Chapter

Melaleuca bracteata var. Revolution Gold (Myrtaceae) Essential Oil: Chemical Composition, Antibacterial, Membrane Damage, Antiplatelet Aggregation and Antiacetylcholinesterase Activities

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Abstract

Melaleuca bracteata var. Revolution Gold (a cultivar of *Melaleuca bracteata*) is an ornamental plant, which has been used in traditional medicine for the treatment of several diseases. Till moment, information is scanty on the biological activities of the essential oil from the plant. The water-distilled essential oil was analyzed by gas chromatography and gas chromatography-mass spectrometry. Antibacterial activity of the oil was evaluated by paper disc diffusion and micro-dilution methods. Cell membrane damage was assay using cytosolic lactate dehydrogenase released method. Platelet aggregation inhibitory activity was separately evaluated on Adenosine diphosphate, collagen, epinephrine and thrombin induced aggregation. Thirteen components representing 95.3% of the total oil were identified from the essential oil. Phenylpropanoids (82.9%) constitute the predominant class of compounds in the oil. On the whole, the oil displayed strong antibacterial action towards *Staphylococcus aureus*, moderate activity on *Bacillus cereus* and some strains of *Escherichia coli*. The lactate dehydrogenase released (0.78–47%) depicted the oil to exhibit low levels of membrane damage. The percentage platelet aggregation inhibition for the four platelet agonists was concentration dependent with thrombin > collagen > ADP > epi-nephrine. The acetylcholinesterase inhibitory activity (9.16%) indicated that the essential oil was not effective against the enzyme.

Keywords: *Melaleuca bracteata* var. revolution gold, Myrtaceae, essential oil, methyl eugenol, biological activity

1. Introduction

Melaleuca bracteata L. (Syns: *Melaleuca daleana* Blakely, *Melaleuca glaucocalyx* Gand. or *Melaleuca monticola* J.M.Black) and commonly known as black tea tree, honey myrtle, golden bottle brush amid other names, belongs to the Myrtaceae family [1]. *Melaleuca bracteata* var. Revolution Gold (popularly known as *Melaleuca bracteata* var. "Johannesburg Gold") is a garden cultivar of *Melaleuca bracteata* and widely found in woodlands, open forests along watercourses and on the edges of swamps as well as garden and urban street ornamental plant in South Africa [1, 2]. *Melaleuca bracteata* var. Revolution Gold is a shrub or medium-size tree growing as tall as 5 m, with dark gray stem-bark. The leaves (*ca* 7 cm by 25 cm) with intact margin are evergreen, alternately arranged, ovate to lanceolate. The flowers in clusters vary from white to pink-red, pale yellow or greenish, with small petals and bundle of stamens. The fruits (2–3 mm) with numerous seeds of about 0.5–0.8 mm long aggregated into cylindrical stacks along the twigs [1, 2]. In traditional medicine, *M. bracteata* var. Revolution Gold has been reported used for treatment and prevention of numerous diseases [1, 2]. Previous studies on different extracts of *M. bracteata* var. Revolution Gold revealed the isolation of betulinic acid, oleanolic acid, maslinic acid and their derivatives, with many possessing antibacterial, anti-inflammatory, antiplatelet aggregation, antifungal, antiulcer antioxidant, anti-sickling and cytotoxic activities [3–7].

As a continuation of our studies on the flora of South African species [8–11], we reports the chemical composition, antibacterial, membrane damage, acetylcholinesterase and antiplatelet aggregation activities of essential oil of *Melaleuca bracteata* var. Revolution Gold collected from KwaDlangezwa area in uThungulu District Municipality, KwaZulu-Natal Province, South Africa.

2. Experimental

2.1 Chemicals and reagents

Analytical grade chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

2.2 Animals

Either sex of Sprague-Dawley rats (between 8 weeks and 220 to 250 kg) were collected from the Department of Biochemistry and Microbiology, University of Zululand animal house. The animals were preserved under standard temperature of 23 ± 2°C and 12 h light dark cycle and had free access to standard pellet feed and enough drinking water. Certificate of ethic clearance number: UZREC 171110–030 PGD 2014/53 was acquired from the Research Animal Ethical Clearance Committee (RAEC) of the University.

