constituents, encompassing 98.5% of the total oil, were identified by GC-MS data. The major components of essential oil were methyl eugenol (86.5%), methyl cinnamate (4.33%), 3,4,5-trimethoxybenzoic acid, methyl ester (1.77%), and germacrene D (1.24%). The chemical compounds were characterized by the presence of three major biochemically-related groups of components. The majority of the volatiles were aromatic compounds, which accounted for 87.1% of the total. The next most abundant group of volatiles was aliphatic compounds (7.60%), followed by the third group that included the terpenoids (3.76%), such as germacrene D, *α*-phellandrene, *p*-cymene, terpinolene, *β*-caryophyllene, bicyclogermacrene, and calamenene.

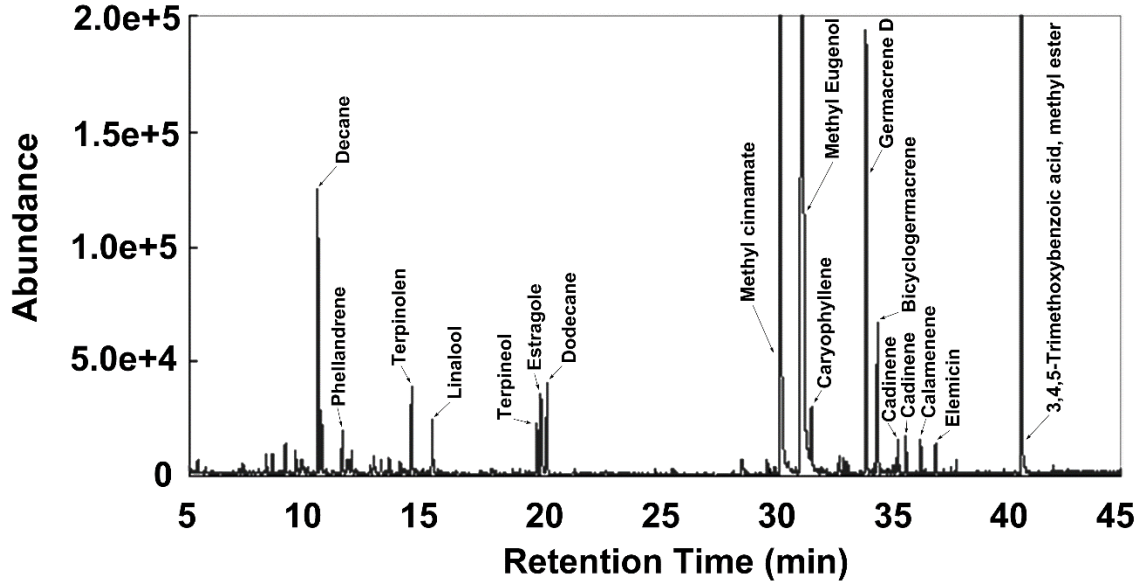

Fig. 3. GC-MS chromatogram of essential oil obtained from *M. bracteata* leaves

Table 4. Chemical Components of the Essential Oil from *M. bracteata* Leaves

No.	RT (min)	Compounds	Molecular Formula	Molecular Weight	Relative Content (%)
1	4.647	Ethylbenzene	C_8H_{10}	106	0.04
2	4.942	p-Xylene	C_8H_{10}	106	0.06
3	5.710	1,3,5-Cyclooctatriene	C_8H_{10}	106	0.03
4	7.310	1-Nonene	C_9H_{18}	126	0.02
5	7.962	3-Ethyl-3-methylheptane	$C_{10}H_{22}$	142	0.02
6	8.303	4-Ethyloctane	$C_{10}H_{22}$	142	0.07
7	8.551	5-Methylnonane	$C_{10}H_{22}$	142	0.07
8	9.117	3-Methylnonane	$C_{10}H_{22}$	142	0.11
9	10.088	1-Decene	$C_{10}H_{20}$	140	0.04
10	10.509	Decane	$C_{10}H_{22}$	142	0.74
11	10.659	α-Phellandrene	$C_{10}H_{16}$	136	0.20
12	11.560	p-Cymene	$C_{10}H_{14}$	134	0.15
13	11.786	D-Limonene	$C_{10}H_{16}$	136	0.05
14	12.756	trans-β-Ocimene	$C_{10}H_{16}$	136	0.02
15	12.912	2-Methyldecane	$C_{11}H_{24}$	156	0.08
16	13.224	γ-Terpinen	$C_{10}H_{16}$	136	0.04
17	14.044	3-Methyldecane	$C_{11}H_{24}$	156	0.04
18	14.529	Terpinolen	$C_{10}H_{16}$	136	0.29
19	15.418	β-Linalool	$C_{10}H_{18}O$	154	0.19
20	19.911	α-Terpineol	$C_{10}H_{18}O$	154	0.16
21	20.073	Estragole	$C_{10}H_{12}O$	148	0.28
22	20.327	Dodecane	$C_{12}H_{26}$	170	0.28
23	21.586	Citronellol acetate	$C_{12}H_{22}O_2$	198	0.05
24	28.777	Eugenol	$C_{10}H_{12}O_2$	164	0.08
25	29.839	Copaene	$C_{15}H_{24}$	204	0.05
26	30.168	β-Bourbonene	$C_{15}H_{24}$	204	0.03
27	30.405	Methyl cinnamate	$C_{10}H_{10}O_2$	162	4.33
28	31.352	Methyl Eugenol	$C_{11}H_{14}O_2$	178	86.5
29	31.728	β-Caryophyllene	$C_{15}H_{24}$	204	0.28
30	32.929	Calarene	$C_{15}H_{24}$	204	0.06
31	33.131	.alpha.-Caryophyllene	$C_{15}H_{24}$	204	0.05
