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# Chemical composition, antioxidant and bioactivities of essential oils from *Melaleuca bracteata* leaves

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**Abstract**: *Melaleuca bracteata* possesses antioxidant, antibacterial, and herbicidal activities. However, the agricultural applications of *M. bracteata* have not been explored yet. The *M. bracteata* leaves were distilled, and the petroleum ether extract of the essential oils (PEEO) was analysed by GC-MS, where methyl eugenol was found to be the most abundant (66.68%). The total polyphenol content (TPC), the total flavonoids content (TFC) of the PEEO were 6.617 ± 0.535 mg gallic acid equivalents/g and 7.086 ± 0.452 mg rutin equivalents/g, respectively. The $IC_{50}$ values of the DPPH, ABTS and $Fe^{3+}$ were 4.180 ± 0.050 mg/ml, 5.400 ± 0.140 mg/ml, and 8.935 ± 0.067 mg/g, respectively. The $EC_{50}$ value of the PEEO was 33.78 ± 2.35 μg/ml against the *Pyricularia oryzae*. The minimum inhibitory concentration and minimum bactericidal concentration values were 0.10 mg/ml and 0.45 mg/ml against *Bacillus subtilis*. The results indicate that *M. bracteata* PEEO possesses excellent antioxidant activities and bioactivities.

**Keywords**: total phenolic content; total flavonoids content; scavenging activity; antifungal activities; antibacterial activities

The increasing problems associated with the safety of synthetic additives in food and crop resistance to chemical pesticides (Chan *et al.* 2012; Kim *et al.* 2015) have contributed to the search for natural alternatives, and have sparked research on the uses of medicinal plants (García-Heredia *et al.* 2016). Many essential oils (EOs) from medicinal plants have been shown to have antioxidant (Riahi *et al.* 2013), antibacterial (Senthilkumar & Venkatesalu 2013), antiviral, antifungal, anti-parasitic (Ribeiro *et al.* 2014) properties. As antioxidants, EOs have exhibited the potential for treating diseases associated with free radical oxidation, such as Alzheimer's disease, hepatitis and liver cirrhosis (Hefnawy & Ramadan 2013).

*Melaleuca bracteata* (F. Mueller) belongs to the Myrtaceae family, whose members' EOs have been reported to possess antibacterial, insecticidal and antioxidant activities (Carson *et al.* 2008; Garozzo *et al.* 2011; Souza *et al.* 2016). For example, the *Medinilla alternifolia* (Blume) EO was clinically applied to treat skin infections (Millar & Moore 2008) and to control *Aeromonas hydrophila* (Chester, 1901) in an aquaculture (Souza *et al.* 2016). The

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*Melaleuca quinquenervia* (Cavanilles) EO was used to control animal intestinal nematodes (Gaínza *et al*. 2015), *Culex quinquefasciatus* (Say, 1823), *Aedes aegypti* (Linnaeus, 1762) and *Aedes albopictus* (Skuse, 1894) (Leyva *et al*. 2016). Hayouni *et al*. (2008) found that the *Melaleuca armillaris* (Sol. ex Gaertn.) EO remarkably reduced the growth rate and biomass of lactic acid bacteria.

Previous research on *M. bracteata* had mainly focused on the breeding technology and cultivation management (Qu *et al*. 2009; Wu *et al*. 2010). In 1948, methyl eugenol (75.0%) was described as the dominant component of the *M. bracteata* EO in Kenya (Cosgrove *et al*. 1948). Subsequent reports by Zhong *et al*. (2009) and Ye *et al*. (2014) confirmed this discovery. Hou *et al*. (2016) optimised the extraction of the total phenolics and flavonoids from *M. bracteata* leaves using the response surface methodology, and found that the extracts showed an excellent antioxidant activity. Adesanwo *et al*. (2009) reported that the stem bark extract of *M. bracteata* containing betulinic acid showed antisecretory and antiulcerogenic activities. Melaleucin A isolated from *M. bracteata* leaves was found to have antibacterial activity against *Staphylococcus aureu*s (Li *et al*. 2017). In addition, the *M. bracteata* EO was shown to possess herbicidal activities (Almarie *et al*. 2016). However, the antioxidant, antifungal and antibacterial activities of *M. bracteata* EOs were not reported, the aim of this research was to explore the practical value of *M. bracteata* for agricultural applications. So, the *M. bracteata* leaves were distilled and extracted, the composition of its EO was analysed. The total polyphenol content (TPC), the total flavonoids content (TFC) and the antioxidative activity of the *M. bracteata* EO *in vitro* and their bio-activities were determined.

## MATERIAL AND METHODS

***Chemicals***. The Folin-Ciocalteu reagent, gallicacid (GA), aluminium chloride ($AlCl_3$), rutin ($C_{27}H_{30}O_{16}$), 1,1-diphenyl-2-trinitrobenzene hydrazine (DPPH), potassium persulfate ($K_2S_2O_8$), 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), potassium sorbate ($C_6H_7KO_2$), methyl eugenol ($C_{11}H_{14}O_2$), sophocarpidine, pyrogallol, Tween 80 ($C_{24}H_{44}O_6$), and the other analytical grade or pure chemicals were purchased from Sigma Company (China). The myclobutanil (99.5%) was purchased from Dr. Ehrenstorfer GmbH (Germany).

***Plant material***. The leaves of *M. bracteata* were collected from the central regions of Hainan province, China (18°46'N, 109°32'E), identified by Prof. Wenxing Long (Hainan University), and washed with water three times, dried naturally, and then cut into pieces of 4–5 mm.

Distillation and extraction of the essential oil. Following a previous protocol (Harkat-Madouri *et al*. 2015), the plant material (150 g) was distilled with steam in a round bottomed flask, controlling the outflow rate of the oil-water mixture at 1–2 drops/s for 3 h, until the outflow mixture became clear and odourless. To separate the EO from the water, the oil-water mixture was extracted with petroleum ether, ethyl acetate, chloroform and *n*-butanol, and then concentrated under vacuum at 45°C to yield the corresponding EOs, abbreviated as PEEO, EAEO, TEEO and BAEO, respectively. The extraction rate was calculated with the following formula (Equation 1):

$$\text{Extraction rate} = \text{weight of EO}/150\text{g} \quad (1)$$

where: EO – essential oil.