2.3 Plant material

Melaleuca bracteata var. Revolution Gold fresh plant materials were collected from the University of Zululand, KwaDlangezwa campus, South Africa. Dr. N. R. Ntuli, a plant taxonomist at the Department of Botany, University of Zululand, identified the

plant material. Voucher specimen (VN 0256) was deposited in the Herbarium of the University.

2.4 Oil isolation

Air dried and squeezed leaves of *M. bracteata* var. Revolution Gold (300 g) were subjected to hydrodistillation in an all glassed Clevenger-type apparatus for 3 h according to an established procedure [12]. The distillate isolated was collected over water in the receiver arm of the apparatus into clean and previously weighed sample bottle, and refrigerated until further analyses.

2.5 Gas chromatography

Gas Chromatography analyses was carried out using an Agilent Gas Chromatography (7890A) equipped with Agilent 190,915 capillary column (30 m × 250 μmid; film thickness 0.25 μm) and FID detector. Oven temperature was programmed from 45°C (after 2 min) to 310°C at 5°C/min and final temperature was held for 10 min. Injection and detector temperatures were 200 and 240°C respectively. Helium was used as the carrier gas at a flow rate of 1 ml/min. Diluted oil (0.1 μl) was injected into the GC and peaks were measured by electronic integration method. *n*-Alkanes were runs at the same condition for retention indices determination.

2.6 Gas chromatography: mass spectrometry

Gas chromatography-mass spectrometry analyses was performed on an Agilent Gas Chromatography (7890A) equipped with an Agilent 190,915 capillary column (30 m × 250 μmid; film thickness 0.25 μm) interfaced with an Agilent mass spectrometer system (5975C VL MSD with Triple Axis Detector). Temperature oven was programmed from 70 to 240°C at the frequency of 5°C/min. Ion source was set at 240°C with electron ionization at 70 eV. Helium was used as the carrier gas at a flow rate of 1 ml/min. Diluted oil in hexane (1.0 μl) was injected into the GC/MS with the scanning ranges between 35 to 425 amu.

2.7 Identification of compounds

Constituents were identified on the basis of their retention times (RT) along with co-injection reference under identical experimental conditions. Comparison of their mass spectra was also check with those of NIST [13]. Furthermore, home-made MS library built up from pure substances and components of known essential oils was compared with literature [14].

3. Antimicrobial activity

3.1 Microorganisms

The acquired test microorganisms from the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa were used in the antimicrobial activity. The microorganisms included referenced *Bacillus cereus* (ATCC 10702), *Staphylococcus aureus* (ATCC

25925), *Aeromonas hydrophila* (ATCC 7966) and seven species of *Escherichia coli*, as well as environmental isolates of *Vibrio vulnificus*, *Vibrio fluvialis* and *Vibrio parahaemolyticus*. The stock cultures were maintained in 20% glycerol at −80°C until use.

3.2 Disc diffusion method

The procedure of agar disc diffusion method [15] was used to determine the antibacterial activity of *M. bracteata* var. Revolution Gold essential oil. Briefly, the microorganisms were grown overnight at 37°C in 20 mL of Mueller-Hinton broth (Oxoid). The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland no. 5 standard (1.0×10^8) $CFU.mL^{-1}$. Standard (90 mm petri dishes (Merck, South Africa) containing 12 mL of sterilized Mueller-Hinton agar (Oxoid) were inoculated with the microbial suspensions. Whatman No.1 (6 mm) sterile discs paper was individually placed on the surface of the seeded agar plates and 10 μL of the oil (5 mg/mL) was applied to the filter paper disk. The plates were incubated at 37°C for 24 h and the diameter of the resulting zones of inhibition (mm) of growth was measured. All tests were performed in triplicates. Ciprofloxacin was used as positive control.