32	33.281	(+)-Aromadendrene	$C_{15}H_{24}$	204	0.05
33	34.078	Germacrene D	$C_{15}H_{24}$	204	1.24
34	34.569	Bicyclogermacrene	$C_{15}H_{24}$	204	0.44
35	35.256	γ-Cadinene	$C_{15}H_{24}$	204	0.04
36	35.378	δ-Cadinene	$C_{15}H_{24}$	204	0.05
37	35.459	Calamenene	$C_{15}H_{22}$	202	0.14
38	35.788	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	$C_{15}H_{24}$	204	0.09
39	36.417	Elemicin	$C_{12}H_{16}O_3$	208	0.11
40	37.948	Hexadecane	$C_{16}H_{34}$	226	0.04
41	40.743	3,4,5-Trimethoxybenzoic acid, methyl ester	$C_{11}H_{14}O_5$	226	1.77
42	53.472	1-Docosene	$C_{22}H_{44}$	308	0.13

Among all the components of BEO, methyl eugenol was the major constituent, similar to previous studies on the major constituents of *M. bracteata* oil from Australia, Egypt (Aboutabl *et al.* 1991), and South Africa (Oyedeji *et al.* 2014), while the major constituent of the oil from Thailand was methyl eugenol ether (Oyedeji *et al.* 2014). The results showed that *M. bracteata* grown in China corresponded to a methyl eugenol

chemotype. However, the results of this study appeared to be somewhat different on the chemotypes of other *bracteata* essential oils. Previous studies demonstrated the dominant component of essential oils from different *Melaleuca* species. Terpinen-4-ol (53.7%) for *M. alternifolia*, viridiflorol (71.0%) for *M. quinquenervia*, methyl eugenol (96.6%) for *M. leucadendra*, 1, 8-cineole for *M. ericifolia* (79.5%), *M. cajuputi* subspecies *cajuputi* (43.7%), *M. cajuputi* subspecies *platyphylla* (41.0%), and *M. armillaris* (80.2%) were grown in Brazil (Silva *et al.* 2007); α-terpinol (34.7%) for *M. leucadendron*, caryophyllene (50%) for *M. styphelioides*, ethyl eugenol (98.5%) for *M. ericifolia*, 1, 8-cineole (57.2%) for *M. quinqueneroia*, and (33.7%) for *M. armillaris* were grown in Egypt (Aboutabl *et al.* 1991; Farag *et al.* 1998); and 1, 8-cineole (64.8%) for *M. trichostachya* was obtained from South Africa (Oyedeji *et al.* 2014). These results suggested that the genetic backgrounds and phenological stage, as well as geographical and environmental factors of the plant, presumably contributed to generating a remarkable chemical composition of *M. bracteata* (Mothana *et al.* 2013). Furthermore, the season of harvest, plant growth location, climate, fertility regime, soil type, the age of the leaves, and extraction methods may also affect the contents of the volatile compounds (Batish *et al.* 2008; Chen *et al.* 2014b).