***Determination of the total phenolic content (TPC)***. The TPC of the EO was determined by the Folin-Ciocalteu (How *et al*. 2016) method, and compared with a gallic acid standard (Dranca & Oroian 2016). The gallic acid standard solutions (64, 32, 16, 8, 4, 2, 1 µg/ml) and sample solutions were prepared with 50% ethanol; the sample solution (1 ml), the Folin-Ciocalteu reagent (0.5 ml) and distilled water (4.5 ml) were blended and shaken for 3 min, then 4 ml of 7.5 (w/v) sodium carbonate solution was added, and the mixture was incubated at room temperature for 30 min in the dark. The absorbance was measured at 765 nm, the gallic acid standard curve was fitted, and the TPC was calculated according to the standard curve.

***Determination of the total flavonoids content (TFC)***. The TFC of the EO was determined as previously described (Harzallah *et al*. 2016). The rutin standard solutions (0.64, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01 mg/ml) and the sample solutions were prepared with 50% ethanol. The sample solution (1 ml) and ultrapure water (4 ml) were added into a tube, into which 0.3 ml 5% (v/w) sodium nitrate was added. The mixture was reacted for 6 min, and then 0.3 ml 10% (v/w) aluminium chloride was added. After 6 min at 25°C, 2 ml of 1 M sodium hydroxide was added to terminate the reaction, and the mixture was diluted

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with ultrapure water to 10 ml. The absorbance was detected at 510 nm, and the TFC was calculated according to the rutin standard curve.

**Determination of the antioxidant activities**

*DPPH scavenging activity*. As described previously (Zhou *et al*. 2013), the EO sample and the positive controls, rutin, BHT and TBHQ were diluted with 50% ethanol. The DPPH solution was prepared with 95% ethanol and placed in the dark. The sample solution (0.5 ml) was added into the DPPH solution (3.5 ml), which was incubated for 30 min at 25°C in the dark. The absorbance was detected at 517 nm, and 50% ethanol was used as the control. The DPPH scavenging rate of the EO was calculated with the following Equation 2:

$$\text{Scavenging rate}(\%) = \frac{A_b - A_s}{A_b} \times 100 \quad (2)$$

where: $A_b$ – absorbance of control; $A_s$ – absorbance of sample.

The experiment was repeated three times.

*ABTS· scavenging activity.* The ABTS· scavenging activity of the EO was tested as previously described (Thoo *et al*. 2010). The ABTS solution (10 ml, 7 mM) and potassium persulfate solution (10 ml, 2.45 mM) were mixed and placed in the dark for 12–16 h at 25°C. The absorbance of the mixed solution (ABTS· solution) was adjusted with 95% ethanol to 0.7 (± 0.02) at 734 nm. Then 0.1 ml of the sample solution or ethanol (as control) was added into the 3.9 ml ABTS· solution, and incubated for 6 min. The absorbance was measured at 734 nm. The ABTS· scavenging activity was calculated using the same formula as for the DPPH· one (Schaich *et al*. 2015). The experiment was repeated three times.

*Ferric-reducing power (FRP).* $Fe^{3+}$-TPTZ is reduced by the antioxidants to form $Fe^{2+}$-TPTZ (blue colour). A strong $Fe^{3+}$ reducing power indicates a high antioxidant activity (Henderson *et al*. 2015). The FRP of the EO was determined according to the amount of rutin equivalents (RE) that reduced the $Fe^{3+}$ in the sample's presence (Miranda *et al*. 2016). The sample solution (0.1 ml) was added to 2.9 ml of the FRP reagent (25 ml of 300 mM pH 6.6 acetate buffer solution, 2.5 ml of 10 mM TPTZ in 40 mM HCl; and 2.5 ml of 20 mM ferric chloride mixed uniformly), incubated in a water bath at 37°C for 30 min, and the absorbance was detected at 593 nm. The FRP was calculated according to the RE standard curve as the mg RE/g EO. The experiment was repeated three times.

*Gas chromatography mass spectrometry (GC-MS) analysis.* GC-MS was carried out on an Agilent 7890B-7000B chromatographer (Agilent Technologies Co. Ltd., USA); equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 μm). The carrier gas: high purity He, 1.0 ml/min; the injection port temperature: 250°C; the sampling method: splitless injection; the injection volume: 1 μl; the temperature programme: starting temperature of 60°C for 1.0 min, then heated at 5.0°C/min to 100°C, after 5.0 min, and heated at 10.0 °C/min to 250°C, keeping it steady for 35.0 min, then with 10.0°C/min to 280°C, keeping it steady for 25 min. the MS conditions: EI source; the electron energy: 70 eV; the interface temperature: 280°C; the ion source temperature: 240°C; the mass spectrum acquisition delay time was 3.5 min; the mass spectrum scanning range: 50–650 amu.

**Biological activities**

*Bioassay of the mycelium growth rate*. The antifungal activities of the PEEO were evaluated by measuring the mycelium growth rate (Xie *et al*. 2015). The target fungi (*Aspergillus oryzae* (Ahlburg) Cohn ATCC 42149), *Pyricularia oryzae* CFCC 85561, *Bipolaris cactivora* (Cavara) ATCC 42591, *Alternaria solani* (Sorauer) ATCC 62094, *Colletotrichum musae* (Berk. & M.A. Curtis) ATCC 32604 and *Fusarium oxysporum* f.sp. *radices* ATCC 52429) were cultured on a potato dextrose agar (PDA) medium. The PEEO solutions (4000, 2000, 1000, 500, 250, 125, 62.5 μg/ml) and positive control myclobutanil solutions (400, 200, 100, 50, 20, 10 μg/ml) were prepared with a 1.0% Tween 80 aqueous solution. Two millilitres of the PEEO solution or positive control (sophocarpidine, myclobutanil) were added into an18 ml sterilised PDA medium to yield a poisoned PDA (Schaich *et al*. 2015). A 1.0% Tween 80 aqueous solution was used as the control ($d_k$). The cakes (Ø 5 mm) of the target fungi punched by a pipette tip were placed in the centre of the poisoned PDA, hyphae side down, and were incubated at 28°C for 24–48 hours. The colony growth diameter (mm) for the samples ($d_s$) and controls ($d_k$) was measured using the cross method, and the inhibition rate (IR) was calculated with the following Equation (3):

$$\text{IR}(\%) = \frac{d_k - d_s}{d_k - 5} \times 100 \quad (3)$$

where: IR – inhibition rate; $d_k$ – controls; $d_s$ – samples.