3.3 Minimum inhibitory concentrations (MIC)

The microbroth dilution method of EUCAST [16] as described by Penduka and Okoh [17] in 96 well microtiter plates was used to determine the MIC of the oil. The test organisms were standardized to match the 0.5 McFarland standard. A starting concentration of 5 mg/ml of the oil was serially diluted in double fold strength Mueller-Hinton Broth to make different test concentrations of the oil in the wells. A volume of 20 μL of the test organisms was introduced to 100 μL of the oil in broth. The plates were incubated at 37°C for 18–24 h, and the results were visually read by adding 40 μL of 0.2 mg/ml of ρ- iodonitrotetrazolium violet (INT) to all the wells. MIC was noted as the lowest concentration of the oil or antibiotic that prevented the growth of the organism after 18–24 h. Ciprofloxacin was used as positive control.

3.4 Minimum bactericidal concentrations (MBC)

The MBC was determined using the method described by Penduka et al. [18] with some minor modifications. Briefly, the oil and antibiotics were serially diluted in double fold strength Mueller-Hinton broth in 96 well microtitre plates to make different test concentrations starting with 8× MIC value of the test antibacterial agent up to its MIC value against each organism. The organisms were standardized to match the 0.5 McFarland standard and 20 μL of the organisms were inoculated into different well containing 100 μL of the antibacterial agent in broth. The plates were incubated for 18–24 h and 15 μL of the mixture from each well was sub cultured and inoculated onto fresh Mueller-Hinton agar plates. The plates were incubated for 18–24 h and MBC was taken as the minimum concentration of the antibacterial agent that barred the growth of viable colonies.

3.5 Cytosolic lactate dehydrogenase assay

The cytosolic lactate dehydrogenase assay of membrane damage was carried out according to the process of Mosman [19] as described by Soyingbe et al. [10] with some amendments. Standardized bacterial cultures similar 0.5 MacFarland standard were grown-up for 18–24 h in a concentration of 4× MIC value of the oil and the

mixture was centrifuged at 5000 × g for 5 mins. About 50 μL of the supernatant was incubated with 50 μL mixed reaction solution of lactate dehydrogenase (LDH) release assay kit (Sigma Aldrich), at room temperature and incubated for 30 mins.

The absorbance of the mixture was determined at 492 nm using a 96 well microplate reader (Biotek Instrument ELx 808 UI). Cultures grown in 3% Triton X-100 were used as the positive control.

The percentage membrane damage (MD) was calculated using the formula:

$$\%MD = (E - C)/(T - C) \times 100. \quad (1)$$

where E = experimental absorbance of the cell cultures incubated with the test essential oil, C = control absorbance of the cell medium and T = 3% Triton X-100 treated cells supernatant.

4. Antiplatelet aggregation activity

4.1 Preparation of blood platelets

A method described previously [20] was used to prepared the blood platelets. The rats were sacrificed by a blow to the head and blood was instantly collected by cardiac puncture. The blood was mixed (5:1 $v.v^{-1}$) with an anticoagulant (acid-dextrose anticoagulant, 0.085 M Trisodium citrate, 0.065 citric acid, 2% dextrose). The platelets were gotten by runs of centrifugation at 1200 rpm for 15 min and at 2200 rpm for 3 min consecutively. The supernatant collected was centrifuged at 3200 rpm for 15 min and the resulting supernatant was discarded, and the platelets suspended in 5 ml washing buffer (pH 6.5). Re-centrifuged again at 3000 rpm for 15 min and finally re-suspended to a buffer solution of pH 7.4; containing 0.14 M NaCl, 15 mM Tris–HCl and 5 mM glucose. The platelets were further diluted with the re-suspending buffer (1:10) and the resulting solution was mixed with calcium chloride (0.4 ml: 10 μL $CaCl_2$).

4.2 Anti-platelet aggregation evaluation

Modified method [21] of anti-platelet aggregation activity was carried out to evaluate the oil action. The oil was solubilized in dimethyl sulfoxide (1:20), with 50 Mm Tris–HCl buffer to a final volume of 1% DMSO concentration. Different concentrations (1–10 $mg.mL^{-1}$) of the oil were used in the assay. The platelet aggregation inhibitory activity of the oil was separately evaluated on ADP (5 mM), collagen (5 mM), epinephrine (10 mM) and thrombin (5 $U.mL^{-1}$) induced aggregation. A 5 min. Pre-incubated platelets (100 μL) mixed with different concentrations of the oil and 20 μL of each platelets agonist was added to the mixture. Aggregation of the oil was determined using Biotek plate reader (Biotek Instrument ELx 808 UI) with Gen5 software following change in absorbance at 415 nm. DMSO (1%) was used as negative control and Aspirin was used as positive control.

