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Combination of polymyxin B and *Aquilaria malaccensis* extract enhanced the killing and inhibited the growth of *Acinetobacter baumannii* and *Klebsiella pneumoniae*

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ABSTRACT

Aims: Polymyxins are an important last-line treatment for infections caused by multidrug-resistant Gram-negative bacteria. Nonetheless, the emergence of polymyxin-resistance and the limiting of polymyxin monotherapy urgently demands its optimisation. *Aquilaria malaccensis* (Agarwood) has been widely used as traditional medicine. Many parts of the plant including leaves exhibit a considerable *in vitro* antibacterial activity against microbial pathogens. Exploiting *A. malaccensis* in combination with polymyxins provides a novel strategy in fighting antimicrobial resistance. The objective of this study was to evaluate the combination effects of *A. malaccensis* extract with polymyxins against *Acinetobacter baumannii* and *Klebsiella pneumoniae*.
Methodology and results: *In vitro* time-kill studies and GC-MS analysis were performed to evaluate the bacterial killing of polymyxin B and extract combination and analyse chemical compounds of the extract, respectively. The combination of polymyxin B (1 mg/L) and *A. malaccensis* extract (32 mg/mL and 64 mg/mL) treatments exhibited enhanced bacterial killing compared to polymyxin B alone at 4 h and 24 h. Combination treatments also inhibited the bacterial growth of both *A. baumannii* and *K. pneumoniae* observed throughout the 24 h. More than sixty compounds including phytol, 9,12-octadecadienal, fatty acid, alkanes and terpenoids were putatively identified as the compounds that likely contributed to the antibacterial activity.
Conclusion, significance and impact of study: This study was the first to report the potential application of *A. malaccensis* extract in combination with polymyxin B in treatment against *A. baumannii* and *K. pneumoniae* and can be further investigated and optimized for the treatment of bacterial infectious diseases.

Keywords: Polymyxin B, *Aquilaria malaccensis*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, combination therapy

INTRODUCTION

Infectious diseases have been a significant cause of death and morbidity, accounting for one third of all deaths in the past decades (Bloom and Cadarette, 2019). Worryingly, the discovery and development of new antibiotics has not been able to match the rapid emergence of multidrug-resistant (MDR) bacteria (O'Neill, 2014; Schäberle and Hack, 2014; Tacconelli *et al.*, 2018). The increase in prevalence of carbapenem-resistance in the last few decades including in *Klebsiella pneumoniae*, *Acinetobacter baumannii* and other carbapenem-resistant Enterobacteriaceae (CRE) has contributed to a high mortality rate of up to 50% (Gupta *et al*., 2011; Ahmad *et al*., 2017; CDC, 2019). Global data showed that about 45% of *A. baumannii* isolates were considered MDR which was four times from the occurrence of MDR *K. pneumoniae* (Giammanco *et al.*, 2017; Harding *et al.*, 2018).

Polymyxins, an old class of cationic antibiotics have become an important last-line treatment for MDR Gram-negative bacteria (Nang *et al.*, 2021). Nevertheless, its early application in clinical setting was restrained due to the nephrotoxicity and neurotoxicity cases reported (Durante-Mangoni *et al.*, 2016; Abboud *et al.*, 2018). In addition, pharmacokinetics/pharmacodynamics (PK/PD) data showed that polymyxin monotherapy may lead to treatment failure as it is not always possible to obtain reliably *in vivo* efficacious plasma exposure (Pogue *et al.*, 2017; Landersdorfer *et al.*, 2018) along with the potential emergence of heteroresistant sub-population (Bergen *et al.*, 2011).

Plant-derived antibacterial agents provide a vast range of pharmacologically active compounds and have

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gained significant interest for the discovery and development of new drug candidates (Pešić, 2015). Unlike synthetic drugs, plant-derived agents are more economical, have a great therapeutic potential, lower risk of side effects and have also been classified as generally recognized as safe (GRAS) substances (Bauer and Brönstrup, 2014; Zhang *et al.*, 2015). The actions of compounds are very complex and tend to include a variety of different mechanisms, such as complementary or synergistic activity against microbial pathogens (Nasri and Shirzad, 2013; Wink, 2015). Plant extracts also have been found to produce MDR inhibitors (Seukep *et al.*, 2020). The compounds act as antibiotic potentiators in that though they may not have any antimicrobial properties alone, but when they are taken concurrently with standard drugs, they are able to enhance the effect of that drug (Khameneh *et al.*, 2019).