Determination of Antioxidant Activity

Considering the complexity of antioxidative mechanisms, complementary approaches were used to assess the total antioxidant activity of the sample (Li *et al.* 2012). A DPPH assay is commonly used as an indicator of the free radical scavenging capacity of the antioxidants (Li *et al.* 2018). The ABTS radical cation decolorization assay satisfactorily measures the antioxidant activity of hydrogen-donation and chain break of the extracted essential oil (Ye *et al.* 2013; Dahmoune *et al.* 2015). The reducing power assay significantly estimates the antioxidant capability of plants by determining the electron donation of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (Huang *et al.* 2009; Gholivand *et al.* 2010). Figure 4 demonstrates the characteristics of UAE-extracted BEO based on DPPH free radical scavenging, ABTS free radical scavenging, and the reducing abilities. The BHT and methyl eugenol were used as the control standards. The scavenging ability of BEO on the DPPH free radical correlated with increasing concentrations, even superior to BHT at a high concentration test, with the IC_{50} of BEO and BHT at 0.15 mg/mL and 0.03 mg/mL, respectively. The BEO exhibited strong dose-dependent scavenging activities on the ABTS radical cation (R^2 = 0.99), but was lower than BHT. The IC_{50} of BEO and BHT on ABTS free radical scavenging were 7.23 mg/mL and 1.07 mg/mL, respectively. The BEO exhibited the reducing ability in a dose-dependent manner (R^2 = 0.98) at the experimental concentration. The IC_{50} value of BEO on the reducing power was 6.39 mg/mL, while that of BHT was 0.61 mg/mL. Thus, the reducing power of BEO was inferior to the strong reducing agent, BHT. However, the antioxidant capability of *M. bracteata* essential oils was superior to several other species (Mothana *et al.* 2013; Ye *et al.* 2013), especially the DPPH free radical scavenging activity.

The *Melaleuca* genus encompasses several members, and their bioactivities have been previously characterized (Benelli *et al.* 2013; Gaínza *et al.* 2015). However, studies on the antioxidant activity of *M. bracteata* essential oil are lacking. The current results suggested that the *M. bracteata* essential oil exhibited significant antioxidant activity, which was superior to the major component of methyl eugenol. Several studies demonstrate that the antioxidant activity of essential oil from plants can be potentially associated with the low content of volatile phenolic components, terpenes, and ketone (Li *et al.* 2012;

Mothana *et al.* 2013). Consequently, the excellent antioxidant activity of BEO might have been attributed to the presence of high content phenolic components.