4.3 Antiacetylcholinesterase (AChE) assay

Antiacetylcholinesterase activity of the essential oil was measured according to Ellman's method [22], using 96-well microplate reader. A mixture of 125 mL of 3 mM DTNB, 25 mL of 15 mM ATCI, and 50 mL of buffer, 25 mL of essential oil sample

dissolved in a buffer containing than 10% methanol were added to the wells. The absorbance was measured using (Biotek Instrument ELx 808 UI with Gen5 software) at 405 nm for every 13 for 65 s. In addition, about 25 mL of 0.22 U/mL of AChE enzyme was added and the absorbance was again read at 415 nm for every 13 for 104 s. Inhibition was calculated by comparing the rates for the oil to the blank (10% MeOH in buffer).

5. Statistical analysis

Statistical analysis of the mean value obtained for experiments was calculated as mean ± standard deviation (SD) of three independent measurements using Microsoft excel program, 2016. One way analysis of variance (ANOVA) was used for the data analysis. While, P values ≤ 0.05 were regarded as significant and P values ≤ 0.01 as very significant.

6. Results and discussion

The hydrodistillation of dried leaves of *Mb*RG gave a colorless oil in a yield of 0.17% yield (w/w), calculated on dry weight basis. **Table 1** indicates the chemical compounds identified in the oil sample, their percentages and retention indices in order of their elution on DB-5 column. Thirteen components accounting for 95.3% of the total oil were identified in the oil. The oil was characterized by a high content of phenylpropanoids (82.9%) of which methyl eugenol (77.6%), a phenylpropene was the major constituent. The minor constituents of the oil were *p*-xylene (7.0%), *trans*-methyl cinnamate (4.4%) *m*-xylene (2.3%) and linalool (2.3%). The high content of methyl eugenol (77.6%) in the present sample is in agreement with the only previously reported samples of which the contents were 82.5 and 84.6% [23]. However, some compounds such as *p*-xylene, *m*-xylene and *trans*-methyl cinnamate that were identified in the present study were not detected in the previous study [23]. Additionally, methyl eugenol was described as a key volatile constituent of essential oils of some *M. bracteata* species [24–29]. There seems to be homogeneity in the main chemical compound of essential oils of *M. bracteata* species [23–29] and the present study.

The results indicate that *Mb*RG essential oil exhibited stronger antibacterial activity against *S. aureus* with ZI, MIC and MBC values of 12.3 mm, 0.63 and 2.5 mg/mL respectively. The essential oil also displayed stout action on the growth of *B. cereus* (ZI, 11.0 mm; MIC, 1.25 mg/mL, and MBC, 5 mg/mL); *E. coli* (DSM 1089), *E. coli* (DSM 10973) and *E. coli* (DSM 9025) with MIC of 1.25 mg/mL. In addition, mild antibacterial activities were observed towards *E. coli* (DSM 8695), *E. coli* (DSM 4618) and *E. coli* (ATCC 23922) with MIC of 1.25 mg/mL and MBC of 5 mg/mL, when compared with the standards. But, reduced antibacterial effects were recorded against other tested organisms such as *A. hydrophila, V. vulnificus, V.* fluvialis and *V. parahaemolyticus* with MIC and MBC values of 5 mg/mL. On the whole, the essential oil of *Mb*RG displayed antibacterial act towards *S. aurues, B. cereus* and some strains of *E. coli*. The most resistant action was recorded by *E. coli* (DSM 10974), *A. hydrophila* and the *Vibrio* species. It could therefore be postulated that the essential oil of *Mb*RG possessed reasonable antibacterial activity. This result is in agreement with the previous report on the antibacterial activities of *Mb*RG essential oil [23] and a number of species of the genus *Melaleuca* [27–30].