Aquilaria malaccensis is a species of agarwood tree, one of the most precious species on earth which produces a valuable fragrant resin-infiltrated wood (Liu *et al.*, 2017). Currently, its applications are not limited to aromatherapy and incense but have been increasingly explored for pharmaceutical purposes. Studies indicated that leaf extracts from the tree showed bioactivities such as antioxidant (Begum, 2016), anti-inflammatory (Eissa *et al.*, 2018) and anti-hyperglycemic (Pranakhon *et al.*, 2011). In particular, the extract of *A. malaccensis* also has been reported to exhibit antibacterial activities against *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Propionibacterium acnes*, *Escherichia coli*, *A. baumannii*, and *K. pneumoniae* (Hendra *et al.*, 2016; Jihadi *et al.*, 2020; Batubara *et al.*, 2021). The potential of agarwood leaves has been attributed predominantly by the presence of many bioactive compounds including alkaloids, tannins, phenols, terpenoids, quinones and flavonoids (Wang *et al.*, 2018).

A combination approach between antibiotics and plant extracts may lead to new ways of antibacterial treatment. A previous study showed that a combination of *Melissa officinalis* and five different antibiotics namely streptomycin, chloramphenicol, tetracycline, amoxicillin and rifamycin possess effective antimicrobial activities against selected drug resistant microorganism namely *Pseudomonas aeruginosa* and *Proteus mirabilis* (Stefanović and Comic, 2012). In addition, a combination of colistin and curcumin extract showed a considerable synergistic inhibitory effect on the *A. baumannii* growth (Kaur *et al.*, 2018). In this study, we aimed to evaluate the antibacterial activity of the combination of crude *A. malaccensis* leaf extracts and polymyxin B against *A. baumannii* and *K. pneumoniae*. The chemical constituents of the extract were then analysed using gas chromatography mass spectrometry (GC-MS) to identify and postulate the candidate of compounds likely to contribute to its antibacterial activity.

MATERIALS AND METHODS

Plant material, polymyxin B and bacterial strains

The *Aquilaria malaccensis* leaves of healthy and non-inoculated plants were acquired from a private agarwood plantation in Bangi, Selangor, Malaysia. Identification of the leaves was based on its morphology characteristics and the voucher specimen (#HBL707 [VS-1]) was deposited at the Herbarium Laboratory at Kulliyyah of Architecture and Environmental Design (KAED), International Islamic University Malaysia. Polymyxin B sulfate (PmB) was purchased from Merck (Germany). The laboratory (reference) strains of Gram-negative bacteria from American Type Culture Collection (ATCC) of *Acinetobacter baumannii* ATCC 19606 and *Klebsiella pneumoniae* ATCC 10031 were cultured in Cation-adjusted Mueller-Hinton broth (CAMHB; 20-25 mg/L Ca^{2+} and 10-12.5 mg/L Mg^{2+}). Dimethylsulfoxide (DMSO) (Chemiz, 100%) was used as the solvent for the preparation of plant crude extract.

Ethanolic leaf extraction

The freshly obtained leaves of 1 kg were watery washed and rinsed with distilled water. The leaves were put in the oven at 50 °C for 24 h for a complete drying. The leaves were grounded into fine powder before subjected to the extraction process and kept at room temperature until further use. The leaves were extracted according to a previous method with modifications (Zainurin *et al.*, 2020). The leaves powder of 20 g was added with 300 mL ethanol (HmbG, 99%) (1:15 of leaves-to-solvent ratio) in the Soxhlet apparatus, and the temperature was set at 80 °C and incubated for 18 h until the colour of solvent became colourless. To avoid degradation of phytochemical compounds and its activities, the extraction process was kept in a dark environment. The filtrate of ethanol solvent was evaporated by the rotary evaporator (Heidolph, Instruments GmbH and Co, Schwabach, Germany) at 45 °C under 100 mbar and the extract was kept at 4 °C until further use.

Static time-kill studies

Bacterial isolates of both *A. baumannii* and *K. pneumoniae* were sub-cultured onto nutrient agar plates and incubated for 16-18 h at 37 °C. A single colony of the respective bacteria was inoculated into 10 mL Mueller Hinton Broth and grown for 16-18 h at 37 °C. The overnight cultures were adjusted to give an inoculum concentration of about 10^8 CFU/mL according to McFarland turbidity standard. Seven samples were evaluated for time-kill assays namely polymyxin B and extract alone and its combinations including the DMSO as a negative control. Two concentrations of extracts employed were according to the MIC and 2× MIC values obtained from our previous study; for *A. baumannii* (MIC was 32 mg/mL and 2× MIC was 64 mg/mL) and *K. pneumoniae* (MIC was 32 mg/mL and 2× MIC was 64

