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RESEARCH ARTICLE

Antibacterial and antibiotic potentiating capabilities of extracts isolated from *Burkillanthus malaccensis*, *Diospyros hasseltii* and *Cleisthanthus bracteosus* against human pathogenic bacteria

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ABSTRACT

Antibiotics which once a boon in medicine and saved millions of lives are now facing an ever-growing menace of antibacterial resistance, which desperately needs new antibacterial drugs which are innovative in chemistry and mode of action. For many years, the world has turned to natural plants with antibacterial properties to combat antibiotic resistance. On that basis, we aimed to identify plants with antibacterial and antibiotic potentiating properties. Seventeen different extracts of 3 plants namely *Burkillanthus malaccensis*, *Diospyros hasseltii* and *Cleisthanthus bracteosus* were tested against multi-drug resistant *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*, Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA). Antibacterial activity of hexane, methanol and chloroform extracts of bark, seed, fruit, flesh and leaves from these plants were tested using, disk diffusion assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Antibiotic potentiating capabilities were tested using time-kill assay. *B. malaccensis* fruit chloroform extract showed the biggest zone of inhibition against MRSA (13.00±0.0 mm) but *C. bracteosus* bark methanol extract showed the biggest inhibition zone against MSSA (15.33±0.6 mm). Interestingly, bark methanol extract of *C. bracteosus* was active against MRSA (8.7±0.6 mm), MSSA (7.7±0.6 mm) (Gram-positive) and *A. baumannii* (7.7±0.6 mm) (Gram-negative). Overall, the leaf methanol and bark methanol extract of *C. bracteosus* warrants further investigation such as compound isolation and mechanism of action for validating its therapeutic use as antibiotic potentiator importantly against MRSA and *A. baumannii.*

Keywords: Antibacterial; antibiotic potentiator; antibiotic resistance; natural plant extracts.

INTRODUCTION

Antibacterial resistance is a never-ending humongous threat faced by the healthcare system, which results in high financial costs, prolonged hospital stays, permanent disabilities as well a huge loss of lives (Marc, 1998). It was reported by CDC (2019), that in the United States (US) the number of illnesses caused by antibiotic resistance rose from approximately two million in 2013 to 2.6 million and, deaths nearly double from 23 000 in 2013 to 44 000, in 20 (Craig, 2019). Health care-associated infections coupled with antibiotic resistance, is a deadly combination. The persistent contact of bacteria with antibiotics contributes greatly to the increase of resistance as seen during the Covid-19 pandemic (Sulayyim *et al*., 2022). The trends are in such manner all over the world, that the World Health Organization (WHO) announced in 2019 that antibiotic resistance is global health and developmental threat which needs multisectoral action (WHO, 2019).

Resistance in some bacteria is innate while others acquire resistance via various means. European Center for Disease Prevention and Control (ECDC), Center for Disease Prevention and Control (CDC), and Infectious Diseases Society of America (IDSA) have classified the acquired resistance into 3 categories: multi-drug resistance (MDR), pan-drug resistance (PDR) and extensive-drug resistance (XDR). The gram-negative *Klebsiella pneumoniae* causes soft tissue infections, pneumonia, bacteraemia, liver abscess, endogenous endophthalmitis and urinary tract infections, among others is found to be the cause of 10% of all nosocomial infections (Ashayeri-Panah *et al*., 2014). Discovered first in Malaysia in