Compounds[a]	RI (Cal.)	RI (Lit.)	%Composition
p-Xylene	867	865	7.0
m-Xylene	871	870	2.3
α-Phellandrene	1002	1002	0.2
o-Cymene	1017	1016	0.8
1,8-Cineole	1028	1026	0.4
α-Terpinene	1015	1014	0.2
Z-Ocimene	1037	1034	0.1
Linalool	1099	1103	1.1
Terpinen-4-ol	1177	1179	0.1
α-Terpineol	1189	1191	0.2
Estragole	1197	1196	0.9
E-Methyl cinnamate	1357	1356	4.4
Methyl eugenol	1405	1407	77.6
Aromatic compounds			9.3
Monoterpene hydrocarbons			1.3
Oxygenated monoterpenes			1.8
Phenylpropanoids			82.9
Total identified			95.3

[a]*Elution order on DB-5 column; RI (Cal.) Experimental retention indices relative to* C_9-C_{24} n-*alkanes; RI (Lit.) literature retention indices.*

Table 1.
Chemical composition of Mb*RG essential oil.*

Lactate dehydrogenase (LDH) release is a suitable, dependable and non-radioactive colorimetric method of cytotoxicity to detect necrosis which is closely associated with inflammatory diseases, based on the leakage of cytosolic enzyme from the damaged cells [17]. **Table 2** displays the lactate dehyrogenase release from the bacterial cell exposed to *Mb*RG essential oil. The result shows the percentage lactate dehydrogenase release of 47, 43, 4–21, and 0.78% for *S. aureus*, *B. cereus, E. coli* species and *A. hydrophila*, respectively. When compared with the cells treated with 3% Triton X-100 (LDH of 96%) under the same experimental conditions, it could be concluded that *Mb*RG oil exhibited moderate membrane damage. However, the oil exhibited noticeable cell membrane disruption more on Gram-positive bacteria than Gram-negative bacteria. Essential oils and their constituents have been reported to exhibit antibacterial and anti-inflammatory activities, with many studies demonstrating membrane damage, removal of harmful stimuli and healing processes [31]. This finding also supports the fact that essential oils have greater activities against Gram-positive bacteria than Gram-negative bacteria [11].

The inhibitory actions of *Mb*RG essential oil against acetyl cholinesterase could be found in **Table 3**. The value of 9.16% was too low to achieve any significant inhibitory action against the enzyme, when compared with the standard drug Tacrine (96.9%). The platelet aggregation inhibitory activity of *Mb*RG essential oil against the four platelet stimulants (ADP, collagen, epinephrine and thrombin) and the standard

Microorganisms	Antibacterial					M.d
	*Mb*rgEO[a]			Ciprofloxacin		% LDH[e]
	ZI[b] (mm)	MIC[c]	MBC[d]	ZI (mm)	MIC	
B. cereus (ATCC 10702)	11.0 ± 0.0	1.25	5	29 ± 2.5	0.63	43
S. aureus (ATCC 25925)	12.3 ± 0.6	0.63	2.5	ND	NA	47
A. hydrophila (7966)	10.3 ± 0.6	5	5	ND	NA	0.78
E. coli (DSM 1089)	12.0 ± 0.0	1.25	5	24 ± 5.7	0.31	12
E. coli (DSM 8695)	10.7 ± 0.6	2.5	—	23 ± 3.5	0.31	4
E. coli (DSM 4618)	12.0 ± 1.0	2.5	5	29 ± 2.0	0.31	7
E. coli (DSM 10973)	12.0 ± 0.0	1.25	5	19 ± 1.2	0.63	ND
E. coli (DSM 10974)	11.0 ± 0.0	5	—	25 ± 3.0	0.63	ND
E. coli (DSM 9025)	11.7 ± 0.6	1.25	—	24 ± 3.5	0.31	ND
E. coli (ATCC 23922)	12.7 ± 0.6	2.5	5	25 ± 1.2	0.31	21
V. vulnificus	12.0 ± 0.0	5	5	26 ± 1.4	2.5	ND
V. fluvialis	11.0 ± 1.0	5	5	34 ± 0.7	0.16	ND
V. parahaemolyticus	11.3 ± 0.6	5	5	33 ± 1.4	0.78	ND

[a]Mb*EO -10 µg/mL.*
[b]*ZI: Inhibition zones diameter (mm) including diameter of sterile disc (6 mm), values are given as mean ± SD (3 replicates).*
[c]*MIC - minimum inhibitory concentration (mg/mL).*
[d]*MBC - minimum bactericidal concentration (mg/mL).*
[e]*%LDH releases in relation to Triton X-100.*
M.d - Membrane damage; ND - Not determined.

