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Identification and Mode of Action of Antibacterial Components from *Melastoma malabathricum* Linn Leaves

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ABSTRACT

The present study aimed to assess the antibacterial activity of the methanol extract of *Melastoma Malabathricum* Leaves (MMML), active fraction of *M. malabathricum* leaves (ML5) and isolated kaempferol-3-*O*-(2",6"-di-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside (Kf) and to describe the preliminary mode of action against Gram-positive and Gram-negative bacteria. The MMML extract was fractionated by Vacuum Liquid Chromatography (VLC) to afford *M. malabathricum* leaves fractions (ML1-ML6) of increasing polarities. Bioassay-guided fractionation (direct-TLC bioautography) revealed that fraction of *M. malabathricum* Leaves (ML5) had the highest antibacterial activity. Therefore, the ML5 was further subjected to Column Chromatography (CC) to afford Kaempferol-3-*O*-(2",6"-di-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside (Kf). The compound structure was elucidated using data obtained from ^{1}H NMR and ^{13}C NMR spectra. Antibacterial activity of MMML, ML5 and bioactive constituents Kf against *Staphylococcus aureus* reference strain, Methicillin-Resistant *S. Aureus* reference strain (MRSA), 11 clinical MRSA isolates, 3 clinical *Pseudomonas aeruginosa* isolates and *P. aeruginosa* reference strain was evaluated through disk diffusion method and Minimum Inhibitory Concentration (MIC). The time kill assay and field emission scanning electron microscopy was used to determine the preliminary mode of action. The data obtained from disk diffusion method and MIC showed that the MMML, ML5 and Kf showed antibacterial activity against all test bacteria with various values**.** Generally, the ML5 and Kf exhibited the highest antibacterial activity with the largest zones of inhibition and lowest MIC values against *S. aureus* and all tested MRSA. The MMML exerted the strongest killing effect against all tested bacteria while the overall performance of ML5 and Kf were significantly more effective against *S. aureus* and MRSA than *P. aeruginosa*. Field emission scanning electron microscopy revealed irregular bacterial surface indicating that the MMML, ML5 and Kf disrupted the integrity of membrane. The results obtained showed that MMML, ML5 and Kf have potential alternative to antibiotics.

Keywords: *Melastoma Malabathricum* Linn Leaves, Flavonoids, Antibacterial and Mode of Action

1. INTRODUCTION

Infectious diseases caused by pathogenic microorganisms such as bacteria, viruses, parasites or fungi affect millions of people worldwide. Discovery of antibiotics was an essential part in combating bacterial infections especially when the diseases remained as the major cause of mortality (Chanda and Rakholiya, 2011). The development of resistance to available antibiotics is a worldwide concern. Antimicrobial Resistance (AMR) is resistance of a microorganism to an antimicrobial medicine to which it was previously susceptible.

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Resistant microorganisms are able to withstand attack by antimicrobial medicines. Though, standard treatments become ineffective and infections persist and may spread to others. AMR is a consequence of the use, particularly the misuse of antimicrobial medicines and develops when a microorganism mutates or acquires a resistance gene. Globally, a high percentage of hospital-acquired infections are caused by highly resistant bacteria such as Methicillin-Resistant *Staphylococcus Aureus* (MRSA) and *Pseudomonas aeruginosa* (WHO, 2012). MRSA is a serious current health care concern with both community-associated MRSA and health care-associated MRSA are growing threats to the immune compromised patients (Broekema *et al*., 2009). Furthermore, it is a major pathogen that causes a wide variety of diseases from common and mostly uncomplicated to severe invasive infections such as intravenous catheter associated infections, ventilator associated pneumonias and surgical wound infections (Uhlemann *et al*., 2013). On the other hand, *P .aeruginosa*is one of the major causes of nosocomial infections that is highly related to opportunistic infections among hospitalized patients. It causes urinary tract infections, respiratory system infections, soft tissue infections, dermatitis, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immune suppressed. With the huge threat posed by these bacterial pathogens and the need to develop safe, more dependable than costly drugs that have no adverse side effect and potent alternatives to existing drugs. However, plant extracts and bioactive constituents isolated from ethnomedicinal plants are considered prolific resources for novel antibacterial substances with various structures and new mechanisms of action (Rios and Recio, 2005).

An increasing interest in the search for chemotherapeutics agents from natural sources including ethno medicinal plants used in traditional medicine (Sharma *et al*., 2010). In many areas, particularly in the tropical countries, traditional medicinal plants remain one of the main sources in prevention and treatment of varying ailments through self-medication (Mahomoodally *et al*., 2012). *Melastoma malabathricum* Linn. (Melastomataceae) is one of the most important herbs or shrubs found in Malaysia and known to Malays as “senduduk”. The plant has been used in traditional Malay medicine for alleviating diarrhea, leucorrhoea, puerperal infection, dysentery; wound healing, post-partum treatment and haemorrhoids (Sirat *et al*., 2010; Zakaria *et al*., 2011).

Several activities have been reported to *M. malabathricum* leaves including antiviral activity (Nazlina *et al*., 2008), antibacterial activity (Sunilson *et al*., 2008; Zulaikah *et al*., 2008; Choudhury *et al*., 2011), antioxidant activity (Sirat *et al*., 2010) and antinociceptive, anti-inflammatory and antipyretic activity (Zakaria *et al*., 2006).

In spite of different medicinal uses and various types of bioactive constituents isolated and identified from *M. malabathricum*, their therapeutics efficacy and mode of action are still unknown (Mohd. Joffry *et al*., 2012). Therefore, the scope of the present study is (1) to evaluate the antibacterial activity of the methanol extract of *M. Malabathricum* Leaves (MMML), active fraction of *M. malabathricum* Leaves (ML5) and isolated Kaempferol-3-*O*-(2",6"-di-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside (Kf) against *S. aureus*, Methicillin-resistant *S. aureus* and *P. aeruginosa*; (2) to describe the preliminary mode of action using time kill assay and field emission scanning electron microscopy.

2. MATERIALS AND METHODS

2.1. Plant Extract and Isolation of Compound

M. malabathricum Linn leaves was purchased from Ethno Herbs Resources Sdn. Bhd (Malaysia) and identified by a botanist with the specimen voucher NI01 deposited in the Herbarium, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. In preliminary extraction, 50 g of powdered plant leaves from *M. malabathricum* was treated with *n*-hexane to remove fats, waxes and chlorophylls. This is followed by extraction with methanol (MeOH) according to Green (2004) using solvent to sample dry weight ratio of 10:1 (v/w). The powdered plant leaves were stirred vigorously in MeOH at ambient temperature for 72 h. After 24 h, the liquid was removed and filtered through Whatman No.1 filter paper. MeOH was again added to the powdered leaves. The method was repeated three times. The filtered extract was dried by rotary evaporator at 40°C to yield the methanol extract of *M. malabathricum* Leaves (MMML). The MMML was fractionated by Vacuum Liquid Chromatography (VLC) on silica gel 60 (Merck, Germany; Art no.1.07747.1000) and eluted with gradient solvent system of *n*-hexane: Ethyl acetate (EtOAC) (9:1, 7:3, 5:5, 3:7 and 1:9) and EtOAC: MeOH (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9) and finally MeOH. Solvents used were analytical grade (Merck, Germany). Fractions were combined based on similar profile using Thin Layer Chromatography (TLC Kieselgel 60PF254; Merck, Germany; Art

no.1.05554.0001) plates to afford six fractions of *M. malabathricum* Leaves (ML1-ML6) according to difference in polarity and type of constituents extracted. Direct TLC-bioautography was performed to determine specific fraction showing the highest numbers of antibacterial components with sufficient amount (Sgariglia *et al*., 2011). The fraction ML5 is then further fractionated by pre-adsorption on silica gel and further subjected to Column Chromatography (CC) on silica gel 60 (Merck, Germany; Art no. 1.09385.1000) and eluted with the gradient solvent system of chloroform: MeOH to yield bioactive flavonoids. The structure of the active compound Kaempferol-3-*O*-(2",6"-di-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside (Kf) was elucidated using data obtained from ^{1}H NMR and ^{13}C NMR spectra.