the year 2004, 88.8% of *K. pneumoniae* that was isolated in the University Malaya Medical Center (UMMC), was found to carry a variety of carbapenemases (Anuar *et al.*, 2013; Lau *et al.*, 2021). The ubiquitous, opportunistic pathogen *Pseudomonas aerunginosa*, has been found to grow increasingly resistant towards antibiotics, with quorum sensing, biofilm formation and toxin secretion abilities (Ribeiro *et al*., 2019). According to an international study, the increasingly resistant *P. aerunginosa* in the ICU is responsible for 16.2% of overall intensive care unit (ICU) cases and causes urinary tract infections (UTIs), ventilator associated-pneumonia (VAP) among others (Vincent *et al*., 2020; Reynolds & Kollef, 2021). As for *Escherichia coli*, the extraintestinal pathogenic *E. coli* (ExPec) emerged during the 2000s, acquired resistance to fluoroquinolones and cephalosporins. In Malaysia, out of 640 isolates collected in water samples from Semenyih river, 81.1% exhibited resistance to 6 types of antibiotics (Al-Badaii & Shuhaimi-Othman, 2015). It was reported long ago that Methicillin-resistant *Staphylococcus aureus* (MRSA) is vancomycin and cefotaxime resistant (Fung-Tomc *et al*., 1988). Historically, health care-associated MRSA (HA-MRSA), the emergence of community-acquired MRSA (CA-MRSA) is a major source of soft tissue and skin infections, in the US and in many countries around the world (Fridkin *et al.*, 2005; Kaplan *et al.*, 2005; Chambers & Deleo, 2009). Here in Malaysia, prevalence of MRSA which ranged at 17% was found to increase to 44.1% in the year 2007 (Rohani *et al*., 2000; Ahmad *et al*., 2009). First detected in the ICU in the 1960s, the persistence of *Acinetobacter baumannii* in the hospital settings and its constant contact with the antibiotics, which has resulted in various clones of the with antibiotic resistance (Gonzalez-Villoria & Valverde-Garduno, 2016). *A. baumannii* was announced to be a major source of concern by the Center for Disease Control (CDC), in the year 2019(Craig, 2019). Although the National Antibiotic Resistance Surveillance Report (NARSR) indicates the Malaysian prevalence of carbapenem-resistant *A. baumannii* to be 50 to 60%, other studies from hospitals in Malaysia shows the prevalence to be higher than this figure (Hanifah *et al*., 2011; MKK *et al*., 2019). The increase in antibiotic resistance have been recorded variously in the Asian region. Carbapenemase-producing Enterobactericeae with virtually all carbapenamase genes being described have been reported in countries like India, Pakistan and other South and Southeast Asian regions (Bushnell *et al*., 2013; Molton *et al*., 2013; Zurfluh *et al*., 2015).

Burkillanthus malaccensis from the citrus family Rutaceae, is native to the Indonesian Sumatra, Peninsula and Sarawak in Malaysia. Known commonly as the 'Malay ghostlime' It grows singly or in small groups in primary and secondary forest habitat (Glen, 2004; Krueger & Navarro, 2007). There are rarely any studies on this plant. Only one study has reported the isolation of Werneria chromene and Dihydroxiacidissimol as secondary metabolites with antimicrobial capabilities (Zulkipli *et al*., 2022). *Cleisthanthus bracteosus* (Phyllanthaceae family) is found in Perak, Malaysia Glen, 2004. The discovery of the plant was recorded in, 'The Journal of the Asiatic Society of Bengal', Volume 75, as early as 1915 (Glen, 2004). The *C. bracteosus* plant has virtually no records of any studies performed by researchers. Therefore, the phytochemical contents or the contents of bioactive compounds are yet to be discovered. However, another species of the same genus, *Cleisthanthus collinus* has been found to have some activities. Elangomathavan *et al*. (2016) mentioned it being used as septic wounds cleansing agent. Beneficial bioactive compounds such as glycosides, aryl naphthalene lignan lactones such as cleistanthin A and B, collinusin and oduvin have been isolated (Fabricant & Farnsworth, 2001). For example, *cleistathin B* has been reported to have potential anticancer properties (Elangomathavan *et al*., 2016; Zulkipli *et al*., 2022). Another plant that has been chosen for this study is *Diospyros hasseltii* (Ebenaceae family), which is mostly found in Southeast Asia, Thailand, Malaysia, and Indonesia. Known as 'merangat' in Malaysia (Delfianti *et al*., 2018). *D. hasseltii* is a species of plants that produce edible seeds and fruits, whereas their cultivation status is recognized as wild (Milow *et al*., 2014). No studies have been done onto *D. hasseltii*, but *D. busifolia* and *D. kaki* (persimmon) have been found to have medicinal properties. Chemicals which are known to be betulin and betulinic acid which is contained in some of the *Diaspyros* plants have been found to have a variety of biological activities such as antiviral, antibacterial, antiplasmodial, anticarcinogenic among others (Nordin & Zakaria, 2016).

