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Enhanced Antibacterial Activity of Meropenem against Extensively Drug-Resistant *Acinetobacter baumannii* by Myrtaceae Plant Extracts[†]

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Abstract

Acinetobacter baumannii (*A. baumannii*) has been known as a major cause of nosocomial bacterial infections worldwide. The bacteria are increasingly associated with a broad spectrum of antibiotic resistance, and this has become a widespread concern in a variety of hospitals.Antibiotic development and alternative treatment have become priorities for the treatment of bacterial infections.This study investigated the efficacy of meropenem in combination with five ethanolic extracts of plants in Myrtaceae against extensively drug-resistant (XDR) *A. baumannii*. The resistant phenotype was previously determined by microdilution method. XDR-*A. baumannii* strains showed resistance to meropenem with the minimum inhibitory concentration (MIC) in a range of 16 - 128 µg/mL, whereas the MIC value of all extracts, including *Calistemon lancealatus*, *Eucalyptus citridora*, *Rhodomytus tomentasa*, *Syzygium cumini*, and *Xanthortemon chrysanthus*, was over 1,000 µg/mL. Interestingly, all extracts potentiated the activity of the antibiotic by reducing the MIC values of the antibiotic. *Xanthortemon chrysanthus* extract displayed excellent synergism against the bacteria by decreasing the MIC value of the drug greater than 8-fold. In addition, the extract, at concentrations of 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL, obviously increased the inhibitory effect of meropenem (1/4×MIC) against *A. baumannii*. The percentage of bacterial growth inhibition by combination was 87.9, 88.8, 91.8, 93.6, 99.9, and 100, respectively. The results supported that the extract could improve the activity of ineffective antibiotics against drug-resistant pathogens.Therefore, the findings may serve as therapeutic options for XDR-*A. baumannii* infections in the future.

Keywords: *Acinetobacter baumannii*, Combination, Meropenem, Myrtaceae

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Introduction

Acinetobacter baumannii (*A. baumannii*), an opportunistic nosocomial pathogen, is responsible for pneumonia, bacteremia, meningitis, and wound-surgery site infection. The bacteria are a cause of serious problems for global public healthcare, according to various drug resistances [1]. Carbapenems are the first-line drug for multidrug-resistant *A. baumannii* infection, but there is currently-emerging resistance to many antibiotics [2]. Carbapenem resistance is a hallmark of extensively drug-resistant phenotypes, being susceptible to only 1 or 2 antimicrobial categories [3]. Extensively drug-resistant *A. baumannii* (XDR-*A. baumannii*) is extremely difficult to treat and poses considerable infection control issues worldwide. The resistance leads to a 45 - 60 % mortality rate in ICUs. In particular, ventilator-associated pneumonia has been found to be the cause of mortality up to 84.3 % [4]. Colistin is a later-optional therapeutic for drug-resistant *A. baumannii* infections. However, the adverse effects, such as nephrotoxicity, must be considered [5]. Combination antibiotic therapy has shown promise, such as meropenem-sulbactam and meropenem-colistin. Adverse effects such as nephrotoxicity and diarrhea remain [6]. Antibiotic development and alternative treatment with low toxicity are thus urgently needed to deal with these threats.

Searching for new antibiotics from natural resources, such as bacteria, fungi, and plants, has been receiving substantial attention. Among the sources of natural products, almost half of FDA-approved natural products have plant origins [7]. Due to their vast chemical diversity, plant metabolites have emerged as alternate antimicrobial agents and bioenhancers. Plants in Myrtaceae are the ninth largest plant family found in tropical areas and include more than 100 genera and 5,600 species. Myrtaceae species have been used in traditional medicine for the treatment of several diseases, such as gastrointestinal disorders, hemorrhagic issues, and infectious diseases [8]. It has been reported anti-microbial, anti-oxidant, and anti-inflammatory activities. Moreover, several extracts from Myrtaceae plants have been proven low toxicity in mouse models [9-11]. Considering these properties, there has been interest in developing it as an alternative agent.

In recent years, the use of bioactive plant extracts to increase antibiotic susceptibility of drug-resistant bacteria has been of growing interest. Synergistic interaction between natural plants and antibiotics could convincingly reverse the antibiotic resistance of the bacteria and increase the efficiency of antibiotics. Moreover, small doses of synthetic medicines tend to decrease side-effects in pateints. The number of plant extracts acting in synergy with synthetic antibiotics against pathogens has been found. However, active extracts against Gram-negative bacteria, in particular XDR-*A. baumannii*, is in a minority [12]. Therefore, this study aimed to investigate the efficacy of meropenem in combination with extracts of Myrtaceae against XDR-*A. baumannii*.

Materials and methods

Bacterial strains and culture conditions

A. baumannii ATCC 19606 were used as a reference strain. Clinical bacterial isolates were taken from the Microbiology Unit, Department of Pathology, Songklanagarind Hospital, Thailand. Antibiotic sensitivity patterns of *A. baumannii* clinical isolates were assessed using disk diffusion susceptibility assay. The diameter of the inhibition zone around the antibiotic disk was measured and interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) [13], **Table S1** in the Supplementary Data.