2.2. Determination of Antibacterial Activity

2.2.1. Bacterial strains and Growth Condition

Staphylococcus aureus reference strain ATCC 11632, Methicillin-Resistant *S. Aureus* reference strain ATCC 43300 (MRSA), 11 clinical MRSA isolates, *Pseudomonas aeruginosa* reference strain ATCC 10145 and 3 clinical *P. aeruginosa* isolates were kindly provided by the Microbiology Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The bacteria cultures were maintained in Brain Heart Infusion Broth (BHIB; Oxoid, UK). Bacteria were cultured at 37°C for 24 h and then sub-cultured on BHI agar (Oxoid, UK) at 37°C for 24 h. For each experiment, bacteria were re-suspended in 0.85% saline to obtain the required densities equivalent to the McFarland 0.5 turbidity standard.

2.3. Disk Diffusion Method

Antibacterial activity of the MMML, ML5 fraction and bioactive compound (Kf) was determined by disk diffusion method according to CLSI (2006). Briefly, 100, 40 and 20 mg mL^{-1} of the MMML, ML5 and Kf were dissolved in 5% Dimethylsulfoxide (DMSO, Merck, Germany). The surface of the Mueller-Hinton Agar (MHA; Oxoid, UK) was inoculated using a 100 µL of 0.5 McFarland standardized inoculum suspension of bacteria and allowed to dry. Sterile filter paper disks (6 mm) were impregnated with 10 µL of each concentration, air dried, placed onto MHA and incubated at 37°C for 24 h. Vancomycin (5 µg $disk^{-1}$) and ciprofloxacin (5 µg $disk^{-1}$) were used as positive control or reference antibiotics whilst 5% DMSO serves as a negative control. The tests were performed in triplicate and the antibacterial activity was expressed as the mean of the inhibition zones diameter in millimeters (mm).

2.4. Minimum Inhibitory Concentration (MIC) Determination

Minimum Inhibitory Concentrations (MICs) were determined according to NCCLS (2000). Various concentrations of the MMML, ML5 fraction and bioactive compound (Kf) were prepared in Mueller-Hinton broth (MHB; Oxoid, UK) to a final volume of 100 µL in round bottom microplate with 96-well. Bacterial suspension adjusted to 0.5 McFarland standards was added to test solutions or antibiotic controls to a final volume of 200 µL $well^{-1}$. The negative control wells received only 200 µL of MHB. All tests were performed in triplicate. The MIC was recorded as the lowest concentration that produced a complete suppression of visible growth after 24 h incubation at 37°C.

2.5. Minimum Bactericidal Concentration (MBC) Determination

To determine minimum bactericidal concentrations (MBCs), an aliquot of 5µl was withdrawn from wells with no bacterial growth and plated onto Nutrient agar (NA; Oxoid, UK) then incubated overnight at 37°C. The lowest concentration which showed no growth on the agar was defined as the MBC.

2.6. Time-Kill Studies

The effects of the MMML, ML5 fraction and Kf on the viability of cells were monitored by Microdilution method according to NCCLS (1999). Overnight culture was inoculated into Nutrient broth (NB, Oxoid, UK) and incubated at 37°C with agitation at 151 rpm (Blue Pard, China). To each round bottom well (96-well), 100 µL of bacterial suspension adjusted to approximately 4×10^{5} colony forming unit (CFU)/ml was added to MHB containing the MMML, ML5 fraction and Kf at different concentrations of 1×, 2× and 4× MIC to a final volume of 200 µL $well^{-1}$. Aliquots were removed at 0, 4, 8, 12 and 24 h. post inoculation. Samples were serially diluted (10^{-1}-10^{-4}) in 0.85% saline. Thereafter, 10 µL of each dilution was spread aseptically onto nutrient agar and incubated at 37°C for 24 h. Colonies were counted after 24 h of incubation. All experiment was carried out in triplicate. Bactericidal activity was defined as a ≥3 $\log_{10}$ reduction in the number of survivors at each time point compared with original inoculum (99.9%) at zero time point after 24 h incubation. Bacteriostatic activity was defined as maintenance of or a <3 $\log_{10}$ decrease in the total colony forming unit CFU/ml from initial inoculums (99.9%). Killing curves were plotted as mean colony counts against time.

2.7. Field Emission Scanning Electron Microscopy (FESEM)

Field emission scanning electron microscope (FESEM) was employed to observe morphological changes in test organisms caused by the MMML, ML5 fraction and bioactive constituent (Kf) following the method modified from Sharifa *et al*. (2007). Inocula of *S. aureus*, MRSA and *P. aeruginosa* were prepared as described previously in time-kill assay. Bacterial growth was incubated with and without MMML, ML5 fraction and bioactive constituent at 37°C on shaker-incubator. Briefly, harvested cells were centrifuged at 3,000 rpm (Beckman Coulter Inc, Germany) for 15 min and then fixed in 2% glutaraldehyde in 0.1M phosphate buffer overnight at 4°C. Cells were collected by centrifugation, washed thrice with Phosphate Buffer Solution (PBS) pH 7.4. Cells were serially dehydrated with 30, 50 and 70% ethanol respectively for 5 min, followed by centrifugation for 10 min at 3000 rpm after each dehydration process. An aliquot of 20 µL of bacterial pellets were applied on Poly-L-Lysine slide (Poly-L-Lysine 0.1% in water; Sigma, France), subjected to gold coating and observed under FESEM (Zeiss, Supra^{TM}55VP, Germany).

3. RESULTS

3.1. Isolation and Evaluation of Active Constituent with Antibacterial Activity

The chromatographic separation of MMML by VLC afforded six *M. malabathricum* Leaves fractions (ML1-ML6). Bioassay-guided fractionation, direct TLC-bioautography revealed that the ML5 fraction showed the highest numbers of antibacterial components with sufficient amount (2.5 g per 1 kg of MMML). The bioactive constituents responsible for antibacterial activity against *S. aureus* and MRSA were detected. This is followed by purification of ML5 by Column Chromatography (CC) which resulted in the isolation of an effective compound. The structure of the compound was elucidated using data obtained from ^{1}H and ^{13}CNMR spectra and identified as Kaempferol-3-*O*-(2",6"-di-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside, Kf (**Fig. 1**).