Despite the focus on researching synthetic pharmaceuticals, there has been a resurgence in interest in medicinal plants. Due to the availability of promising therapeutic components, medicinal plants have been used to cure illnesses for a long time and are frequently employed as sources of novel medications. According to the WHO, there are about 20 000 plant species that are utilized medicinally, and traditional medicine serves as the primary source of care for 80% of the world's population (Dangoggo *et al*., 2012). There have been reports of bactericidal activity in several plant constituents, including phenols, quinines, flavones, flavonoids, flavanols, tannins, terpenoids, essential oils, and alkaloids, among other (Elangomathavan *et al*., 2016).

As Albert Einstein once said, "Look deep into nature, you will understand everything better" (Karatas & Einstein, 2017). Scientists have been looking at nature to provide salvation in the battle against antibiotic resistance. Many natural compounds isolated from natural plants have provided answers to the never-ending battle of discovering novel antibacterial agents. These three plants are novel, and they have not been studied before this although there are some studies with regards to the other species from the same genus.

MATERIALS AND METHODS

Preparation of standardized bacterial inoculum

The bacterial isolates used in this study were archived samples used in a previous study in the Department of Medical Microbiology, University of Malaya, Medical Faculty, where the antibiotic growth resistant profiling has already been done. The following human pathogenic bacteria were used as tested organisms: Gram positive organisms methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *Staphylococcus aureus* (University of Malaya Hospital clinical isolate), and Gram-negative organisms *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15442), *Klebsiella pneumoniae* (University of Malaya Hospital clinical isolate) and meropenem- resistant *Acinetobacter baumannii* (University of Malaya Hospital clinical isolate).

For standardization of bacterial inoculum, quadrant streaking of the isolates were prepared using Mueller-Hinton agar (MHA) (Oxoid Ltd, Ireland). After incubation for 18-24 hours at 37°C, 1-2 colonies produced were taken and suspended in Mueller-Hinton broth (MHB) (Oxoid Ltd, Ireland). The suspension's turbidity was adjusted to 0.5 McFarland Standards in accordance with the recommendations by the Clinical and Laboratory Standard Institute (CLSI, 2018), by measuring the absorbances at a wavelength of 600 nm. The absorbance should range between 0.08 to 0.13, corresponding to inoculum size of roughly 1 x 10x CFU/mL. Serial dilution was done to achieve an approximate 1 x 10v CFU/mL inoculum size (Fadare *et al*., 2022).

Plant extracts

Plant selection was done based on the families of these plants (Phyllanthaceae, Rutaceae and Ebenaceae) being attributed with antibacterial capabilities in previous studies (Khulbe & Sati, 2009; Ngane, 2019; Nematollahi *et al*., 2011; Bitchagno *et al*., 2015; Djarot *et al*., 2020). The plants were collected around the villages in the Manong district in Northern Perak. The bark, stems, leaves, seeds were collected, and herbarium samples were prepared. The plants were identified, and voucher specimens were deposited at Forest Research Institute, Malaysia (FRIM). The bark, stems, leaves,

seeds were separated and were air-dried, under shade at room temperature for a period of two-week. Upon drying, these materials were pulverized using aluminium collection blender (Philips, Guangdong, China) and the powders were weighed with top loading balance (Sartorius AG, Gɤttingen, Germany). Dried and grinded plant powders were mixed with organic solvents of increasing polarity, beginning with hexane, chloroform and methanol. Each extraction was performed by maceration of plant powder to solvent ratio of 1:5 (w/v) for three days, in room temperature, with three consecutive repetitions. The extracts were then filtered using filter paper using an aspirator pump. The filtrates were concentrated to dryness under reduced pressure at 40°C using a rotary evaporator (Buchi Labortechnik AG, Switzerland). The dried extracts were then collected, weighed and stored at -20°C until further use. For our usage, extract was weighed and dissolved in 100% of dimethyl sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, US) to prepare the stock. Due to solubility issues, some were also dissolved in their respective mother solvents such as hexane.