*Determination of the mycelial weight*. One millilitre of the PEEO solution (40, 20, 10, 5, 2.5, 1.25 mg/ml) prepared with a 5% Tween 80 solution was added into the sterilised potato dextrose liquid medium (PD, 99 ml)

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to yield 400, 200, 100, 50, 25, 12.5 µg/ml solutions. The target fungi cakes (Ø = 5 mm) were inoculated into the PD, and cultured for 36 h at 28°C with shaking at 180 rpm. A Tween 80 solution (1 ml, 5%) was used as the blank control instead of the PEEO. The solution was filtered with two layers of the filter paper to collect the mycelia (Amorim *et al*. 2003).

*Superoxide dismutase (SOD) activity in the mycelia*. The SOD activity was determined according to a previously published protocol (Marklund & Marklund 1974) with some modifications. Briefly, the supernatant (0.1 ml) from the mycelia was quickly added into the mixture of the pyrogallol (30 uL 50 mM) and Tris-HCl buffer solution (4.5 ml, 50 mM, pH 8.2). The absorbance was measured at 325 nm. The Tris-HCl buffer solution was used as the blank control, and pyrogallol was used as the positive control. The enzyme activity was calculated according to the Equation (4):

$$\text{SOD activity} = \frac{A_o - A_s}{A_s \times 50\%} \times \frac{4.5 \text{ ml}}{0.1 \text{ ml}} \quad (4)$$

where: $A_o$ – the positive control; $A_s$ – the absorbance.

*Paper-disc agar-diffusion method*. The antibacterial activities of the PEEO were measured by the paper-disc agar-diffusion method (Sacchetti *et al*. 2005). The target bacteria (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus subtilis* ATCC 6633) were cultured on a beef extract peptone agar (BPA) medium at 37°C for 24 hours. The target bacteria (100 µl, $10^6$ CFU/ml) was added onto the BPA medium, spread evenly with sterilised rods, and then incubated for 30 minutes. The PEEO solutions (200, 100, 50, 20 mg/ml) were prepared with a 1.0% Tween 80 aqueous solution, the Rilter paper discs (Ø 5 mm) were immersed in the PEEO solution for 40 min, taken out, placed on the BPA medium, and incubated at 37°C for 24 hours. Each experiment was repeated three times. The inhibition zones were measured by the cross method, and defined according to De Billerbeck (2007) as follows: resistant, D < 6 mm; intermediate, 13 mm > D > 6 mm; sensitive, D > 13 mm.

*Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).* The MIC and MBC were determined as described previously (Said *et al*. 2016) with slight modifications. A beef extract peptone liquid medium (174–195 µl) was added in sequence into wells numbered from 1 to 22 of a 96-well plate, followed by the sequential addition of the PEEO (21-0 µl, 10 mg/ml). The beef extract peptone liquid medium (185 µl) and DMSO (10 µl) were added into well number 12 as the control. The target bacteria (5 µl, $10^6$ CFU/ml) was added into each well, and incubated at 37°C for 24 hours. Each experiment was repeated three times. The MIC of the EO was established as the concentration at which the turbidity of the above solution failed to change in any obvious way (Cherrat *et al*. 2014). The MIC activity standards of the EO were defined as: excellent activity, MIC ≤ 0.1 mg/ml; medium activity, MIC 0.1–0.2 mg/ml (Okem *et al*. 2015).

The mixing solution of 100 µl from the MIC experiment was added into the BPA medium, and cultivated at 37°C for 24 hours. Each experiment was repeated three times. The MBC was recorded when fewer than three colonies were observed.

***Data analysis***. The results were calculated from three parallel trials, and are presented as the mean ± standard deviation. The analysis of variance was performed using an IBM SPSS (version 22.0). Tukey's multiple range test (HSD) was used to compare the means of each parameter. The evaluations were based on a significance level of $P < 0.05$.

## RESULTS

***The yield, TPC and TFC of the M. bracteata essential oil***. The EOs were extracted with petroleum ether, ethyl acetate, chloroform and *n*-butanol to yield four kinds of EOs. The colours of the EOs changed from canary yellow to deep yellow, and all had a refreshing scent. The extraction rates of the PEEO, EAEO, TEEO and BAEO were 8.82 ± 26, 26.45 ± 0.69, 13.54 ± 0.60 and 12.33 ± 0.61 mg/g, respectively (Table 1).

The TPCs of the PEEO, EAEO, TEEO and BAEO were calculated (Table 1) based on the gallic acid standard curve Equation (5):

$$Y = 0.0113X + 0.0164 \quad (5)$$

($r^2 = 0.9993$, 1–64 µg/ml). Among them, the TPC of the PEEO was the highest [6.617 ± 0.535 mg of the gallic acid equivalents (GAE)/g EO], and was significantly different from the TPCs of the EAEO, TEEO and BAEO ($P < 0.05$).