3.2. Evaluation of Antibacterial Activity

The MMML, ML5 and Kf from *M. malabathricum* were evaluated for their antibacterial activity against selected bacteria. The zones of inhibition, Minimum Inhibitory Concentration (MIC) values and Minimum Bactericidal Concentration (MBC) values of the MMML, ML5 and Kf for selected bacteria are shown in the **Table 1**. The MMML displayed potent antibacterial activity with larger zones of inhibition diameter equal to 19±0 mm for *S. aureus* between 18±0 to 20.33±0.58 mm for MRSA and from 15.33±0.58 to 20±0 mm for *P. aeruginosa* at concentration 1000 µg disk^{-1} (**Fig. 2**). The zones of inhibition of the ML5 were 15.67±0.58 mm for *S. aureus*, between 15.33±0.58 to 17±0 mm for tested MRSA and from 13±0 to 15±0 mm for all tested *P. aeruginosa* at 400 µg disk^{-1}. The antibacterial activity of Kf is presented in **Table 1**. Generally, Kf showed different pattern of antibacterial activity in Gram-positive and Gram-negative bacteria. Inhibition towards *P. aeruginosa* was less than inhibition towards *S. aureus* and MRSA.

In order to obtain more quantitative and concise results, the disk diffusion method was compared with MIC and MBC values. Higher MIC and MBC values of MMML against all tested bacteria were observed compared to the ML5 values (**Table 1**). In general, the MMML exhibited antibacterial activity with the MIC values between 0.78±0 to 3.13±0 mg mL^{-1} against all tested pathogenic bacteria whereas the MIC values of ML5 against *S. aureus* and MRSA were found to be relatively lower than *P. aeruginosa* and ranged from 0.125±0 to 0.25±0 mg mL^{-1} (**Table 1**). The data obtained from MIC values showed that Kf at concentration ranging from 0.125±0 to 0.25±0 mg mL^{-1} inhibit the growth of *S. aureus* and MRSA while the MIC values varied between 0.5±0 and 1±0 mg mL^{-1} against *P. aeruginosa*. Kf showed almost the same MIC values with several MRSA strains and *S. aureus* strain (**Table 1**).

3.3. Time kill assays

The time-kill studies were used to assess the time needed by MMML, ML5 and Kf to act on bacterial cells viability of *S. aureus*, MRSA and *P. aeruginosa*. The time kill curves for *S. aureus*, MRSA and *P. aeruginosa* of MMML are shown in **Fig. 3A-C**. As shown in **Fig. 3**, treatment of *S. aureus* with MMML at concentrations 1×, 2× and 4× MIC was successful in killing bacterial cells within 8 h. However, the treatment of MRSA with MMML at concentrations 2× and 4× MIC showed lethal effect after 8 h. Complete lethality occurred at 1×, 2× and 4× MIC within 12, 12 and 8 h. Respectively after treating *P. aeruginosa* with MMML.

Table 1. Antibacterial activity of the methanol extract of *Melastoma malabathricum* Linn leaves (MMML), *M. malabathricum* leaves fraction (ML5) and Kaempferol-3-*O*-(2",6"-di-*O*-*trans*-*p*-coumaroyl)-*β*-D-glucopyranoside (Kf) against tested bacteria species (reference strains and clinical isolates), determined by disk diffusion method and minimum inhibitory concentration. The values are the means of replicates ± standard deviation.

	Plant extract/ML5 fraction/Bioactive compound Kf											
	MMML				ML5				Kf			
	Susceptibility of Bacteria (mg/ml)											
	Zone of inhibition (mm)				Zone of inhibition (mm)				Zone of inhibition (mm)			
Test bacteria	500 µg/disk	1000 µg/disk	MIC mg/ml	MBC mg/ml	200 µg/disk	400 µg/disk	MIC mg/ml	MBC mg/ml	100 µg/disk	200 µg/disk	MIC mg/ml	MBC mg/ml
Gram-positive bacteria												
S. aureus	16.67±0.58	19±0	1.56±0	3.13±0	13.67±0.58	15.67±0.58	0.25±0	0.5±0	15.67±0.58	17.67±0.58	0.25±0	0.5±0
MRSA	16.67±0.58	18.67±0.58	1.56±0	3.13±0	13.33±0.58	15.33±0.58	0.25±0	0.5±0	15.67±0.58	17.67±0.58	0.25±0	0.5±0
Clinical MRSA												
M 01	17±0	20±0	0.78±0	1.56±0	14±0	16.33±0.58	0.125±0	0.25±0	15.67±0.58	18±0	0.125±0	0.25±0
M 02	17.33±0.58	19.33±0.58	0.78±0	1.56±0	14.33±0.58	16±0	0.125±0	0.25±0	16±0	17.67±0.58	0.125±0	0.25±0
M 03	17.33±0.58	19±0	0.78±0	1.56±0	14.67±0.58	16.33±0.58	0.125±0	0.25±0	15.33±0.58	17.33±0.58	0.125±0	0.25±0
M 04	16.33±0.58	19.67±0.58	0.78±0	1.56±0	14.33±0.58	16.33±0.58	0.125±0	0.25±0	15.33±0.58	17.33±0.58	0.125±0	0.25±0
M 05	17±0	20.33±0.58	0.78±0	1.56±0	15±0	17±0	0.125±0	0.25±0	15.67±0.58	18±0	0.125±0	0.25±0
M 06	17.33±0.58	20.33±0.58	1.56±0	3.13±0	13.33±0.58	15.33±0.58	0.25±0	0.5±0	15.33±0.58	17±0	0.25±0	0.5±0
M 07	16.33±0.58	18.33±0.58	0.78±0	1.56±0	14±0	17±0	0.125±0	0.25±0	15.67±0.58	17.67±0.58	0.25±0	0.5±0
M 08	16.33±0.58	19±0	0.78±0	1.56±0	14±0	16.67±0.58	0.125±0	0.25±0	15.67±0.58	17.67±0.58	0.125±0	0.25±0
M 09	17±0	19±0	1.56±0	3.13±0	13.67±0.58	16.33±0.58	0.25±0	0.5±0	15±0	17±0	0.25±0	0.5±0
M 10	16.67±0.58	19.33±0.58	0.78±0	1.56±0	15.33±0.58	17±0	0.125±0	0.25±0	15.67±0.58	17.67±0.58	0.25±0	0.5±0
M 11	16±0.58	18±0	3.13±0	6.25±0	14.67±0.58	16.67±0.58	0.25±0	0.5±0	15.67±0.58	17.33±0.58	0.25±0	0.5±0
Gram-negative bacteria												
P. aeruginos	14.33±0.58	19±0	1.56±0	3.13±0	12.33±0.58	14.33±0.58	0.5±0	1±0	12.33±0.58	14.33±0	0.5±0	1±0
Clinical *P.aeruginosa*												
P01	16.33±0.58	20±0	0.78±0	1.56±0	13±0	15±0	0.5±0	1±0	12.33±0.58	15±0	0.5±0	1±0
P02	14±0	16.33±0.58	3.13±0	6.25±0	10.67±0.58	13±0	1±0	2±0	10.67±0.58	12±0	1±0	2±0
P03	13.33±0.58	15.33±0.58	1.56±0	3.13±0	8.67±0.58	15±0	0.5±0	1±0	12.67±0.58	14.67±0.58	0.5±0	1±0