Kirby-Bauer Disc diffusion antibacterial screening

All the plant extracts in this study are subjected to Kirby Bauer Disc Diffusion test according to the CLSI (2018) guidelines as an initial screening for antibacterial effect against tested isolates. Firstly, 1 mg of extract was impregnated into paper discs and left to dry. A lawn culture was performed by immersing a cotton swab in 1 x 10v CFU/mL bacterial inoculum and swabbing 90° in 4 directions onto a clean Mueller-Hinton agar. The paper discs that had been impregnated with 1 mg plant extract were placed onto the agar. The plates were incubated for 24 hours at 37°C. Diameters of the inhibition zone produced by the extract were observed and measured using a millimeter ruler. The test was repeated in duplicate to get the mean of measurement. Doxycycline (Sigma-Aldrich, St. Louis, US) was used as the positive control, while paper disks added and dried with DMSO were used as negative controls (Hudzicki, 2009). As an initial pre-screening stage, in this section, only Doxycycline was used as positive control as it is known to be active against both gram-positive and gram-negative bacteria (Cunha *et al*., 1982; Holmes & Charles, 2009). The breakpoints used are according to CLSI guidelines (CLSI, 2018). Therefore, no antibiotic was used as standard drug against Gram-negative bacteria specifically.

Broth microdilution assay

Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial that inhibits microbial growth. MIC was identified by using the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2018). 50 uL of plant extract starting from 10 000 ug/mL, serially diluted until 8 different concentrations are begotten. A standardized 1 x 10v CFU/mL *A. baumannii* suspension was prepared. A volume of 50 uL of bacterial suspension was added into 96-well u-bottom microtiter plate vessels. The plate was incubated for 24 hours at 37°C, and the suspension with no visible growth was observed and taken as the MIC. The Minimum Inhibitory Concentration (MIC) and two concentrations above it were subcultured onto a clean Mueller-Hinton agar (MHA) to determine the Minimum Bactericidal Concentration (MBC). MBC is the lowest concentration of an antimicrobial that kills the bacteria. After 24 hours incubation at 37°C, the highest dilution suspension culture that did not show any growth was taken as the MBC. Both MIC and MBC values were calculated as the mean of triplicate experiments. To determine whether the effect was bactericidal or bacteriostatic, the ratio of MBC to MIC was used. A ratio of d" 4 indicates bactericidal effect, while a ratio of >4 indicates bacteriostatic effects (Krishnan *et al*., 2010). Once all the bacteria in this study were subjected to MIC/MBC assay, multi-drug resistant (MDR), extensively-drug resistant (XDR) and pan-drug resistant (PDR) strains of *A. baumannii* were further tested for the same assay as it is a critically important bacteria to the current medical needs. Microbroth dilution assay was used to further screen the antimicrobial activity against bacteria that showed positive results in the disk diffusion assay. Therefore, in addition to Doxycycline used in disk diffusion assay, another broad spectrum antibiotic which is Ceftazidime was added as positive control. The breakpoints for MIC was used according to CLSI guidelines (CLSI, 2018).

Time-kill assay

A. baumannii (1 ×10^6 CFU/ml) was incubated with the following concentrations of plant extract and antibiotic respectively, in Mueller-Hinton broth (MHB) at 37°C to obtain viable colonies. Bacteria count Log_{10} values were calculated as the mean of triplicate experiments (Giacometti *et al.*, 1999; Rios & Recio, 2005). In this assay, instead of Doxycycline and Ceftazidime as mentioned in the disk diffusion and microbroth dilution assays, Colistin (Himedialabs, Mumbai, India) was used as the positive control. This is because, Colistin is one of the drugs of choice to treat *A. baumannii* infections in the hospitals. (Holmes & Charles, 2009; Rizek *et al*., 2015; Zakuan & Suresh, 2018; Piperaki *et al*., 2019). As time kill assay was done to investigate the kinetics of the plant extract and also the antibiotic potentiating capabilities, our extract was tested against Colistin.

The breakpoints for Colistin were used according to EUCAST guidelines (EUCAST, 2019). DMSO 4% with broth is used as control in this assay as all the combinations of *C. bracteosus* bark methanol extract used contained DMSO 4%. Additionally, from the MIC/MBC tests, DMSO 4% did not show any killing activity against *A. baumannii* at all.