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mg/mL), respectively (Jihadi *et al.*, 2020). The concentration of polymyxin B used in this study was at a clinically relevant dose of 1 µg/mL based on its susceptibility breakpoints at MIC of ≤2 µg/mL (Behera *et al.*, 2010). Polymyxin B (1 µg/mL) and different concentrations of the extract, alone or in combination were loaded into the culture and incubated at 37 °C in a shaking incubator. An aliquot of 200 µL of the culture were taken at the time intervals of 0, 1, 4 and 24 h. The aliquots were inoculated aseptically onto a nutrient agar plate and incubated at 37 °C for 24 h. The time-kill curves were constructed by plotting the $\log_{10}$ CFU/mL versus time (h). A reduction of ≥1 $\log_{10}$ CFU/mL below the initial inoculum (i.e. ~10^8 CFU/mL) indicates activity, synergy is defined as a change in $\log_{10}$ CFU/mL value ≥2 $\log_{10}$ CFU/mL lower than the most active monotherapy, additive is defined as those that achieve a change in $\log_{10}$ CFU/mL value that is between >1 and <2 $\log_{10}$ CFU/mL lower than the most active monotherapy (Bergen *et al.*, 2011).

Gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) analysis

The crude extract of 0.01 g was dissolved in 1 mL of ethanol (HmbG, 99%). The mixture of 200 µL was then further dissolved in 1.8 mL of ethanol. Prior to the GC-MS analysis, the extracts were filtered by using filter vials. The samples were analysed using gas chromatography (GC) system based on methods by Hashim *et al.* (2014). Agilent 7890B GC (Agilent Technologies Inc., Santa Clara, CA, USA) system equipped with LECO Pegasus HT time-of-flight mass spectrometer (LECO, USA) and autosampler was used in the study. A volume of 1 µL ethanolic-dissolved extract was injected into the GC inlet by the autosampler and eluted by Helium gas at 2 mL/min flow rate through a Hewlett Packard HP-5MS silica capillary column (Agilent Technologies Inc., Santa Clara, CA, USA) (30 mm × 0.32 mm × 25 µm film thickness) in the GC oven to separate individual compounds. The GC oven was programmed at 80 °C for 2 min and raised to 250 °C at 10 °C/min. The separated compounds were detected by a quadrupole mass spectrometer, which was set at a range of 32-500 amu. The detected compounds were identified by comparing individual mass spectra against mass spectral library of the National Institute of Standards and Technology (NIST) 2014.

RESULTS

Static time-kill studies

Polymyxin B (1 mg/L) and *A. malaccensis* extract (32 mg/mL and 64 mg/mL) alone and in combination were evaluated in the time-kill assays against *A. baumannii* ATCC 19606 (Figure 1a) and *K. pneumoniae* ATCC 10031 (Figure 1b). Log changes in CFU/mL at 1, 4 and 24 h were compared to the starting inoculum (~10^8 CFU/mL) at 0 h for polymyxin B and extract alone and in combination against both bacteria tested (Table 1). At 1 h, the log changed values of polymyxin B alone demonstrated rapid drop in the viable count with ≥1 $\log_{10}$ CFU/mL reduction against *A. baumannii* and *K. pneumoniae* (Table 1). The results showed that polymyxin B alone was unable to produce bactericidal activity (a reduction of ≥3 $\log_{10}$ CFU/mL below the initial inoculum) and considerable bacterial regrowth occurred at 4 h (≥1 $\log_{10}$ CFU/mL regrowth) (Figure 1a and 1b). At 24 h, the bacteria continued to grow as similarly observed as the growth control.

Aquilaria malaccensis extract alone at 32 mg/mL exhibited minimal bacterial killing activity at 1 h and up to 24 h of treatment with ≤1 $\log_{10}$ CFU/mL reduction compared to the starting inoculum at 0 h against *A. baumannii* (Figure 1a) and *K. pneumoniae* (Figure 1b). The log changed values of extract alone at 64 mg/mL against *A. baumannii* demonstrated more apparent reductions in the viable count at 1 h and 4 h with 1.18 $\log_{10}$ CFU/mL and 1.50 $\log_{10}$ CFU/mL, respectively compared to *K. pneumoniae* (Table 1). Interestingly, a reduction of ≥3 $\log_{10}$ CFU/mL below the initial inoculum at 24 h was observed in both *A. baumannii* and *K.*

Table 1: The changes in $\log_{10}$ CFU/mL of polymyxin B (1 mg/L) and *A. malaccensis* extract alone and in combinations at 1, 4 and 24 h compared to 0 h against *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 10031.