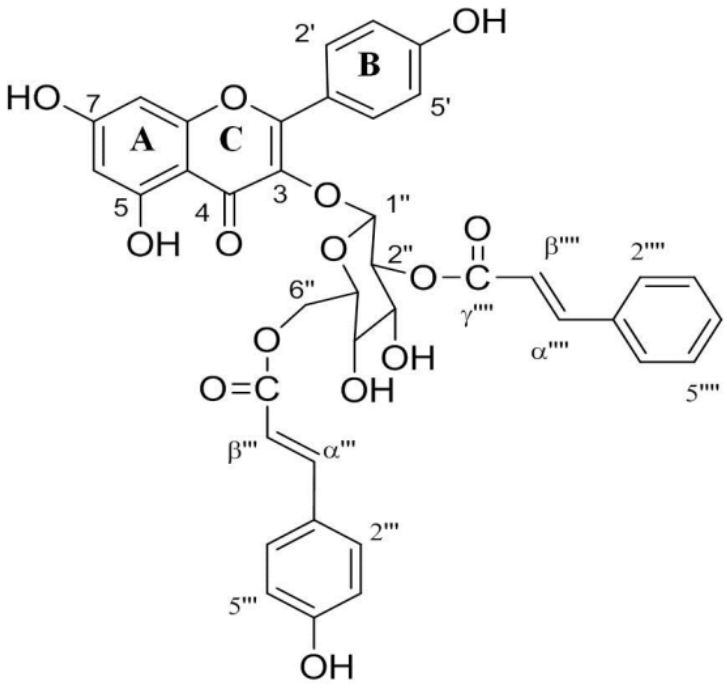


Fig. 1. Structure of Kaempferol-3-*O*-(2",6"-di-*O*-*trans*-*p*-coumaroyl)-*β*-D-glucopyranoside isolated from *M. malabathricum* Linn leaves extract

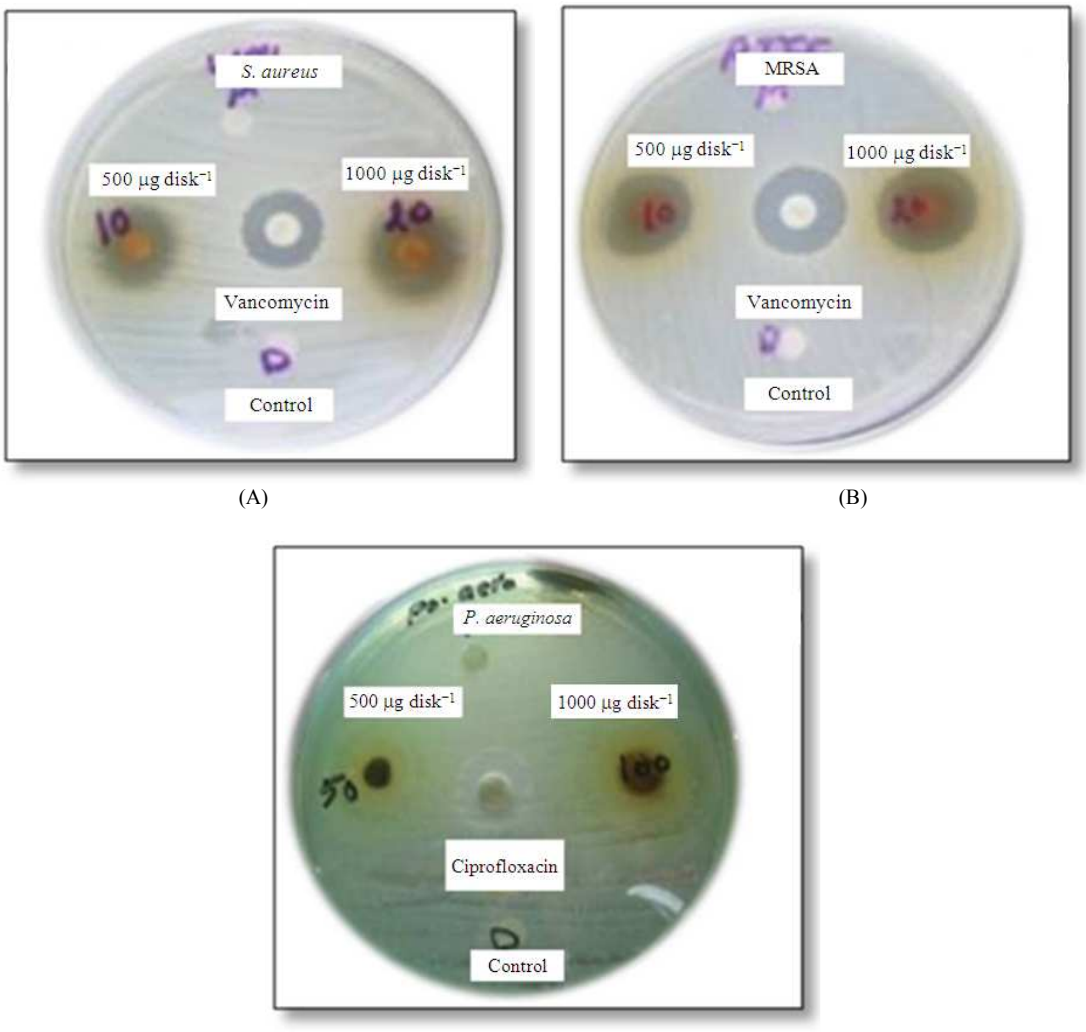
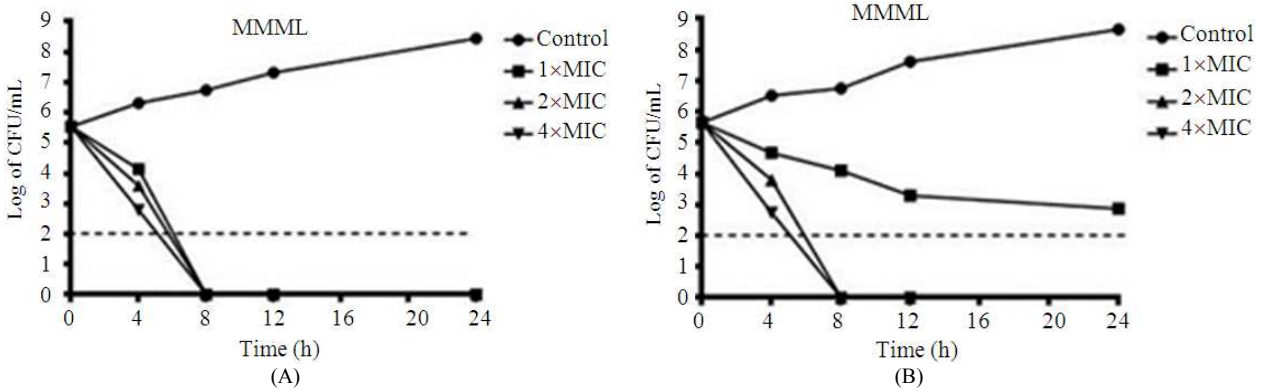


Fig. 2. (A): Zones of inhibition of *S. aureus*, (B): Zones of inhibition of methicillin resistant *S. aureus* and (C): Zones of inhibition of *P. aeruginosa* caused by the methanol extract of *M. Malabathricum* Leaves (MMML).