1. 4×MIC of extract
2. 2×MIC of extract
3. 1×MIC of extract
4. 4% DMSO (Negative control)
5. 1×MIC of Colistin
6. 0.55×MIC of Colistin
7. 2×MIC of Colistin
8. 0.55×MIC of Colistin + 4×MIC of extract
9. 2×MIC of Colistin + 2×MIC of extract

RESULTS

Kirby-Bauer Disc diffusion antibacterial screening

The resistance of ESKAPE-E (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aerunginosa*, *Enterobacter* spp. and *Escherichia coli*) pathogens are a major source of concern. We screened all the 17 plant extracts against MRSA, MSSA, *A. baumannii*, *E. coli*, *K. Pneumonia* and *P. aerunginosa* which are also part of the ESKAPE-E category, using the Kirby Bauer disk diffusion method. As seen in Table 1, none of the plants showed any activity towards *E. coli*, *K. Pneumonia* and *P. aerunginosa*. *B. malaccensis* bark chloroform extract was active against both MRSA and MSSA with the zone of inhibition 7.00±0.00 mm for both organisms. The leaf methanol extract from the same plant inhibited MRSA and MSSA with the inhibition zones of 10.00±0.0 mm and 9.70±0.6 mm respectively. Out of the five extracts from *D. hasseltii*, four extracts were active against both MRSA and MSSA, with an average zone of inhibition of 13.00±0.4. *C. bracteosus* bark methanol extract was active against gram-positive MRSA, MSSA and gram-negative *A. baumannii* with the inhibition zones of 8.7±0.6 mm, 7.7±0.6 mm and 7.7±0.6 mm respectively. The highest zone of inhibition among all tested plants was shown by *C. bracteosus* leaf methanol extract against MSSA (15.33±0.6 mm).

Broth microdilution assay

The result for this assay is provided in Table 2. *C. bracteosus* leaf methanol extract showed highest activity against MSSA and MRSA with MIC values of 19.5/19.5 ug/mL and 19.5 ug/mL respectively,

Table 1. Average zone of inhibition for plant extracts against human pathogenic bacteria

Plant	Part	Solvent	Mean zone of inhibition diameter (mm)[a]					
			MRSA[b]	MSSA[b]	A.b[b]	K.p[b]	P.a[b]	E.c[b]
Burkillanthus malaccensis	*Seed*	Hexane	–	–	–	–	–	–
	Flesh	Chloroform	–	–	–	–	–	–
	Leaf	Chloroform	–	–	–	–	–	–
	Bark	Chloroform	7.00±0.0	7.00±0.0	–	–	–	–
	Leaf	Methanol	10.00±0.0	9.70±0.6	–	–	–	–
	Flesh	Methanol	–	–	–	–	–	–
Diospyros hasseltii	Leaf	Chloroform	12.7±0.6	13.3±0.6	–	–	–	–
	Leaf	Methanol	8.0±0.0	9.7±0.6	–	–	–	–
	Leaf	Hexane	10.7±0.6	9.7±0.6	–	–	–	–
	Fruit	Chloroform	13.0±0.0	13.0±0.0	–	–	–	–
	Fruit	Hexane	–	–	–	–	–	–
Cleisthanthus bracteosus	Leaf	Methanol	–	15.33±0.6	8.7±0.0	–	–	–
	Leaf	Chloroform	–	–	–	–	–	–
	Bark	Chloroform	8.7±0.6	–	–	–	–	–
	Leaf	Hexane	–	–	–	–	–	–
	Bark	Hexane	9.7±0.6	–	–	–	–	–
	Bark	Methanol	8.7±0.6	7.7±0.6	7.7±0.6	–	–	–
Antibiotic:		Doxycycline	23.0±0.0	22.0±0.0	30.0±0.0	29.0±0.0	29.0±0.0	30.0±0.0

[a] Values are given as mean of triplicate. – : no zone of inhibition.
[b] MRSA: methicillin-resistant *Staphyloccocus aureus*; MSSA: methicillin-susceptible *Staphyloccocus aureus*; A.b: *Acinetobacter baumannii*; K.p: *Klebsiella pneumoniae*; P.a: *Pseudomonas aerunginosa*; E.c: *Escherichia coli*.

Table 2. Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) by broth microdilution assay against MRSA, MSSA and *A. baumannii*

Plant	Part	Solvent	Mean MIC/MBC (ug/mL)[a]		
Burkillanthus malaccensis	*Seed*	Hexane	MRSA[b]	MSSA[b]	A.b[b]
	Flesh	Chloroform	–	–	–
	Leaf	Chloroform	–	–	–
	Bark	Chloroform	–	1250/2500 (+)	–
	Leaf	Methanol	2500/>2500	1250/2500 (+)	–
	Flesh	Methanol	–	625/1250 (+)	–
Diospyros hasseltii	Leaf	Chloroform	–	–	–
	Leaf	Methanol	625/1250 (+)	156.25/315.50 (+)	-
	Leaf	Hexane	–	–	–
	Fruit	Chloroform	–	–	–
	Fruit	Hexane	–	–	–
Cleisthanthus bracteosus	Leaf	Methanol	19.5/39 (+)	19.5/19.5 (+)	5000/>10000
	Leaf	Chloroform	–	–	–
	Bark	Chloroform	156.25/156.25 (+)	–	–
	Leaf	Hexane	–	–	5000/10000 (+)
	Bark	Hexane	–	–	
	Bark	Methanol	78.32/78.32 (+)	–	5000/5000 (+)
Antibiotic:		Ceftazidime	N/A	N/A	4
		Doxycycline	2	2	N/A