Strain	Time (h)	ΔCFU [$\log_{10}(CFU_t)$ - $\log_{10}(CFU_0)$]						
		CTRL	PMB (1 µg/mL)	AME 32 mg/mL	AME 64 mg/mL	PMB + AME 32	PMB + AME 64	DMSO
A. baumannii ATCC 19606	1	0.59	-1.82	-0.30	-1.18	-1.30	-1.46	0.38
	4	0.64	0.02	-0.47	-1.50	-1.39	-2.27	0.49
	24	0.99	1.13	-0.63	-3.72	-1.80	-4.27	0.86
K. pneumoniae ATCC 10031	1	0.16	-1.18	-0.21	-0.76	-0.65	-0.99	0.20
	4	0.37	-0.44	-0.66	-0.93	-0.89	-1.37	0.31
	24	0.70	0.67	-0.75	-5.63	-1.78	-5.77	0.69

The grey background indicates the activity, a reduction of ≥1 $\log_{10}$ CFU/mL below the initial inoculum; the blue background indicates bactericidal activity, a reduction of ≥3 $\log_{10}$ CFU/mL below the initial inoculum; the green background indicates additive, a ≥1 $\log_{10}$ decrease in the number of CFU/mL with the combination compared to its most active component. PMB: Polymyxin B; AME: *A. malaccensis* extract; CTRL: Growth positive control.

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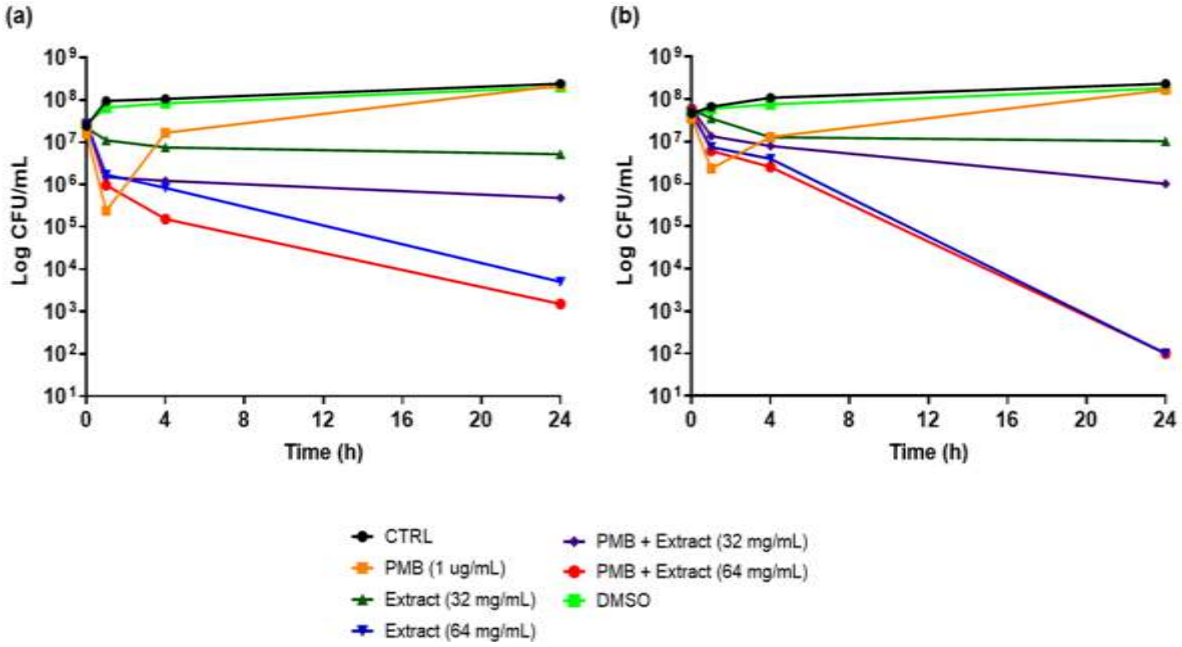

Figure 1: Time-kill curves of polymyxin B and *A. malaccensis* extract alone and the combination against (a) *A. baumannii* ATCC 19606 and (b) *K. pneumoniae* ATCC 10031. PMB: Polymyxin B; CTRL: Growth positive control.

pneumoniae isolates by the treatment with extract alone at 64 mg/mL. The results indicated that there were no regrowth of *A. baumannii* and *K. pneumoniae* colonies observed due to the treatment of *A. malaccensis* extract alone at both concentrations across the 24 h.

The combination of polymyxin B and *A. malaccensis* extract at 32 mg/mL and 64 mg/mL demonstrated no synergy effect against both *A. baumannii* and *K. pneumoniae* (Figure 1). Notwithstanding with the results, the combination treatments enhanced the bacterial killing compared to polymyxin B alone at 4 h and 24 h. At 24 h, the combination of polymyxin B and *A. malaccensis* extract (32 mg/mL) showed additive effect by a reduction of ≥1 $\log_{10}$ compared to its most active component against both *A. baumannii* and *K. pneumoniae.* Meanwhile, with *A. malaccensis* extract at 64 mg/mL, bactericidal effect was observed in both isolates by the combination effects at 24 h with approximately 4 to 5 $\log_{10}$ reduction from its initial inoculum (Table 1). Although the addition of extract did not increase the extent of early activity of bacterial killing seen with polymyxin B alone particularly in *K. pneumoniae*, the combinations at both extract concentrations were able to minimize or inhibit the bacterial growth at 4 h and 24 h.

Gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) analysis

The GC-MS analysis of the crude ethanolic extract of *A. malaccensis* leaves has led to the detection and identification of various compounds. Figure 2 depicted a chromatogram of detected peaks which were labelled with their retention times. On comparison of the mass spectra of the constituents with the NIST library, the compounds detected from the extract were identified (Table 2). Overall, the analysis putatively identified more than sixty components with phytol and 9,12-octadecadienal detected as the most abundant compounds and both with 19.25%. Approximately about 4 to 5% of compounds identified were 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, oleic acid and 9,12-octadecadienoic acid. In addition, a number of other compounds were identified including fatty acid and alkanes such as oleic acid, n-decanoic acid, n-hexadecanoic acid and terpenoids such as squalene and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (Table 2).

DISCUSSION

We previously reported on the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the crude leaf extract of *A. malaccensis* on *A. baumannii* and *K. pneumoniae* (Jihadi *et al.*, 2020). The present study highlights *A. malaccensis* crude extract as a potential agent to be used in combination with polymyxin B in fighting against both Gram-negative bacteria. The extract alone at 64 mg/mL showed a bactericidal effect (a reduction of ≥3 $\log_{10}$ CFU/mL below the initial inoculum) compared to at 32 mg/mL, particularly at 24 h against both *A. baumannii* and *K. pneumoniae*. Notably, samples with the extract alone at 32 mg/mL and 64 mg/mL showed no regrowth of the bacteria across the 24 h. The extract's higher hydrophobicity and concentration likely induced it to be more permeable to occupy an adequate number of binding sites thus causing the death of the bacteria (Ramli

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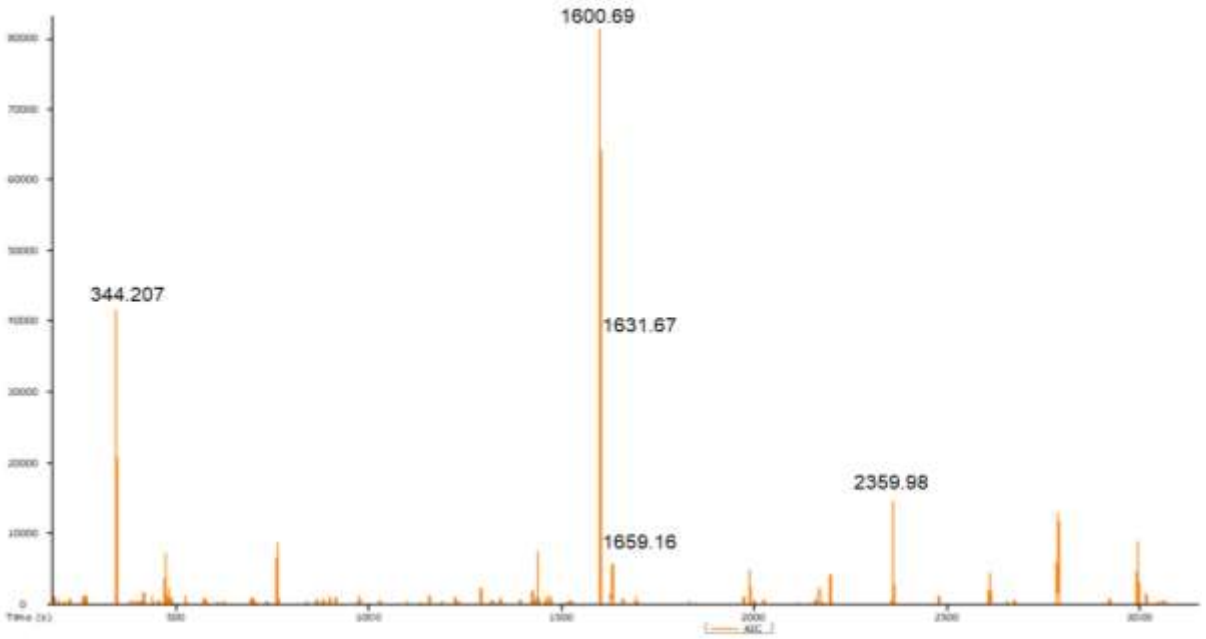

Figure 2: GC-MS chromatogram of ethanolic extracts of *A. malaccensis* leaves. The peak at 1600.69 is identified as phytol, peak at 344.207 is 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; peak at 1631.67 is oleic acid; peak at 1659.16 is 9,12,15-octadecatrienoic acid and peak at 2359.98 is squalene.

Table 2: Putative compounds identified in the ethanolic extract of *A. malaccensis* leaves.