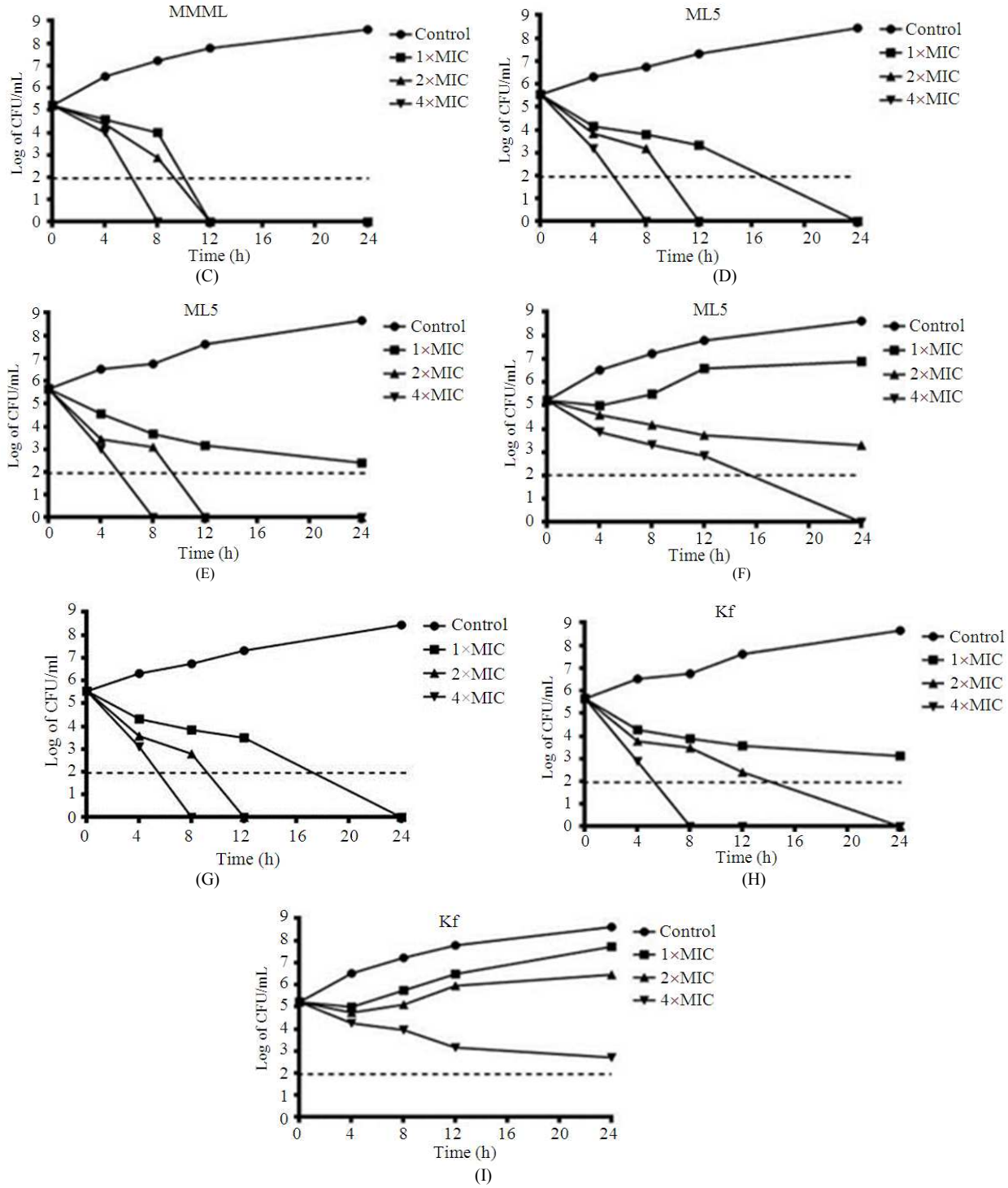


Fig. 3. Time-kill curves of *Staphylococcus aureus* (A-C), methicillin-resistant *S. aureus* (D-F) and *Pseudomonas aeruginosa* (G-I) after treatment with (1): The methanol extract of *M. Malabathricum* Leaves (MMML), (2): *M. malabathricum* leaves fraction (ML5) and (3): Kaempferol-3-*O*-(2",6"-di-*O*-*trans*-*p*-coumaroyl)-*β*-D-glucopyranoside (Kf)