Due to their high antioxidant activity, flavonoids are normally used as an important evaluation index. As shown in Table 1, the TFC of the PEEO

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Table 1. The yield, TPC, TFC and antioxidant activity of the *M. bracteata* EOs

| Samples | Yield (mg/g) | TPC (mg GAE/g EO) | TFC (mg RE/g EO) | FRP (mg RE/g EO) | $IC_{50}$ (mg/ml) | |
|---|---|---|---|---|---|---|
| | | | | | DPPH· | ABTS· |
| PEEO | 8.82 ± 0.26 | 6.617 ± 0.535 | 7.086 ± 0.452 | 8.935 ± 0.067 | 4.180 ± 0.050 | 5.400 ± 0.140 |
| EAEO | 26.45 ± 0.69 | 5.253 ± 0.459 | 3.081 ± 0.281 | 5.979 ± 0.061 | 4.690 ± 0.110 | 7.950 ± 0.090 |
| TEEO | 13.54 ± 0.60 | 5.277 ± 0.281 | 1.461 ± 0.599 | 5.194 ± 0.147 | 8.340 ± 0.320 | 8.340 ± 0.320 |
| BAEO | 12.33 ± 0.61 | 5.784 ± 0.619 | 3.475 ± 0.419 | 7.520 ± 0.080 | 9.920 ± 1.040 | 5.230 ± 0.180 |
| Rutin | – | – | – | – | 0.060 ± 0.002 | 0.113 ± 0.001 |
| BHT | – | – | – | – | 0.132 ± 0.004 | 0.075 ± 0.004 |
| TBHQ | – | – | – | – | 0.130 ± 0.003 | 0.277 ± 0.003 |

TPC – total phenol concentration; TFC – total flavonoids concentration; $IC_{50}$ – half maximal inhibitory concentration; GAE – gallic acid equivalents; RE – rutin equivalents; DPPH – 1,1-diphenyl-2-trinitrobenzene hydrazine; ABTS – 2,2-Azinobis-(3--ethylbenzthiazoline-6-sulfonate; FRP – ferric-reducing power; EO – essential oil; PEEO – petroleum ether fraction of the essential oil; EAEO – ethyl acetate fraction of the essential oil; TEEO – used for symmetry; BAEO – *n*-butanol fraction of the essential oil; BHT – butylated hydroxytoluene; TBHQ – tertiary butylhydroquinone; "–" – not tested; values – mean ± standard deviation

was the highest [7.086 ± 0.452 mg rutin equivalents (RE)/g EO], amounting to 4.85 times that of the TFC of the TEEO. It is likely that the polarity and solubility of the organic solvent were the major factors influencing the TFC of the EOs.

**Antioxidant activities**

*DPPH· scavenging activity*. DPPH· is a stable free radical at room temperature, but it changes colour upon accepting electrons or hydrogen ions as shown in Figure 1A, the DPPH· scavenging rate increased with the concentration of all the EOs. At concentrations above 4 mg/ml, the scavenging rates of the PEEO and EAEO were higher than those of the TEEO and BAEO. The half maximal inhibitory concentration ($IC_{50}$) values of the DPPH from the PEEO (4.180 ± 0.050 mg/ml) and EAEO (4.690 ± 0.110 mg/ml) were significantly different from those of the other EOs ($P < 0.05$, Table 1).

*ABTS· scavenging activity*. The ABTS· scavenging activity measurement has been widely applied *in vitro* to evaluate the antioxidant activities. The ABTS· scavenging rates of the *M. bracteata* EOs are shown in Figure 1B. As with the DPPH·, the ABTS· scavenging rates increased with the increasing concentrations. The order of the $IC_{50}$ values was BAEO PEEO > EAEO > TEEO. The $IC_{50}$ values of the PEEO (5.400 ± 0.140 mg/ml) and BAEO (5.230 ± 0.180 mg/l) were not significantly different from each other ($P > 0.05$). However, the scavenging activity of the *M. bracteata* EOs was weaker than that of the chemical antioxidants rutin, BHT and TBHQ (Table 1).

*$Fe^{3+}$ reducing power (FRP)*. The FRP of the *M. bracteata* EOs increased with the increasing concentration (Figure 1C), as the corresponding curves did not cross at any point, the antioxidants from the EOs were deemed very stable. The order of the FRP for the *M. bracteata* EOs was PEEO > BAEO > EAEO > TEEO (Table 1). The FRP of the PEEO was 8.935 ± 0.067 mg RE/g EO, which was significantly different from those of the other *M. bracteata* EOs ($P < 0.05$). This order of potency reflected that of the TPC and the TFC, indicating that the FRP was closely associated with the TPC and TFC. Thus, the *M. bracteata* PEEO exhibited good antioxidant activities, which indicated that petroleum ether should be used to extract the oil-water mixture of *M. bracteata* to yield the natural antioxidants.

*Chemical composition of the PEEO*. The total ion chromatogram of each *M. bracteata* PEEO is shown in Figure 2. Each peak was identified through a comparison with the NIST98 standard MS database. The content of each peak was calculated by the area normalisation method. Twenty-eight different chemical components were identified.

The information of the chemical composition of the *M. bracteata* PEEO is shown in Table 2. The major component of the PEEO was methyl eugenol (66.68%). The other components included, among others, 3,4,5-trimethoxy-benzoic acid methyl ester (8.06%), 1-methoxy-4-propenyl-benzene (6.04%), 3,7-dimethyl-octa-1,6-dien-3-ol (3.52%), and coniferaldehyde methyl ether (2.35%).

**Biological activities**

*Antifungal activities*. The antifungal activities of the *M. bracteata* PEEO were tested against five pathogenic fungi by the mycelial growth method,