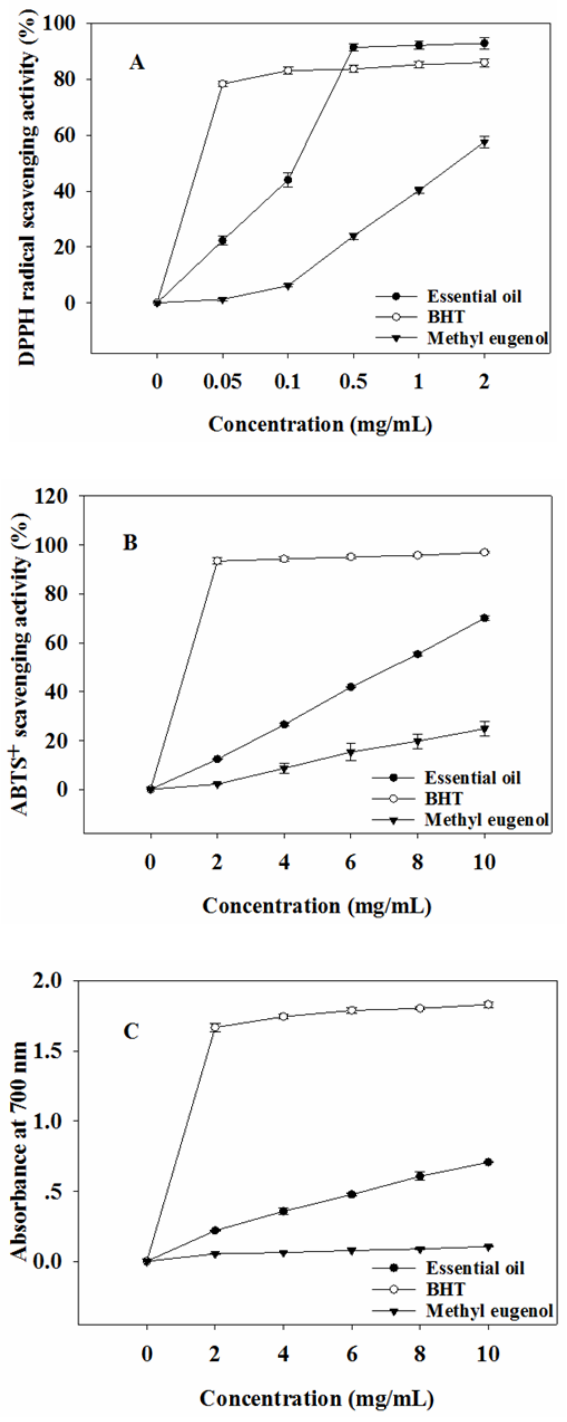

Fig. 4. Antioxidant activities of the essential oil isolated from *M. bracteata* leaves; (A) DPPH radical scavenging activity; (B) $ABTS^+$ scavenging activity; and (C) reducing power; Data are represented as means ± SE (n = 3)

In vitro Antimicrobial Activity

The UAE-based BEO was qualitatively and quantitatively tested for antimicrobial activity against several pathogenic microorganisms by the IZ, MIC, and MBC assays (Table 5). The results indicated that BEO exhibited an effective BEO concentration-dependent inhibitory effect against both Gram-positive and Gram-negative bacteria. The BEO exerted a broad antimicrobial spectrum and showed the strongest inhibition against *C. violaceum* ATCC31532 with the largest inhibition zone of 18.53 mm, the lowest MIC value of 2.5 mg/mL, and a MBC value of 5.0 mg/mL. Additionally, the BEO showed a high antimicrobial effect on *P. aeruginosa* PAO1, *S. marcescens* MG1, and *S. marcescens* H30 at different levels. However, subjecting *S. aureus* ATCC25933 to BEO exposure resulted in a moderate inhibition zone of 8.01 mm followed by identical MIC and MBC values of 5 mg/mL and 20 mg/mL, respectively.

Interestingly, coupling this result with the component of the essential oil showed that the antimicrobial activity could have been primarily due to the high content of compositions, such as oxygenated monoterpenes, menthone, piperitone, and pulegone, with known antimicrobial activity (Mothana *et al.* 2013). In the present study, the positive control of methyl eugenol, which contained an -OH group attached to an aromatic ring that induces the antimicrobial activity (Farag *et al.* 1998), showed similar antimicrobial results on all the investigated microorganisms, but results that were weaker than BEO. A comparison of the results of this study to those of previous literature revealed a strong antibacterial effect of *M. bracteata* that might have been attributed to the high percentage of methyl eugenol as well as some oxygenated monoterpenes, such as β-linalool and α-terpineol, and their putative synergistic effect on the bacteriostatic capability.