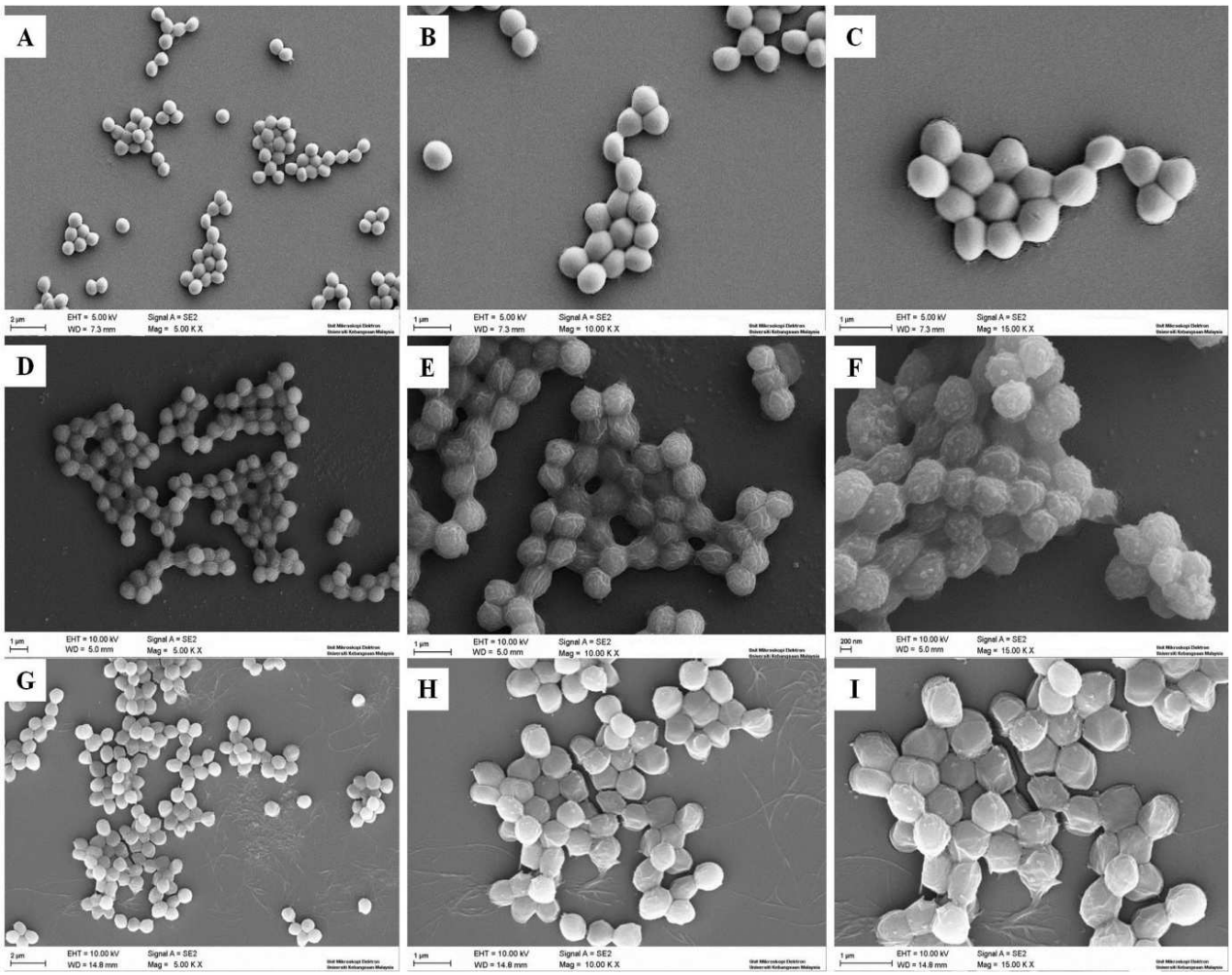
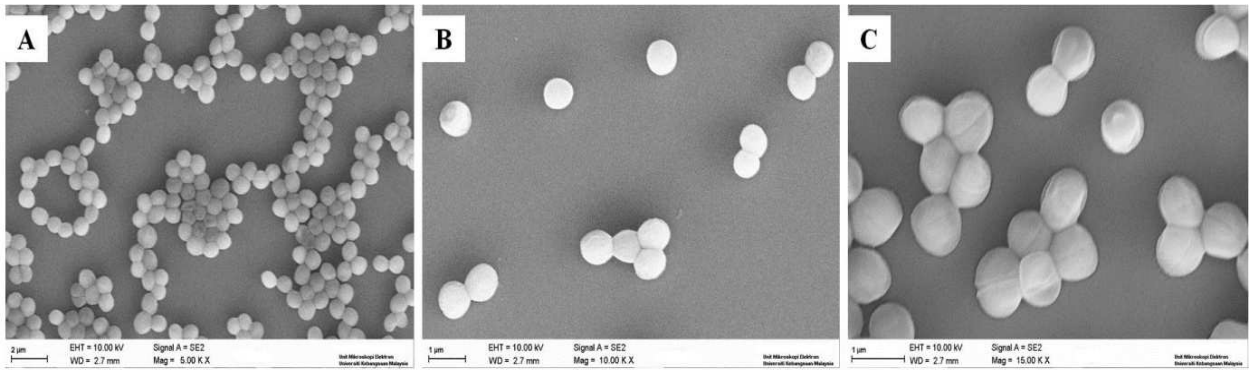


Fig. 4. External morphology of *Staphylococcus aureus* observed by Field Emission Scanning Electron Microscopy (FESEM). (A-C) untreated cells (control) incubated in Mueller-Hinton broth for 8 h at 37°C,(D-F) treated cells incubated in Mueller-Hinton broth containing 0.5 mg mL^{-1} *M. malabathricum* leaves fraction (ML5) for 8 h at 37°C and (G-I) treated cells incubated in Mueller-Hinton broth containing 1.56 mg mL^{-1} the methanol extract of *M. Malabathricum* Leaves (MMML) for 6 h at 37°C



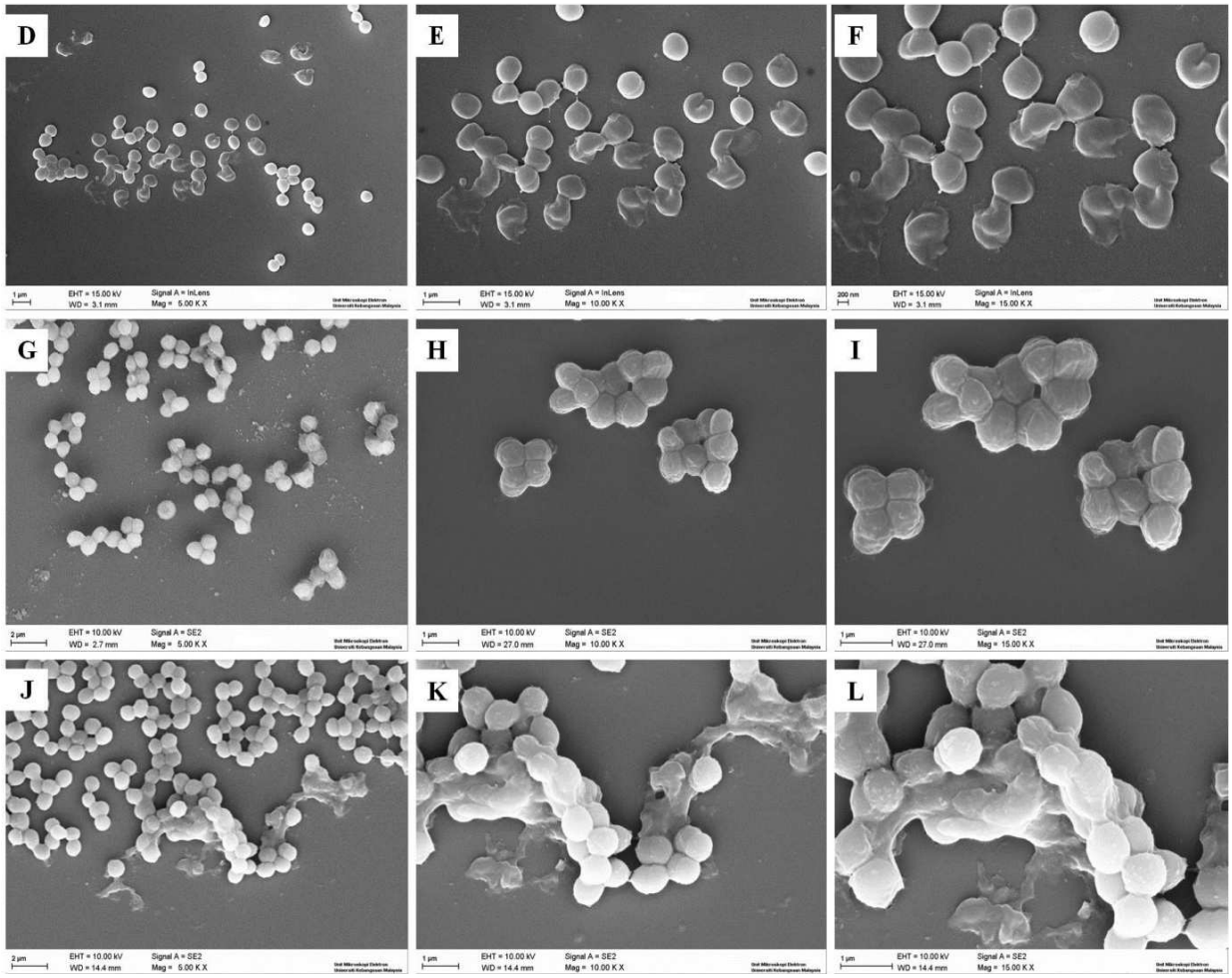
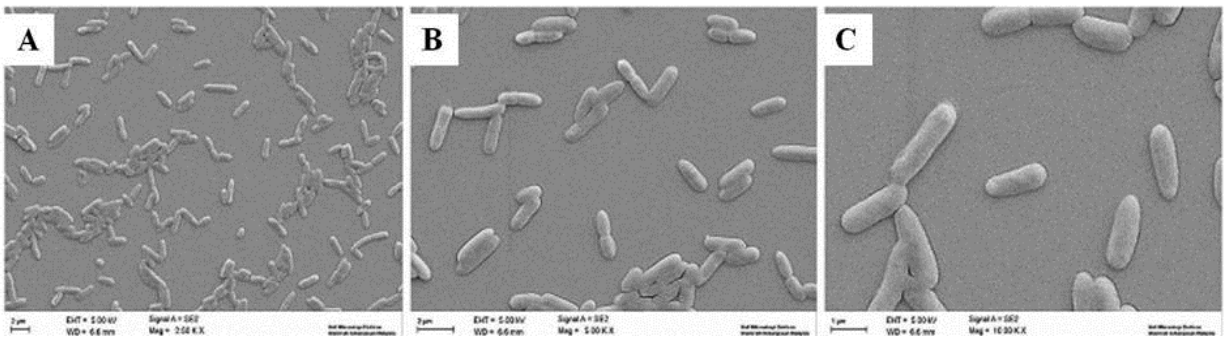