[a] Values are given as mean of triplicate with the effect of MBC of whether static (*) or cidal (+). Bacteria with no MIC in either of the 17 extracts is excluded from this table. No inhibition zone is indicated with –. N/A: Not Applicable.
[b] MRSA: methicillin-resistant *Staphyloccocus aureus*; MSSA: methicillin-susceptible *Staphyloccocus aureus*; A.b: *Acinetobacter baumannii*.

with bactericidal effects. The same extract was also found to inhibit the gram-negative *A. baumannii* at 5000 ug/mL. The bark methanol extract of the same plant inhibited MRSA at 78.32 ug/ml with a cidal effect. Apart from this, only leaf methanol extract of *B. malaccensis* show MIC value of 2500 ug/mL with its effect being undecided. But bark methanol, leaf methanol and flesh methanol extracts from the same plant were found to have MIC values of 1250 ug/ml, 1250 ug/mL and 625 ug/mL with cidal effect respectively, against MSSA. For *D. hasseltii* the leaf methanol extract showed 625 ug/mL and 156.25 ug/mL MIC values for MRSA and MSSA respectively. Further MIC and MBC assays were conducted against XDR, PDR and MDR strains of *A. baumannii* with the bark methanol extract of *C. bracteosus*. The results are in Table 3. Consistently, the bark methanol extract of this plant actively inhibits the PDR, XDR and MDR strain isolates 5000 ug/mL, with bactericidal effect.

Time-Kill and Synergy Assay

Meanwhile Time Kill Assay results as shown in Figure 1 suggests the 1×MIC and 2×MIC concentrations the *C. bracteosus* bark methanol extract shows slight decrease of CFU and its continuity throughout the time points. More interestingly, 4×MIC concentrations is found to kill the bacteria entirely at 8 hours, similar with the positive control colistin at 1×MIC. A simultaneous synergy study also has been done with the same time kill assay, using combinations of Colistin with the *C. bracteosus* bark methanol extract. While the combination of 2×MIC bark methanol extract and 2×MIC Colistin eliminated the bacteria at 2 hours, the combination of 4×MIC bark methanol extract and 0.55× MIC Colistin is found to kill the bacteria at 6 hours.

DISCUSSION

Plants are an essential source of both curative and preventative medicinal remedies. According to the WHO, there are about 20 000 plant species that are utilized medicinally, and traditional medicine serves as the primary source of care for 80% of the world's population (Dangoggo *et al*., 2012). Among all three plants, the various extracts of *D. hasseltii* were found to be most active against MRSA and MSSA. There are no previous studies on this plant to match this data. However, *D. kaki* for the same Ebenaceae family was found to be inhibit *S. aureus* via disk diffusion assay and MIC (Shah *et al*., 2012). In our study, most of the plant extracts of all three plants were found to inhibit gram-positive bacteria (MRSA and MSSA). The lack of susceptibility against gram-negative bacteria is attributed to the existence of outer hydrophilic lipo-polysaccharide (LPS). The LPS hinders the infiltration of hydrophobic materials and blocks its build-up in membrane of the bacteria (Mann *et al*., 2000). Interestingly in our study, *A. baumannii* was found to be inhibited by *C. bracteosus* leaf and bark methanol extracts. From Table 1, none of the extracts prepared using hexane but the *C. bracteosus* bark hexane extract was found to inhibit bacteria. This is probably because hexane is immiscible in DMSO. Although the extract was diluted in DMSO, it doesn't mean it is homogenously soluble.