Table 5. Antimicrobial Activity of the Essential Oil from *M. bracteata* Leaves

Strains	Inhibition Zones (mm)						Essential Oil (mg/mL)	
	Essential Oil (mg/mL)				Methyl Eugenol[a]	Ampicillin[b]	MIC[c]	MBC[d]
	10	20	30	40				
S. marcescens H30	12.5 ± 0.3[e]	12.8 ± 0.6	12.8 ± 0.4	12.9 ± 0.2	12.5 ± 0.2	13.8 ± 0.6	5	10
S. marcescens MG1	12.9 ± 0.5	13.1 ± 0.20	13.2 ± 0.3	13.3 ± 0.4	12.8 ± 0.4	6.71 ± 0.20	5	10
P. aeruginosa PAO1	13.0 ± 0.3	13.3 ± 0.2	13.3 ± 0.5	13.6 ± 0.4	12.3 ± 0.8	-[f]	5	10
C. violaceum ATCC31532	15.3 ± 0.9	16.5 ± 1.0	17.8 ± 0.6	18.5 ± 0.6	15.6 ± 0.4	7.08 ± 0.66	2.5	5
S. aureus ATCC25933	5.51 ± 0.08	6.28 ± 0.11	7.33 ± 0.09	8.01 ± 0.18	5.63 ± 0.07	46.2 ± 0.4	5	20

[a] Methyl eugenol (10 mg/mL) was used as the positive control; [b] Ampicillin (10 mg/mL) was used as the positive control; [c] Minimum inhibitory concentrations; [d] Minimum bactericidal concentrations; [e] Each value is expressed as means ± SE (n = 3); [f] No activity observed

CONCLUSIONS

1. The ultrasound-assisted extraction method resulted in a higher essential oils yield with 4.55% from *M. bracteata* leaves in comparison to that of 1.02% through using conventional hydrodistillation extraction methods.
2. The GC/MS analysis of *M. bracteata* essential oil revealed that the essential oil contained a high content of methyl eugenol (86.5%). Methyl cinnamate, 3,4,5-trimethoxybenzoic acid, methyl ester, and germacrene D were identified as the major compounds.
3. *Melaleuca bracteata* essential oil possessed remarkable antioxidant activity, which included DPPH scavenging, ABTS scavenging, reducing power activity, and strong antimicrobial ability.

ACKNOWLEDGMENTS

This study was financially supported by the National Natural Science Foundation of China (Grant Nos. 31501694 and 81673542), the Foundation of Fujian Science and Technology Committee (Grant No. 2018N0003), and the Student's Platform for Innovation and Entrepreneurship Training Program (Grant Nos. 201710389013 and 201810389070). Yongyu Li was also funded by the China Scholarship Council.

REFERENCES CITED

Aboutabl, E. A., El Tohamy, S. F., De Footer, H. L., and De Buyck, L. F. (1991). "A comparative study of the essential oils from three *Melaleuca* species growing in Egypt," *Flavour Frag. J.* 6(2), 139-141. DOI: 10.1002/ffj.2730060209

Adesanwo, K. J., Shode, F. O., Aiyelaagbe, O. O., Rabiu, O. O., Oyede, R. T., and Oluwole, F. S. (2009). "Antisecretory and antiulcerogenic activities of the stem bark extract of *Melaleuca bracteata* and isolation of principles," *J. Med. Plants Res.* 3(10), 822-824.

Al-Abd, N. M., Mohamed Nor, Z., Mansor, M., Azhar, F., Hasan, M. S., and Kassim, M. (2015). "Antioxidant, antibacterial activity, and phytochemical characterization of *Melaleuca cajuputi* extract," *BMC Complem. Altern. Med.* 15(1), 1-13. DOI: 10.1186/s12906-015-0914-y

Batish, D. R., Singh, H. P., Kohli, R. K., and Kaur, S. (2008). "Eucalyptus essential oil as a natural pesticide," *Forest Ecol. Manage.* 256(12), 2166-2174. DOI: 10.1016/j.foreco.2008.08.008

Ben Ahmed, Z., Yousfi, M., Viaene, J., Dejaegher, B., Demeyer, K., Mangelings, D., and Heyden, Y. V. (2016). "Determination of optimal extraction conditions for phenolic compounds from *Pistacia atlantica* leaves using the response surface methodology," *Anal. Methods* 8(31), 6107-6114. DOI: 10.1039/C6AY01739H