Fig. 5. External morphology of Methicillin-resistant *Staphylococcus aureus* observed by Field Emission Scanning Electron Microscopy (FESEM). (A-C) untreated cells (control) incubated in Mueller-Hinton broth for 8 h at 37°C, (D-F) treated cells incubated in Mueller-Hinton broth containing 0.5 mg mL^{-1} Kaempferol-3-*O*-(2",6"-di-*O*-*trans*-*p*-coumaroyl)-*β*-D-glucopyranoside (Kf) for 8h at 37°C, (G-I) treated cells incubated in Mueller-Hinton broth containing 0.5 mg mL^{-1} *M. Malabathricum* Leaves Fraction (ML5) for 8h at 37°C and (J-L) treated cells incubated in Mueller-Hinton broth containing 1.56 mg mL^{-1} the methanol extract of *M. Malabathricum* Leaves (MMML) for 6 h at 37°C



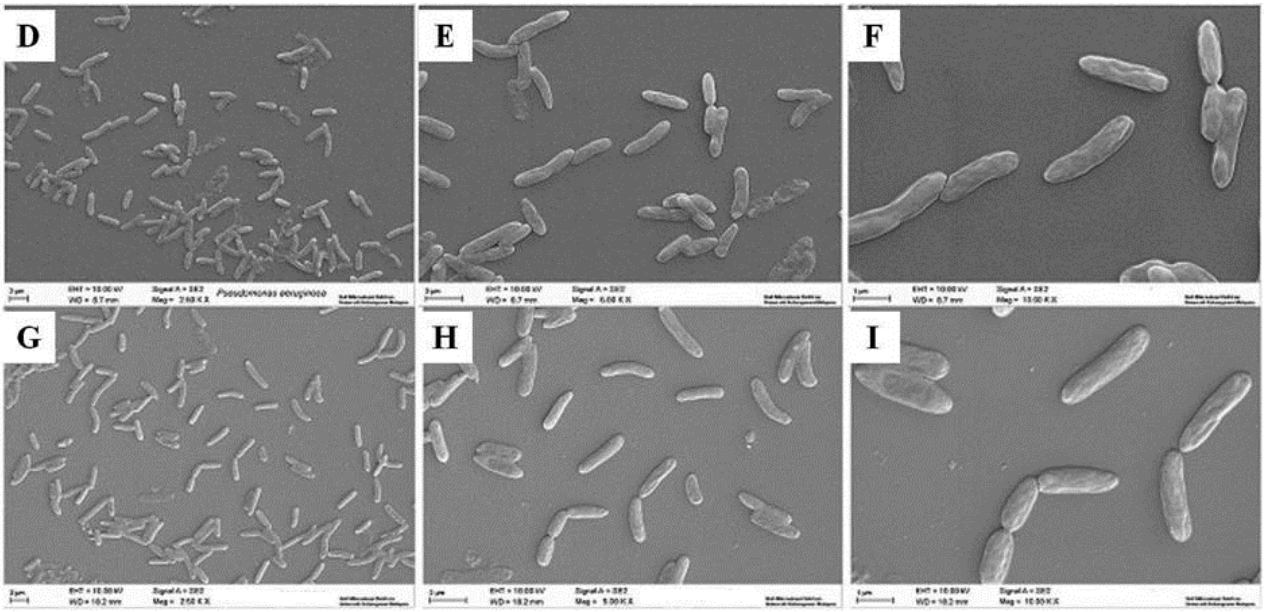


Fig. 6. External morphology of *Pseudomonas aeruginosa* observed by Field Emission Scanning Electron Microscopy (FESEM). (A-C) untreated cells (control) incubated in Mueller-Hinton broth for 8 h at 37°C, (D-F) treated cells incubated in Mueller-Hinton broth containing 2 mg mL^{-1} *M. malabathricum* Leaves Fraction (ML5) for 8 h at 37°C and (G-I) treated cells incubated in Mueller-Hinton broth containing 1.56 mg mL the methanol extract of *M. Malabathricum* Leaves (MMML) for 8h at 37°C

Table 2. The MBC/MIC ratios of the methanol extract of *M. malabathricum* leaves (MMML), *M. malabathricum* leaves fraction (ML5) and Kaempferol-3-*O*-(2",6"-di-*O*-*trans*-*p*-coumaroyl)-*β*-D-glucopyranoside (Kf). No. of strains. 1: *Staphylococcus aureus*, 2-13: Methicillin-resistant *S.aureus* and 14-17: *Pseudomonas aeruginosa*

Antibacterial agents	No. of strains with MIC/MBC ratios																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
MMML	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
ML5	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Kf	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

The time-kill curves of ML5 against *S. aureus*, MRSA and *P. aeruginosa* are presented in **Fig. 3D-F**. The overall performance of ML5 was more effective against *S. aureus* and MRSA than *P. aeruginosa*. Complete inhibition of growth was achieved at concentrations 2× and 4×MIC within 12 and 8 h. Respectively for both *S. aureus* and MRSA, whereas the treatment of *P. aeruginosa* with ML5 at concentration 4× MIC showed lethal effect on *P. aeruginosa* cells after 24 h.