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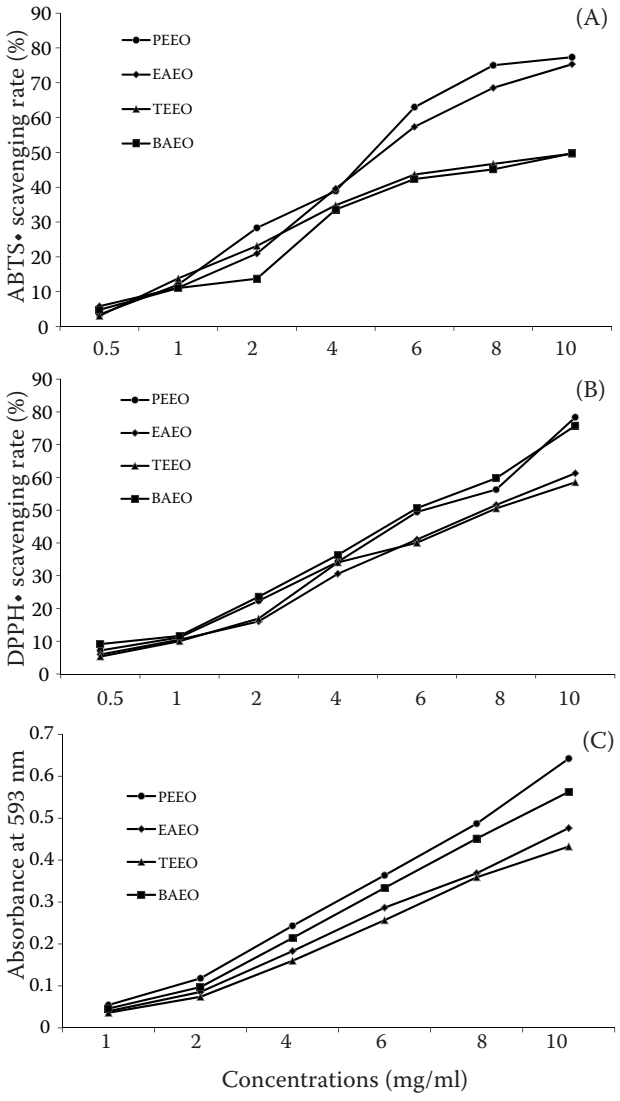


Figure 1. The antioxidant activities of the M. bracteata EOs. (A) the DPPH· scavenging activity, (B) the ABTS· scavenging activity, (C) the $Fe^{3+}$ reducing power

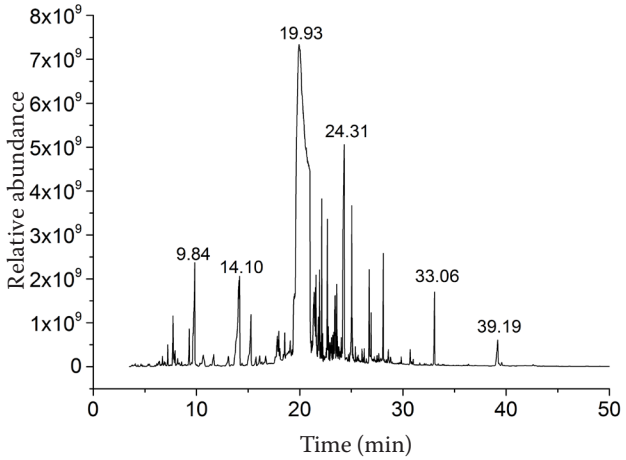


Figure 2. The total ion chromatograms of the *M. bracteata* PEEO

Table 2. The chemical compositions of the *M. bracteata* PEEO by GC-MS

| tR (min) | Compound name | Cont. (%) | Molecular formula |
|---|---|---|---|
| 6.71 | α-myrcene | 0.14 | $C_{10}H_{16}$ |
| 7.22 | α-phellandrene | 0.38 | $C_{10}H_{16}$ |
| 7.72 | benzene,1-methyl-4-(1-methylethyl)- | 0.74 | $C_{10}H_{14}$ |
| 7.81 | D-Limonene | 0.16 | $C_{10}H_{16}$ |
| 7.93 | eucalyptol | 0.27 | $C_{10}H_{18}O$ |
| 9.29 | 1,3-cyclohexadiene,1-methyl-4-(1-methylethyl)- | 0.65 | $C_{10}H_{16}$ |
| 9.84 | 3,7-dimethyl-octa-1,6-dien-3-ol | 3.52 | $C_{10}H_{18}O$ |
| 10.66 | phenylethyl alcohol | 0.57 | $C_8H_{10}O$ |
| 11.66 | 6-octenal,3,7-dimethyl- | 0.43 | $C_{10}H_{18}O$ |
| 13.08 | 3-cyclohexen-1-ol,4-methyl-1-(1-methylethyl)- | 0.36 | $C_{10}H_{18}O$ |
| 14.1 | 1-methoxy-4-propenyl-benzene | 6.04 | $C_{10}H_{12}O$ |
| 15.28 | 6-octen-1-ol, 3,7-dimethyl- | 1.74 | $C_{10}H_{12}O$ |
| 17.83 | methyl cinnamate | 0.64 | $C_{10}H_{10}O_2$ |
| 17.97 | 2,6-octadienoic acid,3,7-dimethyl-,methyl ester | 0.5 | $C_{11}H_{18}O_2$ |
| 19.93 | methyl eugenol | 66.68 | $C_{11}H_{14}O_2$ |
| 23.57 | α-cadinol | 0.85 | $C_{15}H_{26}O$ |
| 24.03 | ledene oxide-(II) | 0.22 | $C_{15}H_{24}O$ |
| 24.31 | 3,4,5-trimethoxy-benzoic acid methyl ester | 8.06 | $C_{11}H_{14}O_5$ |
| 25.05 | coniferaldehyde methyl ether | 2.35 | $C_{11}H_{12}O_3$ |
| 25.12 | 1-(1-hydroxybutyl)-2,5-dimethoxybenzene | 0.18 | $C_{12}H_{18}O_3$ |
| 26.26 | hexadecanoic acid, methyl ester | 0.09 | $C_{17}H_{34}O_2$ |
| 26.73 | dibutyl phthalate | 0.96 | $C_{16}H_{22}O_4$ |
| 26.92 | hexadecanoic acid, ethyl ester | 0.45 | $C_{18}H_{36}O_2$ |
| 28.08 | phytol | 1.21 | $C_{20}H_{40}O$ |
| 28.59 | ethyl 9,12,15-octadecatrienoate | 0.25 | $C_{20}H_{34}O_2$ |
| 30.7 | 9-ctadecenamide (Z)- | 0.22 | $C_{18}H_{35}NO$ |
| 33.06 | 1,2-benzenedicarboxylic acid, diisooctyl ester | 1.26 | $C_{24}H_{38}O_4$ |
| 39.19 | erucamide | 1.08 | $C_{22}H_{43}NO$ |

GC-MS – gas chromatography mass spectrometry; tR – retention time

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and the results were compared to the commercially available methyl eugenol, sophocarpidine and myclobutanil. The median effective concentration ($EC_{50}$) values were calculated based on the relationship between the concentration and the inhibition rate and are listed in Table 3. The antifungal activity of the PEEO was classified in the following order: *P. oryzae* > *C. musae* > *B. cactivora* > *A. solani* > *F. oxysporum*. Compared with the botanical pesticide sophocarpidine, *M. bracteata* PEEO methyl eugenol had stronger antifungal activity against *P. oryzae* and *C. musae*, indicating that this EO could serve as an effective plant fungicide. The $EC_{50}$ values of myclobutanil ranged from 0.98 to 10.37 μg/ml, and were superior to those of the PEEO, methyl eugenol and sophocarpidine.