Benelli, G., Canale, A., Flamini, G., Cioni, P. L., Demi, F., Ceccarini, L., Macchia, M., and Conti, B. (2013). "Biotoxicity of *Melaleuca alternifolia* (Myrtaceae) essential oil against the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), and its

parasitoid *Psyttalia concolor* (Hymenoptera: Braconidae)," *Ind. Crop. Prod.* 50, 596-603. DOI: 10.1016/j.indcrop.2013.08.006

Chen, J. Y., Ye, Z. M., Huang, T. Y., Chen, X. D., Li, Y. Y., and Wu, S. H. (2014a). "Identification of volatiles in leaves of *Alpinia zerumbet* 'Variegata' using headspace solid-phase microextraction-gas chromatography-mass spectrometry," *Nat. Prod. Commun.* 9(7), 999-1001.

Chen, J., Wu, S., and Li, Y. (2014b). "Chemical composition, antioxidant and antibacterial activities of essential oil from leaves of *Alpinia zerumbet* 'Variegata'," *Res. J. Biotechnol.* 9(8), 50-57.

Dahmoune, F., Nayak, B., Moussi, K., Remini, H., and Madani, K. (2015). "Optimization of microwave-assisted extraction of polyphenols from *Myrtus communis* L. leaves," *Food Chem.* 166, 585-595. DOI: 10.1016/j.foodchem.2014.06.066

Farag, R. S., Daw, Z. Y., Sidky Mahassen, M. A., and Mohamed Saffaa, H. (1998). "Biochemical and biological studies on some tea trees (*Melaleuca* spp.) essential oils," *Adv. Food Sci.* 20(5-6), 153-162.

Gaínza, Y. A., Domingues, L. F., Perez, O. P., Rabelo, M. D., López, E. R., and Chagas, A. C. d. S. (2015). "Anthelmintic activity *in vitro* of *Citrus sinensis* and *Melaleuca quinquenervia* essential oil from Cuba on *Haemonchus contortus*," *Ind. Crop. Prod.* 76, 647-652. DOI: 10.1016/j.indcrop.2015.07.056

Gholivand, M. B., Rahimi-Nasrabadi, M., Batooli, H., and Ebrahimabadi, A. H. (2010). "Chemical composition and antioxidant activities of the essential oil and methanol extracts of *Psammogeton canescens*," *Food Chem. Toxicol.* 48(1), 24-28. DOI: 10.1016/j.fct.2009.09.007

Huang, W., Xue, A., Niu, H., Jia, Z., and Wang, J. (2009). "Optimised ultrasonic-assisted extraction of flavonoids from *Folium eucommiae* and evaluation of antioxidant activity in multi-test systems *in vitro*," *Food Chem.* 114(3), 1147-1154. DOI: 10.1016/j.foodchem.2008.10.079

Kardinan, A. K., and Hidayat, P. (2013). "Potency of *Melaleuca bracteata* and *Ocimum* sp. leaf extracts as fruit fly (*Bactrocera dorsalis* complex) attractants in guava and star fruit orchards in Bogor, West Java, Indonesia," *J. Dev. Sustain. Agr.* 8(2), 79-84. DOI: 10.11178/jdsa.8.79

Kowalski, R., Kowalska, G., Jamroz, J., Nawrocka, A., and Metyk, D. (2015). "Effect of the ultrasound-assisted preliminary maceration on the efficiency of the essential oil distillation from selected herbal raw materials," *Ultrason. Sonochem.* 24(4), 214-220. DOI: 10.1016/j.ultsonch.2014.12.008

Li, X.-J., Wang, W., Luo, M., Li, C.-Y., Zu, Y.-G., Mu, P.-S., and Fu, Y.-J. (2012). "Solvent-free microwave extraction of essential oil from *Dryopteris fragrans* and evaluation of antioxidant activity," *Food Chem.* 133(2), 437-444. DOI: 10.1016/j.foodchem.2012.01.056