No	RT (s)	Name	Molecular formula	Molecular weight (g/mol)	Peak area (%)
1	1600.69	Phytol	$C_{20}H_{40}O$	296	19.25
2	1600.93	9,12-octadecadienal	$C_{18}H_{32}O$	264	19.25
3	344.207	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	$C_6H_8O_4$	144	4.93
4	1631.67	Oleic acid	$C_{18}H_{34}O_2$	282	4.53
5	1631.84	9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	280	4.53
6	2359.98	Squalene	C_3OH_{50}	410	3.60
7	2996.46	α-sitosterol	$C_{29}H_{50}O$	414	2.82
8	1437.62	n-decanoic acid	$C_{10}H_{20}O_2$	172	2.41
9	2199.22	2,3-dihydroxypropyl ester octadecanoic acid	$C_{21}H_{42}O_4$	358	2.02
10	3198.88	1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetramethyl-6-ethenye-10,14-dimethylene-pentadec-4-enyl) cyclohexane	$C_{33}H_{56}$	452	1.79
11	1437.95	n-hexadecanoic acid	$C_{16}H_{32}O_2$	256	1.34
12	1341.4	3,7,11,15-tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	1.29
13	1988.47	2-hydroxy-1-(hydroxymethyl)ethyl ester hexadecanoic acid	$C_{19}H_{38}O_4$	330	0.99
14	437.665	α-[α-methoxy-(tetrahydrofuran-5-on-2-ylmethoxy)]-tetrahydrofuran-5-on-2-methanol	$C_{11}H_{16}O_7$	260	0.95
15	184.814	2-hexenoic acid	$C_6H_{10}O_2$	114	0.92
16	761.863	4'-hydroxy- Acetophenone	$C_8H_8O_2$	136	0.82
17	1291.1	3,7,11,15-tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	0.69
18	3329.72	Dodecanoic acid	$C_{32}H_{48}O_6$	528	0.68
19	1659.16	9,12,15-octadecatrienoic acid	$C_{18}H_{30}O_2$	278	0.68
20	3050.9	5α-iodomethyl-1α-isopropenyl-4α,5α-dimethyl-6α bicyclo [4.3.0] nonane	$C_{15}H_{25}I$	332	0.68

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No	RT (s)	Name	Molecular formula	Molecular weight (g/mol)	Peak area (%)
21	3064.42	2-hydroxy-1-(hydroxymethyl)ethyl ester, hexadecanoic acid	$C_{19}H_{38}O_4$	330	0.65
22	2611.17	5-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-4H-1-benzopyran-4-one	$C_{16}H_{12}O_5$	284	0.59
23	2198.79	2,3-dihydroxypropyl ester octadecanoic acid	$C_{21}H_{42}O_4$	358	0.58
24	736.565	3-phenyl-2-propenoic acid	$C_9H_8O_2$	148	0.53
25	2788.72	DL-α-Tocopherol	$C_{29}H_{50}O_2$	430	0.43
26	481.821	(+)-diethyl l-tartrate	$C_8H_{14}O_6$	206	0.43
27	1233.77	5-tridecene	$C_{13}H_{26}$	182	0.42
28	2479.76	5-hydroxy-7-methoxy-2-(4-methoxyphenyl)- 4H-1-benzopyran-4-one	$C_{17}H_{14}O_5$	298	0.41
29	2000.19	Glycerol 1-palmitate	$C_{19}H_{38}O_4$	330	0.40
30	1974.39	1-heneicosanol	$C_{21}H_{44}O$	312	0.39
31	488.461	Thioacetic acid	$C_2H_4O_5$	76	0.38
32	180.266	Lycopene	$C_{40}H_{56}$	536	0.38
33	2655.33	ç-tocopherol	$C_{28}H_{48}O_2$	416	0.37
34	881.25	Ethylparaben	$C_9H_{10}O_3$	166	0.33
35	472.226	1-acetate 1,2,3-propanetriol	$C_5H_{10}O_4$	134	0.33
36	2171.2	methyl ester 11,14,17-eicosatrienoic acid	$C_{21}H_{36}O_2$	320	0.32
37	578.5	2-methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	0.27
38	2922.12	Stigmasterol	$C_{29}H_{48}O$	412	0.26
39	383.051	3-methyl-1H-pyrrole	C_5H_7N	81	0.24
40	1134.63	4-hydroxymethyl-2-methyl-5-methylsufanylmethyl-pyridin-3-ol	$C_9H_{13}NO_2S$	199	0.23
41	183.054	2-butene ozonide	$C_4H_8O_3$	104	0.23
42	396.364	1-(2-furanylmethyl)-1H-pyrrole	C_9H_9NO	147	0.22
43	455.228	5-hydroxymethylfurfural	$C_6H_6O_3$	126	0.22
44	900.639	3-ethyl-1-heptyne-3-ol	$C_9H_{16}O$	140	0.21
45	914.716	2-methyl-1H-pyrrole	C_5H_7N	81	0.21
46	974.775	cyclobutyl octadecyl ester oxalic acid	$C_{24}H_{44}O_4$	396	0.21
47	194.907	3-chloro-acetate1-propanol	$C_5H_9CLO_2$	136	0.20
48	260.41	diethyl ester propanedioic acid	$C_7H_{12}O_4$	160	0.18
49	1393.63	methyl ester hexadecanoic acid	$C_{17}H_{34}O_2$	270	0.18
50	573.984	3,4-dihydroxytetrahydro 2-Furanone	$C_4H_6O_4$	118	0.17
51	2675.54	ç-tocopherol	$C_{28}H_{48}O_2$	416	0.17
52	2160.65	(Z)6,(Z)9-pentadecadien-1-ol	$C_{15}H_{28}O$	224	0.16
53	1157.37	4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	0.14
54	2168.05	9-octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	$C_{21}H_{40}O_4$	356	0.14
55	1223.28	4-methoxyphenyl ester heptanoic acid,	$C_{14}H_{20}O_3$	236	0.13
56	2167.55	1-hexadecyne	$C_{16}H_{30}$	222	0.13
57	1461.62	1-(2,4,6-trihydroxyphenyl) 2-pentanone	$C_{11}H_{14}O_4$	210	0.12
58	3227.69	1-(2,6,6-trimethyl-3-cyclohexen-1-yl)-2-buten-1-one	$C_{13}H_{20}O$	192	0.12
59	3227.96	1(22),7(16)-diepoxy- Tricyclo[20.8.0.0(7,16)]triacontane	$C_{30}H_{52}O_2$	444	0.12
60	1471.94	ethyl ester decanoic acid,	$C_{12}H_{24}O_2$	200	0.11
61	693.77	4-dodecene	$C_{12}H_{24}$	168	0.11
62	1029.59	2-chloro-6-(2-furanylmethoxy)-4-(trichloromethyl)-pyridine	$C_{11}H_7CL_4NO_2$	325	0.10
63	1191.24	3,4-dihydro-2,5-dimethyl-2H-pyran-2-carboxaldehyde	$C_8H_{12}O_2$	140	0.10