Generally, the antifungal activity of the *M. bracteata* PEEO was better than that of methyl eugenol against the same fungus (Table 3). The $EC_{50}$ value of the PEEO was 3.02 times lower than that of methyl eugenol against *P. oryzae.* A spider chart can visually reflect the difference between two or more agricultural chemicals. Therefore, the PEEO and methyl eugenol (200 μg/ml) were tested against the target fungi. The inhibitory activities showed that methyl eugenol was located in the inner ring of the spider chart, while the PEEO was on the outside (Figure 3). *P. oryzae* was the most successfully inhibited species (92.80%), whereas *B. cactivora* differed by 38.32% between the PEEO and the methyl eugenol. Thus, the antifungal activities of the *M. bracteata* PEEO were significantly higher than those of methyl eugenol. Although the latter was the major component of the *M. bracteata* PEEO, the antifungal activity of the PEEO was clearly not ascribed to methyl eugenol alone.

To further study the antifungal activity of the *M. bracteata* PEEO, the mycelial weight was measured. With the increasing concentration of the PEEO, the mycelial weights of the target fungi decreased (Figure 4A). Compared with the control 200 μg/ml, the PEEO exerted the highest inhibition on the *P. oryzae* mycelial weight (82.58%), confirming the result from Figure 3. Even though the initial mycelial weight of *C. musae* was higher than that of the other target fungi, it decreased as the PEEO treatment concentration increased. Therefore, the PEEO exhibited good antifungal activity, especially on *P. oryzae*.

The SOD activity of the *M. bracteata* PEEO was also evaluated. With the increasing concentration of the PEEO, the SOD activities of all the target fungi decreased as shown in Figure 4B. In summary, our data demonstrate that the PEEO exhibited a good antifungal activity.

*Antibacterial activities*. The inhibitory zones of the *M. bracteata* PEEO were determined against

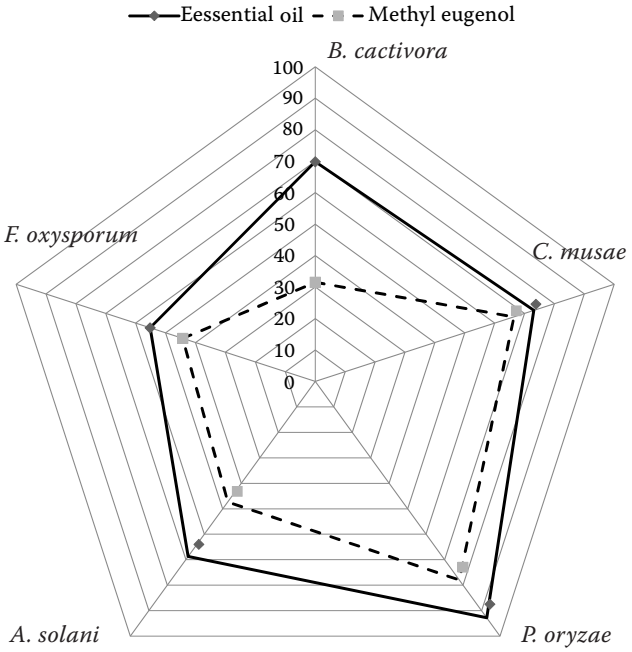


Figure 3. The spider chart of the antifungal activity of the PEEO and methyl eugenol (200 μg/ml)

Table 3. The $EC_{50}$ values (mean ± standard deviation) of the *M. bracteata* PEEO, methyl eugenol, sophocarpidine and myclobutanil

| Fungus | PEEO $EC_{50}$ (μg/ml) | Methyl eugenol $EC_{50}$ (μg/ml) | Sophocarpidine $EC_{50}$ (μg/ml) | Myclobutanil $EC_{50}$ (μg/ml) |
|---|---|---|---|---|
| *P. oryzae* | 33.78 ± 2.35 | 102.06 ± 2.74 | 65.42 ± 2.83 | 3.49 ± 0.47 |
| *B. cactivora* | 94.32 ± 5.43 | 252.13 ± 4.67 | 54.87 ± 3.65 | 0.98 ± 0.06 |
| *A. solani* | 138.25 ± 9.72 | 264.06 ± 6.42 | 83.85 ± 4.32 | 1.47 ± 0.09 |
| *C. musae* | 85.37 ± 5.01 | 142.88 ± 3.11 | 97.86 ± 5.96 | 2.02 ± 0.15 |
| *F. oxysporum* | 205.76 ± 14.70 | 217.95 ± 4.67 | 122.86 ± 7.13 | 10.37 ± 0.97 |

$EC_{50}$ – the median effective concentration; PEEO – the petroleum ether fraction of the essential oil