Table 3. Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) by broth microdilution assay against XDR, MDR, and PDR *A. baumannii*

A. Baumannii	Mean MIC/MBC (ug/mL)[a]
MDR:	
Isolate 1	5000/10000 (+)
Isolate 2	5000/10000 (+)
Isolate 3	5000/10000 (+)
XDR:	
Isolate 1	5000/5000 (+)
Isolate 2	5000/5000 (+)
Isolate 3	5000/5000 (+)
PDR:	
Isolate 1	5000/5000 (+)
Isolate 2	5000/5000 (+)
Isolate 3	5000/5000 (+)
ATCC 15308	5000/5000 (+)
Antibiotic: Ceftazidime	4/4

[a] Values are given as mean of triplicate with the effect of MBC of whether static (*) or cidal (+). – : no inhibition.

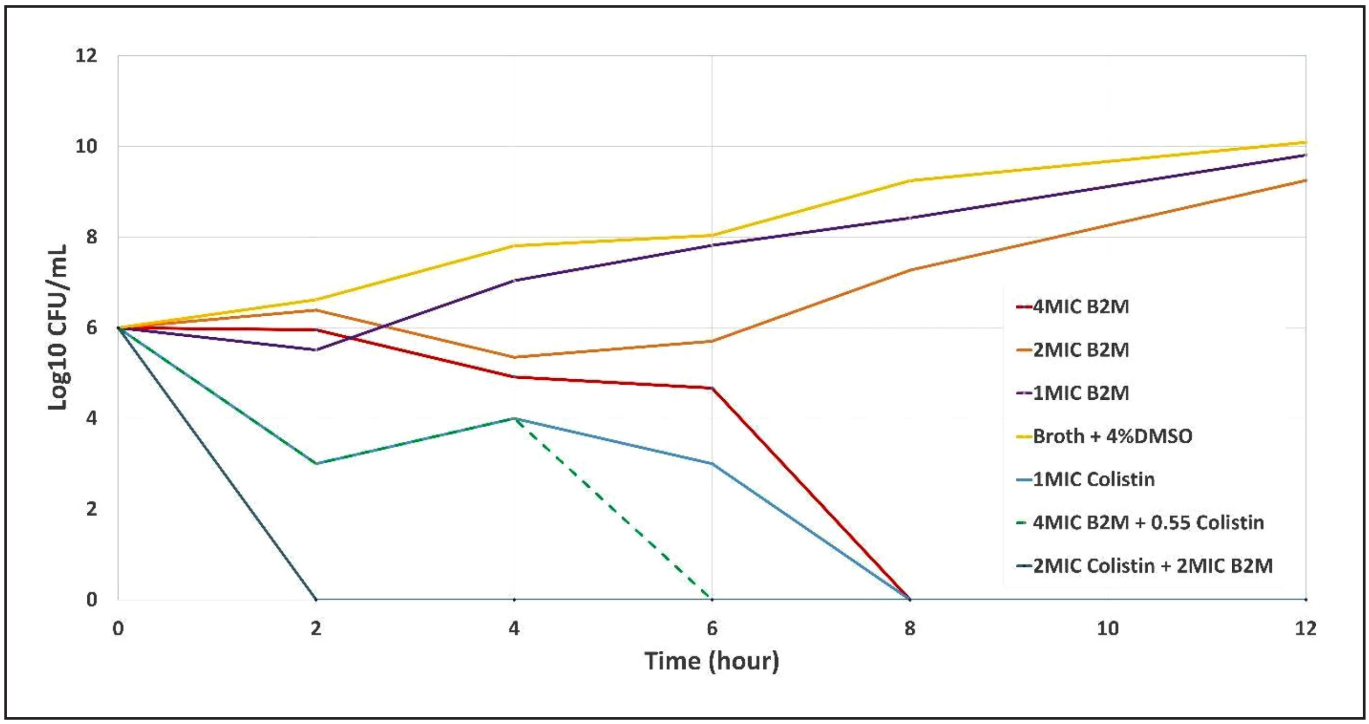

Figure 1. Time- Kill assay of *Cleisthanthus bracteosus* bark methanol extract against multi-drug resistant *A. baumannii*.