Li, Y., Chen, J., Cao, L., Li, L., Wang, F., Liao, Z., Chen, J., Wu, S., and Zhang, L. (2018). "Characterization of a novel polysaccharide isolated from *Phyllanthus emblica* L. and analysis of its antioxidant activities," *J. Food Sci. Technol.* 55(7), 2758-2764. DOI: 10.1007/s13197-018-3199-6

Mothana, R., Al-Said, M., Al-Yahya, M., Al-Rehaily, A., and Khaled, J. (2013). "GC and GC/MS analysis of essential oil composition of the endemic soqotraen *Leucas virgata* Balf.f. and its antimicrobial and antioxidant activities," *Int. J. Mol. Sci.* 14(11), 23129-23139. DOI: 10.3390/ijms141123129

Naidu, B. P. (2003). "Production of betaine from Australian *Melaleuca* spp. for use in agriculture to reduce plant stress," *Aust. J. Exp. Agr.* 43(9), 1163-1170. DOI: 10.1071/EA02223

Osunsanmi, F. O., Soyingbe, O. S., Ogunyinka, I. B., Ikhile, R. A. M. M. I., Ngila, J. C., Shode, F. O., and Opoku, A. R. (2015). "Antiplatelet aggregation and cytotoxic activity of betulinic acid and its acetyl derivative from *Melaleuca bracteata*," *J. Med. Plants Res.* 9(22), 647-654. DOI: 10.5897/JMPR2015.5826

Oyedeji, O. O., Oyedeji, A. O., and Shode, F. O. (2014). "Compositional variations and antibacterial activities of the essential oils of three *Melaleuca* species from South Africa," *J. Essent. Oil Bear. Pl.* 17(2), 265-276. DOI: 10.1080/0972060X.2013.813221

Samaram, S., Mirhosseini, H., Tan, C. P., Ghazali, H. M., Bordbar, S., and Serjouie, A. (2015). "Optimisation of ultrasound-assisted extraction of oil from papaya seed by response surface methodology: Oil recovery, radical scavenging antioxidant activity, and oxidation stability," *Food Chem.* 172, 7-17. DOI: 10.1016/j.foodchem.2014.08.068

Sereshti, H., Rohanifar, A., Bakhtiari, S., and Samadi, S. (2012). "Bifunctional ultrasound assisted extraction and determination of *Elettaria cardamomum* Maton essential oil," *J. Chromatogr.* 1238, 46-53. DOI: 10.1016/j.chroma.2012.03.061

Silva, C. J., Barbosa, L. C. A., Maltha, C. R. A., Pinheiro, A. L., and Ismail, F. M. D. (2007). "Comparative study of the essential oils of seven *Melaleuca* (Myrtaceae) species grown in Brazil," *Flavour Frag. J.* 22(6), 474-478. DOI: 10.1002/ffj.1823

Tian, Y., Xu, Z., Zheng, B., and Martin Lo, Y. (2013). "Optimization of ultrasonic-assisted extraction of pomegranate (*Punica granatum* L.) seed oil," *Ultrason. Sonochem.* 20(1), 202-208. DOI: 10.1016/j.ultsonch.2012.07.010

Wilkinson, J. M., and Cavanagh, H. M. A. (2005). "Antibacterial activity of essential oils from Australian native plants," *Phytother. Res.* 19(7), 643-646. DOI: 10.1002/ptr.1716

Yatagai, M., Ohira, T., and Nakashima, K. (1998). "Composition, miticidal activity and growth regulation effect on radish seeds of extracts from *Melaleuca* species," *Biochem. Syst. Ecol.* 26(7), 713-722. DOI: 10.1016/S0305-1978(98)00034-9

Ye, C.-L., Dai, D.-H., and Hu, W.-L. (2013). "Antimicrobial and antioxidant activities of the essential oil from onion (*Allium cepa* L.)," *Food Control* 30(1), 48-53. DOI: 10.1016/j.foodcont.2012.07.033

Article submitted: June 21, 2018; Peer review completed: August 25, 2018; Revised version received: September 11, 2018; Accepted: September 12, 2018; Published: September 26, 2018.
DOI: 10.15376/biores.13.4.8488-8504