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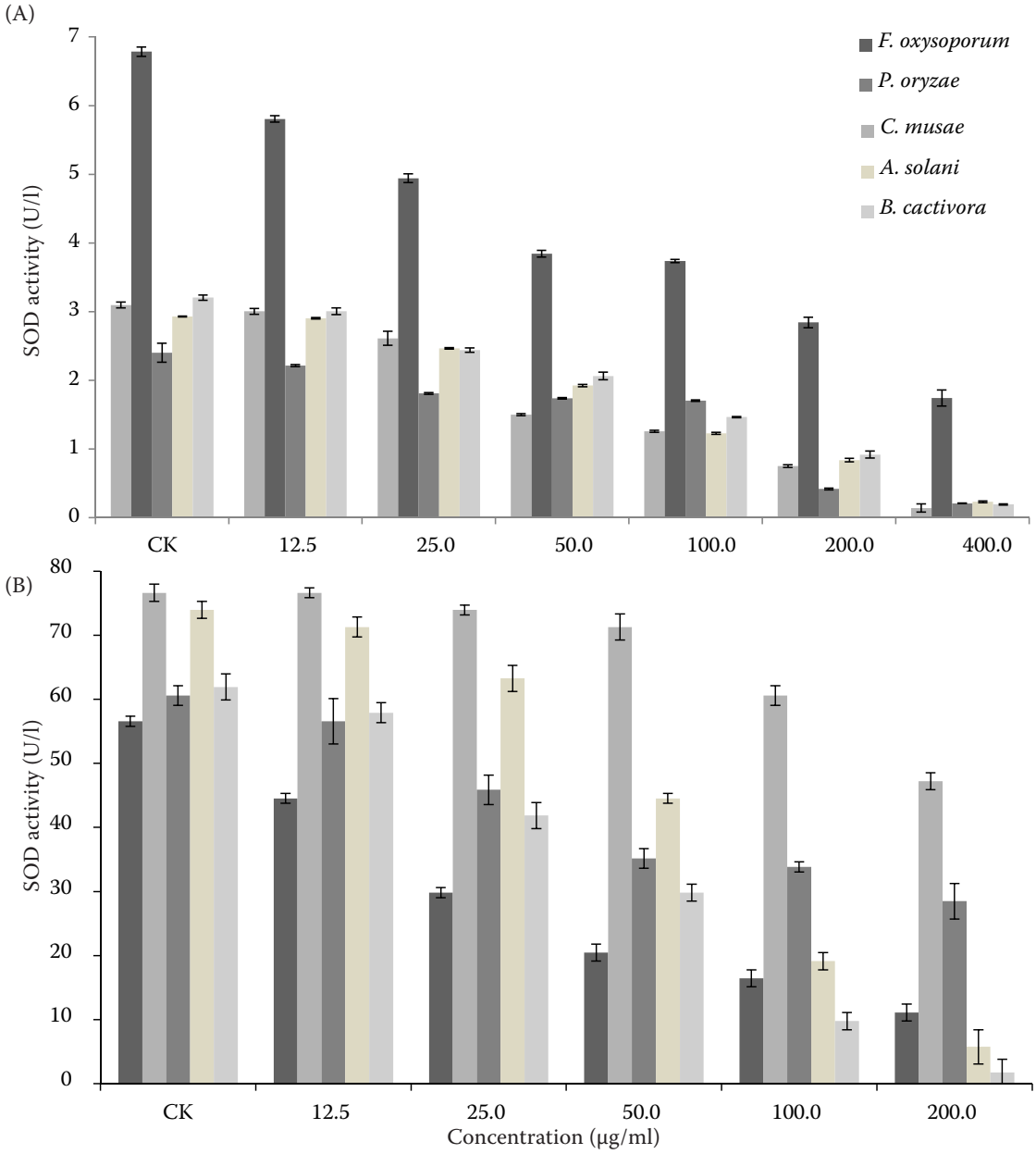


Figure 4. The antifungal effect of the *M. bracteata* PEEO

(A) the mycelial weight (B) the SOD activity

the target bacteria. Most inhibitory zones ranged from 6 mm to 13 mm at the applied experimental concentrations, except *P. aeruginosa* at 20 mg/ml (Table 4). This result indicates that the PEEO exhibited a good inhibitory activity against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis*. The effect of the PEEO differed significantly from that of the potassium sorbate and methyl eugenol controls ($P < 0.05$). With the increasing concentration of the *M. bracteata* PEEO, the inhibitory zones became bigger. At a concentration > 100 mg/ml, the inhibitory activity of the PEEO on the bacteria was *E. coli* > *B. subtilis* > *S. aureus* > *P. aeruginosa*. This result suggests that the *M. bracteata* PEEO could be used as an antimicrobial agent in food.

***MIC and MBC***. The MIC and MBC of the *M. bracteata* PEEO were tested against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* (Figure 5). The MIC of the PEEO was 0.1 mg/ml against *B. subtilis*, which showed that the PEEO had an excellent antibacterial activity. The *M. bracteata* PEEO had a medium activity against *E. coli* (MIC = 0.2 mg/ml), while those against *S. aureus* and *P. aeruginosa* were 0.35 mg/ml and 0.4 mg/ml, respectively.

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Table 4. The inhibitory zones of the *M. bracteata* PEEO, methyl eugenol and potassium sorbate (mm)

| Strains | Concentrations of PEEO (mg/ml) | | | | Potassium sorbate (1 mg/ml) | Methyl eugenol (200 mg/ml) |
|---|---|---|---|---|---|---|
| | 200 | 100 | 50 | 20 | | |
| *E. coli* | 11.58 ± 0.28 | 10.70 ± 0.13 | 10.15 ± 0.20 | 7.42 ± 0.13 | 5.00 ± 0.00 | 5.00 ± 0.00 |
| *S. aureus* | 9.18 ± 0.06 | 8.83 ± 0.10 | 8.68 ± 0.10 | 8.60 ± 0.05 | 5.00 ± 0.00 | 5.00 ± 0.00 |
| *P. aeruginosa* | 8.65 ± 0.10 | 7.58 ± 0.08 | 6.60 ± 0.26 | 5.42 ± 0.03 | 6.73 ± 0.22 | 5.00 ± 0.00 |
| *B. subtilis* | 11.05 ± 0.28 | 9.28 ± 0.08 | 8.18 ± 0.13 | 7.42 ± 0.13 | 5.00 ± 0.00 | 5.00 ± 0.00 |

PEEO – the petroleum ether of the essential oil; the values shown – mean ± standard deviation