When the microbroth dilution method was used to determine the MIC, the first thing to be noted was that there were inconsistencies. Plant extracts which were previously found to be active against the bacteria in the disk diffusion assay, didn't show inhibition in the microbroth dilution assay. Similarly, some extracts which didn't show antibacterial activity were found to be active in the MIC assay. This is probably because, while preparing the paper disks with plant extracts, 100% DMSO was used, with which there were minimal solubility issues. However, in preparing the extracts with water based MHB for microbroth dilution assay, we encountered numerous solubility issues which suggests that the distribution of active plant material may not be uniformed in all plant extracts which has resulted in this type of inconsistency. In a study in 1998, Fabry *et al*. has specified plant extracts as being active when their MIC values are < 8 000 ug/mL (Fabry *et al*., 1998). Subscribing to this, all three plants had active antibacterial extracts against MRSA, MSSA and *A. baumannii.* The lowest MIC towards both MRSA and MSSA was found with leaf methanol extract of *C. bracteosus* (19.5 ug/mL). The same extract has been shown to have some activity against *A. baumannii* (5000 ug/mL). According to another stipulation by Rios and Recio (2005), crude plant extracts with MIC values below 1000 ug/mL can be deemed as active (Rios & Recio, 2005). Following categorization defined by Krishnan *et al*. (2010), all but two plant extracts in Table 2 are found to be cidal. It is not unusual for plant extracts to exhibit antibacterial activity at high concentrations. In a study in 2010, *Cassia fistula* seed methanol extract was found to have MIC values of 12.5 mg/mL against *S. aureus* and *Bacillus thuringiensis* and 50 mg/mL against *E. coli* (Subramanion *et al*., 2010). In another instance, the leaf methanol extract of *Muntingia calabura* was found to inhibit *S. aureus* ATCC25923, *S. aureus* ATCC33591, *Campylobacter coli* and *P. aeruginosa* at 12.5 mg/mL, 2.5 mg/ML, 5 mg/mL and 5 mg/mL respectively (Zakaria *et al*., 2010). Such high concentrations are common for crude extracts as there are many phytoconstituents which require high concentrations of active compounds in the crude extracts to show antibacterial activity. While the plants have been found to show interesting MIC values for MRSA and MSSA, our focus shifts to *C. bracteosus* activity against *A. baumannii* as the strain used in this study is carbapenem resistant. Hence, any activity against it will be valuable to the knowledge bank as it is a novel plant with zero prior studies.

Meanwhile time kill assay results as shown in Figure 1 suggests the 1×MIC and 2×MIC concentrations the *C. bracteosus* bark methanol extract shows slight decrease of CFU and its continuity throughout the time points. In the MIC and MBC assays, this extract was found to kill the bacteria entirely at its MIC value. However, it may not be so for the time kill-assay as the final volume of reaction is much smaller with the microbroth dilution method (100 uL final volume) compared to the time-kill assay reaction volume (1 mL final volume). There is limited nutrient for the growth of bacteria in a microbroth plate that the centrifuge tube which may contribute to the increase in MIC concentration in the time-kill assay. More interestingly, 4×MIC concentrations is found to kill the bacteria entirely at 8 hours, similar with the positive control colistin at 1×MIC. A simultaneous synergy study also has been done with the same time kill assay, using Colistin. Colistin methasulfonate (CMS) as it is one of the drug of choice being used in the hospitals, to treat carbapenem-resistant *A. baumannii* (Qureshi *et al*., 2015). Due to this, colistin resistance is also being seen in recent times (Trebosc *et al*., 2019). In the present study, it is interesting to see, the 2 combinations: 2×MIC bark methanol extract and 2×MIC Colistin, the combination of 4×MIC bark methanol extract and 0.55× MIC Colistin, eliminating the bacteria at 2 hours and 6 hours respectively, much faster than the control of 1×MIC Colistin. However, further investigation is needed to ascertain how the mechanism of action of the bark methanol extract of *C. bracteosus* against *A. baumannii.*

CONCLUSION

Due to the unending battle with antibiotic resistance, there has been a stall in the momentum of new antibiotic discovery in recent years. However, the crippling state of public health around the world due to antibiotic resistance screams for immediate discovery of novel modalities with antibiotic capabilities. Our study has shown that the plants in this study have novel and valuable antibacterial capabilities against MRSA, MSSA and CRAB. The bark methanol extract of the *C. bracteosus* plant has also been found to have antibiotic potentiator capabilities especially against multi-drug resistant *A. baumannii*. Further investigation into the phytoconstituents of this extract is needed to understand *its* antibacterial and antibiotic potentiator qualities.

Conflict of interest

The authors declare they have no conflict of interests to declare.

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