Table 2.
Antibacterial and membrane damaging activities of Mb*RG essential oil.*

Sample		Acetyl cholinesterase[b]	Anti-platelet aggregation			
			ADP	Collagen	Epinephrine	Thrombin
*Mb*RG	1	9.16 ± 0.00	19.0 ± 3.3	37.0 ± 4.5	43.4 ± 1.9	32.1 ± 0.1
	3		44.0 ± 2.3	51.1 ± 3.1	45.0 ± 0.5	56.3 ± 2.5
	5		69.3 ± 0.7	62.0 ± 1.2	48.1 ± 3.4	73.0 ± 8.9
	10		79.0 ± 1.4	64.0 ± 1.1	59.6 ± 2.2	86.2 ± 6.0
	IC_{50}[c]		3.52	2.88	5.78	2.58
Aspirin	1	—	36.6 ± 0.4	27.0 ± 0.7	12.2 ± 0.6	46.1 ± 3.1
	3		55.2 ± 0.2	37.0 ± 3.2	37.1 ± 2.1	75.3 ± 2.0
	5		58.0 ± 0.7	59.0 ± 5.0	39.6 ± 1.6	77.6 ± 3.4
	10		61.0 ± 0.5	69.1 ± 1.1	57.6 ± 1.0	53.3 ± 1.2
	IC_{50}[c]		2.34	4.20	8.18	1.88
Tacrine[d]		96.90 ± 0.00	—	—	—	—

[a]*Values are given as mean ± SD (3 replicates).*
[b]*Percentage platelet aggregation inhibition.*
[c]IC_{50} *values (mg/mL).*
[d]*dcholinesterase inhibitor.*

Table 3.
Acetyl cholinesterase and antiplatelet aggregation activities of Mb*RG essential oil.*[a]

are summarized in **Table 3** as percentage platelet aggregation inhibition and lethal concentration (IC_{50}). The results are concentration dependent. As concentrations of the oil increases, percentage platelet aggregation inhibition against the four platelet agonists was significantly. However, the lethal concentration (IC_{50}) values of the oil for the four platelet agonists showed the highest strength in the order thrombin (IC_{50}: 2.58 mg/mL) > collagen (IC_{50}: 2.88 mg/mL) > ADP (IC_{50}: 3.52 mg/mL) > epinephrine (IC_{50}: 5.78 mg/mL). It can be concluded that the essential oil of *Mb*RG showed potential anti-platelet aggregation inhibitory activity.

The observed biological activities displayed by the essential oil of *Mb*RG may be attributed to the effect of methyl eugenol. This compound was known to be an inhibitor of the enzyme acetylcholinesterase [31]. In addition, essential oils containing high contents of methyl eugenol have demonstrated cytotoxicity against cancer lines [32], antimicrobial and antioxidant [33] activities. Methyl eugenol is also most effective in terms of knockdown activity, as well as repelling and killing effects, apart from larvicidal activity against *Spodoptera litura* [31].

Acknowledgements

The authors (OAL, FOO, RAM and ARO) were grateful to Medicinal Research Council, South Africa, University of Zululand Research committee and National Research Fund, South Africa. OAL and KOA are also indebted to Lagos State University, Ojo, Lagos, Nigeria.

Authors' contribution

Author OAL designed the study and wrote the final draft of the manuscript. Authors OAL, RAM and FOO performed the experiments and analyzed the data. Authors KOA, RAM and FOO wrote part of the manuscript and manage the literature search. Authors OAL and ARO supervised the work. All authors read and approved the final manuscript.

Conflict of interest

Authors declared that there are no competing interests.

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