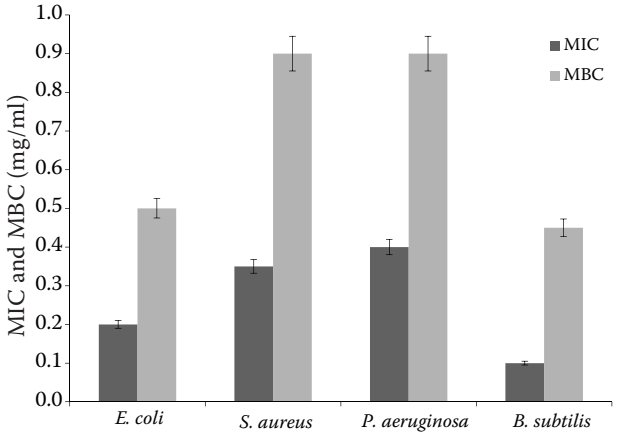


Figure 5. The minimum inhibitory concentration and minimum bactericidal concentration of the *M. bracteata* PEEO

MIC – the minimum inhibitory concentration; MBC – the minimum bactericidal concentration

The MBC of the PEEO was 0.45 mg/ml and 0.50 mg/ml against *B. subtilis* and *E. coli* respectively, consistent with the MIC result. The *M. bracteata* PEEO displayed good antibacterial activities against *E. coli*, and particularly *B. subtilis*

## DISCUSSION

The highest extraction rate of the *M. bracteata* EO was obtained with ethyl acetate (2.645%), similarly to what had been reported previously (Al-Matani *et al.* 2015). The differences in the extraction rate might be caused by the different polarity and solubility of the organic solvents. At 2.645%, The extraction rate of the EAEO was higher than that reported by Zhong *et al.* (2009), Li *et al.* (2009) and Qin *et al.* (2014) (1.13%, 0.913%, 0.69%, respectively), but lower than that reported by Ye *et al.* (2014) (4.51%).

Almarie *et al.* (2016) reported that the yield of the *M. bracteata* EO by hydrodistillation was 0.42%, whereas Siddique *et al.* (2017) reported a value of 0.14%. The latter was calculated by comparing the volume of the EO to the weight of fresh leaves (v/w), and, thus, differed from our calculation method. In general, the yields were lower than ours (Table 1). In our experiment, a milky liquid was formed when the *M. bracteata* EO entered the water by hydrodistillation, which indicated that the EO and water were emulsified. It is likely that the components dissolved in the water were also extracted, hence the higher yield.

The TPCs and TFCs of the EO are reportedly lower by hydrodistillation than by the ethanol extraction (Hou *et al.* 2016). The *M. bracteata* EOs become volatile by hydrodistillation, due to the presence of the lower boiling components. The ethanol extract of *M. bracteata* contained many components, which could explain why the TPCs and TFCs were higher following the ethanol extraction. The TPC of the PEEO was even higher than that of the *M. alternifolia* EO (1.85 ± 0.008 mg GAE/g EO) and the *Melaleuca leucadendron* (Linnaeus) EO (0.47 ± 0.036 mg GAE/g EO) (Mazzarrino *et al.* 2015). Thus, the *M. bracteata* EO may be used as a natural source of plant polyphenols.

When the polarity of the solvents increased, the DPPH· scavenging activity decreased (Fernández-Agulló *et al.* 2013). This result showed that petroleum ether was suitable to extract components with scavenging activities from the EOs. Owing to its high boiling point, it was difficult to concentrate *n*-butanol, and the volatile components tended to disappear during the concentration process. This may explain why the DPPH· scavenging activity of the BAEO was lower than with rutin, BHT and TBHQ (Table 1).

Methyl eugenol was the main component of the *M. bracteata* EO, although the amount (66.68%) was slightly lower than previously reported (Almarie *et al.* 2016; Cosgrove *et al.* 1948; Hou *et al.* 2016; Siddique *et al.* 2017; Ye *et al.* 2014; Zhong *et al.* 2009).

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Methyl eugenol has been found in 450 different kinds of plants from 80 families, especially in spices and medicinal plants, and has extensive applications (Takahashi *et al*. 2012). 3,4,5-Trimethoxy-benzoic acid methyl ester is used as a key intermediate to synthesise medicines to treat stomach pain, heart disease and anxiety (Godfrey *et al*. 2013). 1-Methoxy-4-propenyl-benzene is a common volatile substance in plants (Ma *et al*. 2015). Here, the bioactivities of essential oil were generally higher than those of methyl eugenol alone.

The content of methyl cinnamate was 0.68%, which was lower than previously reported (11.4%) (Siddique *et al*. 2017). The discrepancy could be explained by losses during extraction, or to the different source of our *M. bracteata* strain. Siddique *et al*. (2017) also found that the methyl cinnamate content differed between *M. bracteata, Melaleuca fulgens* (Robert Brow) and *Melaleuca leucodendron* (Linnaeus).

Twenty-eight components of *M. bracteata* EO were classified into ethers (72.72%), esters (12.21%), alcohols (8.43%), aldehydes (2.78%), alkenes (1.33%) and amides (1.28%). Ethers were the main component of the *M. bracteata* EO chiefly due to methyl eugenol.

The *M. bracteata* PEEO exhibited an excellent antifungal activity by the mycelium growth rate method, but its agricultural applications have been scarcely reported. Here, the *M. bracteata* PEEO was measured against foodborne bacteria also. Our results indicated that the *M. bracteata* PEEO's antibacterial activity was similar to those previously reported against *S. aureus* and *P. aeruginosa* (MIC 4.0 mg/ml) (Dannenberg *et al*. 2016; Van Vuuren *et al*. 2014).

## CONCLUSION

In this study, we extracted the *M. bracteata* EO using four different solvents and found that the PEEO had the highest TPC and TFC than the EOs extracted by other solvents. The PEEO also showed good antioxidant activities. There were 28 components in the PEEO by GC-MS analysis, with methyl eugenol as the dominant compound. Compared to the pure methyl eugenol, the PEEO showed an excellent antifungal activity, especially against *P. oryzae*. Additionally, the PEEO displayed a good antibacterial activity against *B. subtilis*. In conclusion, the *M. bracteata* PEEO can be potentially developed into an antioxidant and applied as an antifungal and antibacterial agent.

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