RT: Retention time.

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et al., 2017). This demonstrates that the extract alone is a promising drug candidate and can be further investigated for the treatment against resistant bacteria.

Furthermore, polymyxin B alone showed a rapid killing at 1 h with regrowth at 4 h and 24 h against both bacteria (Figure 1). The action of polymyxin is well explained via the 'self-promoted uptake' pathway as it binds and pass through the lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria which then increase the membrane permeability and cellular leakage, to finally induce cell death (Nang *et al.*, 2021). The regrowth at 4 h and 24 h likely occurred as the bacteria can develop resistance mechanism to polymyxin B. Cases of polymyxin resistance have been increasingly reported in many studies in bacterial infections (Srinivas and Rivard, 2017; Zavascki *et al.*, 2018). A number of proposed mechanisms associated with polymyxin B resistance have been elucidated and one of the main mechanisms is the alteration of bacteria LPS which reduces its binding to polymyxins, thereby leading to resistance (Moffatt *et al.*, 2019).

To the best of our knowledge, the present work is the first to report on the bacterial killing activity of polymyxin B in combination with crude ethanolic leaf extract of *A. malaccensis* against *A. baumannii* and *K. pneumoniae*. The combination of polymyxin B and *A. malaccensis* extract demonstrated enhanced activity over the polymyxin B monotherapy against both *A. baumannii* and *K. pneumoniae* (Figure 1). Previous studies have shown the potential of plant medicinal extracts in combination with first line antibiotics for the treatment of MDR bacteria (Haroun and Al-Kayali, 2016; Saquib *et al.*, 2019). A study by Bazzaz *et al.* (2018) evaluated the effect of *Lippia citriodora* leaf extract in combination with gentamicin against *S. aureus* and *E. coli* strains. The extract alone showed no inhibitory activity, but in concomitant intake with gentamicin, they were able to increase bacterial killing by significantly lowering the MIC value (Bazzaz *et al.*, 2018). Time-kill analysis of the combination of tannic acid and quercetin with several antibiotics such as fusidic acid, minocycline and rifampicin against methicillin resistant *S. aureus* (MRSA) showed that almost all the combinations produced a high killing rate and synergistic effect with more than ≥3 $\log_{10}$ reduction compared to its most active component (Kyaw *et al.*, 2012). This suggested that broad range of phytochemicals were able to potentiate various antibiotics in suitable combinations.