The data obtained from bioassay-guided fractionation (direct TLC-bioautography) revealed that the ML5 had the highest antibacterial activity in comparison with other fractions. Purification of the ML5 by Column Chromatography (CC) resulted in the isolation of Kf. According to the time-kill curves of Kf against *S. aureus*, MRSA and *P. aeruginosa,* a gradual decline in the number of viable cells of *S. aureus* treated with 1× MIC after 24 h was observed, treatment with Kf achieved complete inhibition in growth when treated with 2× and 4× MIC after 12 and 8h respectively (**Fig. 3G**). As shown in **Fig. 3H**, Kf exerted re-growth of MRSA at 1× MIC after 4h., while complete inhibition of growth occurred at concentrations 2× and 4×MIC within 8 and 24 h. respectively. It is worth to note that Kf was not effective against *P. aeruginosa* at all concentrations, consistent re-growth was observed after 8h causing a loss of bactericidal activity (**Fig. 3I**).

3.4. Field Emission Scanning Electron Microscopy (FESEM) study

The effect of MMML, ML5 and Kf on the membrane morphology of *S. aureus* reference strain ATCC 11632,

MRSA reference strain ATCC 43300 and *P. aeruginosa* reference strain ATCC 10145 was investigated using Field Emission Scanning Electron Microscopy (FESEM). Only Kf was chosen to be examined for its effect on MRSA by FESEM. **Figure 4-6** showed the effect of MMML and ML5 on the membrane morphology of *S. aureus*, MRSA and *P. aeruginosa.* Untreated bacteria retained their morphology and showed a normal outer cell membrane. Following incubation with MMML and ML5 at concentration 1.56 and 0.5 mg mL^{-1} respectively, irregularities and physical damage were observed on the cell membrane (irregular shape and ruptured walls) of *S. aureus*, MRSA (**Fig. 4 and 5**). For MRSA treated with 0.5 mg mL^{-1} Kf, the membranes and shapes were dramatically disrupted (**Fig. 5D-F**). It should be noted that the treated bacteria were however, distinguishable from untreated bacteria.

FESEM images of *P. aeruginosa* (**Fig. 6**) showed that 1.56 and 2 mg mL^{-1} of MMML and ML5 respectively had little effect compared with *S. aureus* and MRSA. *P. aeruginosa* cells that incubated with MMML and ML5appear as distorted shapes and lost their integrity.

4. DISCUSSION

The problem of antibiotic-resistant organisms is established in both the healthcare setting and in community. Antibiotic-resistant organisms are capable of causing serious, life-threatening infections which are difficult to manage. These organisms are resistant to multiple classes of antimicrobial agents and therefore called multidrug-resistant organisms (Mulvey and Simor, 2009). Plants represent the most important source of antimicrobial compounds. Their usage in traditional medicine as remedies for several diseases is most popular for 80% of world population in Asia, Africa and South America (Gautam *et al*., 2012; Brusotti *et al*., 2013). The *M. malabathricum* leaves have been reported to possess antibacterial activity (Sunilson *et al*., 2008; Choudhury *et al*., 2011). Antibacterial activity of *M. malabathricum* was evaluated against standard and clinical isolates of Gram-positive and Gram-negative pathogenic bacteria by disk diffusion method and minimum inhibitory concentration (**Table 1**). Zakaria *et al*. (2011) reported that the methanolic extract of *M. malabathricum* possesses antimicrobial activity and this activity was ascribed to flavonoids. Flavonoids are the most known group of polyphenolic compounds for their pharmacological properties including antibacterial activity (Dulger and Hacioglu, 2009; Marzouk *et al*., 2010). A bio-guided study of ML5 resulted in isolation of Kaempferol-3-*O*-(2",6"-di-*O*-*trans*-*p*-coumaroyl)-*β*-D-glucopyranoside. Teffo *et al*. (2010) reported that kaempferol and its glycosides are well known for their pharmacological activities including antibacterial activity. It is worth to note, the presence of 4' hydroxyl substitution in the B ring, 5,7-dihydroxyl substitutions in the A ring and the substitution with 3-*O*-acyl chains in the C ring enhanced the anti-staphylococcal activity of flavonoids and that support the earlier findings of Otsuka *et al*. (2008).

S. aureus and MRSA to both ML5 and Kf were completely inhibited by 0.25±0 mg mL^{-1}. The ratio of MBC to the MIC has been used to differentiate between the bactericidal and bacteriostatic activity. If the ratio > 4 the antimicrobial agent is considered to be bactericidal while if it is more than <4, the antimicrobial agent is considered to have bacteriostatic properties (Forlenza *et al*., 1981). The ratio of MICs to the MBCs for MMML, ML5 and Kf of each of the 17 isolates was shown in **Table 2**. In this study the MBCs/MICs ratios of MMML, ML5 and Kf were > 4, indicating that MMML, ML5 and Kf have a bactericidal activity rather than bacteriostatic activity.

The time-kill studies were used to assess the time needed by MMML, ML5 and Kf to act on bacterial cells viability of *S. aureus*, MRSA and *P. aeruginosa*. The MMML, ML5 showed bactericidal effects not only against *S. aureus* and tested MRSA but also against *P. aeruginosa* (**Fig. 3A-F**) whereas, the bioactive component Kf exerted bactericidal effect against *S. aureus* and tested MRSA and bacteriostatic effect against *P. aeruginosa* (**Fig. 3G-I**). In review of Bylka *et al*. (2004), it was stated that the antibacterial activity against Gram-negative bacteria including *P. aeruginosa* is higher with flavones, while flavonoid compounds having two or three hydroxyl groups in rings A or B were active against Gram-positive bacteria. Groups Data shown in **Fig. 3** indicate that the bactericidal effect of MMML, ML5 and Kf on the viability of *S. aureus*, MRSA started before 4h.and it takes longer time when a lower concentration is used.

The field emission scanning electron microscopy study of bacterial cells treated with MMML, ML5 and Kf revealed irregular bacterial surfaces and physical damage indicating that the MMML, ML5 and Kf disrupted the integrity of the membrane. Overall, the antibacterial activity of the tested samples was noticeable more effective against the Gram-positive bacteria compared to the Gram-negative bacteria. In fact, Gram-negative bacteria are typically more resistant to antimicrobial agents than Gram-positive bacteria and this

occurrence has been explained by the presence of an outer-membrane permeability barrier, which limits access of the antimicrobial agents to their targets in the bacterial cell (Martins *et al*., 2013).

5. CONCLUSION

The MMML, ML5 and bioactive component Kf exerted potent inhibitory effects against all tested pathogenic bacteria. The *M. malabathricum* leaves fraction ML5 and Kaempferol-3-*O*-(2",6"-di-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside exhibited statistic antibacterial activity against *S. aureus* strain and all tested MRSA. The MMML, ML5 exertedbactericidal effect against all tested bacteria. Field emission scanning electron microscopy showed that the MMML, ML5 and Kf disrupted the integrity of membrane. Therefore, the results obtained exhibited that MMML, ML5 and Kf have a potential alternative to antibiotics.

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