Plant antimicrobial compounds exhibit the ability to complement antibiotics by enhancing the effect of the drugs when they are taken concurrently (Cheesman *et al.*, 2017). A combination of polymyxin and curcumin showed considerable synergy against *A. baumannii* which may have resulted from the changes induced by polymyxin to increase permeability of cell membrane and thereby promote the penetration of greater concentration of curcumin into intracellular targets (Kaur *et al.*, 2018). A study by Kamonwannasit *et al.* (2013) reported on the antibacterial action of *Aquilaria crassna* leaf extract against *S. epidermidis* and proposed that the extract induced swelling and distortion of cells, inhibited biofilm formation and finally caused rupture of bacterial cell wall at 24 h (Kamonwannasit *et al.*, 2013). Coincidently, our results for the extract alone at 64 mg/mL was consistent with the substantial killing of the bacteria at 24 h. Based on the present findings, we hypothesized that the changes observed in the combination was due to the initial effect of polymyxin B that likely enhanced the uptake of *A. malaccensis* extract into the intracellular components of the bacteria to exert its effect particularly at 24 h. Furthermore, phytochemicals are excellent resistance-modifying agents which can function as MDR inhibitors hence leading to synergy with antibiotics (Stefanović and Comic, 2012; Seukep *et al.*, 2020). Phytochemical compounds can act to minimize the emergence of resistance by blocking the activity of efflux pump in bacteria, thus allowing the antibiotics to reach maximum bactericidal effect (Seukep *et al.*, 2020). Cameroonian medicinal plants, *Piper nigrum* and *Telfairia occidentalis* in combination with several major first line antibiotics were shown to exhibit synergistic effects against majority of the tested MDR bacteria which suggested that they acted as efflux pump inhibitors (Noumedem *et al.*, 2013).

GC-MS analysis of *A. malaccensis* ethanolic crude leaf extract identified more than sixty constituents with major component of phytol and 9,12-octadecadienal (Table 2 and Figure 2). The result was consistent with the recent reports by Hashim *et al.* (2014) and Eissa *et al.* (2020) which demonstrated the presence of phytol (26.93%). Phytol has been shown to induce bactericidal killing by oxidative stress response which result in extreme damage in DNA and lead to cell death in *Pseudomonas aeruginosa* (Lee *et al.*, 2016). Previous studies indicated that agarwood extract obtained from different parts of the plant constitute a variety of active compounds including fatty acid and alkanes such as oleic acid, 9-octadecenoic acid, n-hexadecanoic as well as terpenoids such as squalene (Khalil *et al.*, 2013; Wang *et al.*, 2018) which were also found in the present study. These components have been known for antibacterial effects and potential pharmacological activities against a wide range of microorganism (Wang *et al.*, 2018). Hendra *et al.* (2016) reported that the antibacterial activity of *A. malaccensis* leaf extract fraction on *S. aureus* and *E. coli* was potentially due to alkaloid and terpenoid. Hexadecanoic acid was found as the major antibacterial compound in the hexanoic extract of *Albizia adianthifolia* which explains its efficacy in certain illnesses and infections (Abubakar and Majinda, 2016). Dilika *et al.* (2000) reported that oleic acid isolated from dichloromethane extract of *Helichrysum pedunculatum* leaves exerted antibacterial activity against *Bacillus subtilis*, *Micrococcus kristinae* and *S. aureus*. In addition, Guimarães *et al.* (2019) reported on a promising antibacterial effect of terpenoid, a compound commonly found in essential oils against tested Gram-negative bacteria.

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CONCLUSION

In conclusion, although no synergy effect was observed, the combination of polymyxin B and *A. malaccensis* extract were able to enhance the bacterial killing compared to polymyxin B alone. Notably, the crude extract alone and its combination with polymyxin B minimized and inhibited the bacteria growth over the 24 h. GC-MS analysis successfully identified compounds that likely to contribute to the antibacterial activity of the extract including phytol, 9,12-octadecadienal, oleic acid, n-decanoic acid, n-hexadecanoic acid and squalene. Overall, this study highlights the significant potential of *A. malaccensis* leaf extract as a promising candidate of antibacterial agent either to be used alone or in combination with polymyxins for the treatment against MDR Gram-negative bacterial infections particularly *A. baumannii* and *K. pneumoniae*. Further study is warranted to investigate pure bioactive compounds from the extract of *A. malaccensis* leaves.

AUTHORS' CONTRIBUTIONS

Mohd Hafidz Mahamad Maifiah: Conceptualization, Writing - Review and Editing. Nurul 'Izzati Mohd Jihadi: Conducted the experiment, Writing - Original Draft. Khairunnisa Mohd Kamal, Nusaibah Abdul Rahim, Yumi Zuhanis Has-Yun Hashim and Muhamad Shirwan Abdullah Sani: Writing - Review and Editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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