Medicinal plant materials

Five Thai medicinal plants in the Myrtaceae family, *Calistemon lancealatus* (*C. lancealatus*), *Eucalyptus citridora* (*E. citridora*), *Rhodomytus tomentasa* (*R. tomentosa*), *Syzygium cumini* (*S. cumini*), and *Xanthortemon chrysanthus* (*X. chrysanthus*), were selected and collected in Nakhon Ratchasima province. Leaves were dried at 60 °C for 3 days and then extracted with 95 % ethanol. The crude extracts were concentrated under reduced pressure in a rotary evaporator until complete dryness. The extracts were dissolved in dimethylsulfoxide before use.

Antimicrobial susceptibility and synergism testing

Susceptibility testing was performed using a broth microdilution method following CLSI guidelines [13]. Minimal inhibitory concentrations (MICs) for meropenem and extracts were determined using resazurin. Subcultures were performed on Mueller-Hinton agar medium to estimate minimal bactericidal concentrations (MBCs).

Synergism testing of meropenem and extracts was assessed. 2-fold dilutions of the drug were carried out with the initial concentration as the MIC value. The concentration of the extracts was tested at 500 µg/mL. MICs of combination were observed using resazurin. Interpretation was done by calculating the fractional inhibitory concentration index (FICI), according to the equation:

$$\text{FICI} = \frac{\text{the MIC of drug and extract}}{\text{the MIC of drug.}} \quad (1)$$

Resistant modifying ability of the extract

Resistant modifying ability of the extract was observed by growth inhibition assays. *A. baumannii* ATCC 19606 was grown in a combination of meropenem at a concentration of 1/4×MIC, and *X. chrysanthus* at varying concentrations. After 16 h of incubation, bacterial cells were enumerated using a drop plate method. The percentage of growth inhibition was calculated from the following equation:

$$\%\ \text{growth inhibition} = \frac{100 \times (\text{No.control} - \text{No.test})}{\text{No.control}} \quad (2)$$

No. control is the number of bacterial cells treated with meropenem, and No. test is number of bacterial cells treated with meropenem and plant extracts. Statistical analysis was performed by Student's paired t-test, with a 2 tailed distribution. All tests were conducted with a significance level of 0.01 ($p < 0.01$).

Results

The minimum inhibitory concentration (MIC) of meropenem was in a range of 16-128 µg/mL and the minimum bactericidal concentration (MBC) was in a range of 32 - 256 µg/mL (**Table 1**). Meanwhile, the MIC value of all extracts of *C. lancealatus*, *E. citridora*, *R. tomentasa*, *S. cumini*, and *X. chrysanthus* was over 1,000 µg/mL. In combination with meropenem, all extracts showed synergistic activity and reduction of the MIC values of the antibiotic by various folds (**Table 2**). Interestingly, *X. chrysanthus* extract displayed excellent synergism against the bacteria by a decrease in the MIC value of the drug greater than 8-folds (**Table 3**). Furthermore, the extract, at concentrations of 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL, notably increased the inhibitory effect of meropenem (1/4×MIC) against *A. baumannii*. Combination therapy illustrated significant reduction ($p < 0.01$) of bacterial growth compared to meropenem alone (**Table 4**). The percentage of bacterial growth inhibition by combination was 87.9, 88.8, 91.8, 93.6, 99.9, and 100, respectively (**Figure 1**).

Table 1 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of meropenem on XDR-*A. baumannii*.

Concentration of meropenem	ATCC 19606	Clinical 1	Clinical 2	Clinical 3	Clinical 4	Clinical 5
MIC (µg/mL)	32	128	128	64	32	64
MBC (µg/mL)	64	128	> 256	128	64	64

Table 2 Effects of meropenem in combination with extracts against XDR-*A. baumannii*.

Extracts	Concentration of meropenem (fold reduction)					
	ATCC 19606	**Clinical 1**	**Clinical 2**	**Clinical 3**	**Clinical 4**	**Clinical 5**
C. lancealatus	8 (4)	32 (4)	≤ 16 (≥ 8)	32 (2)	16 (2)	64 (0)
E. citridora	8 (4)	32 (4)	≤ 16 - 32 (≥ 4)	16 - 32 (≥ 2)	16 (2)	32 (2)
R. tomentasa	8 (4)	32 (4)	≤ 16 (≥ 8)	16 -32 (≥ 2)	16 (2)	64 (2)
S. cumini	16 (2)	32 (4)	32 (4)	32 (2)	32 (0)	≤ 8 - 16 (≥ 4)
X. chrysanthus	8 (4)	≤ 16 (≥ 8)	≤ 16 (≥ 8)	≤ 8-16 (≥ 2)	16 (2)	≤ 8 - 32 (≥ 2)
Meropenem	32	128	128	64	32	64

Table 3 Fractional inhibitory concentration index (FICI) of combination against XDR-*A. baumannii*.

Extracts	Fractional inhibitory concentration index (FICI)					
	ATCC 19606	**Clinical 1**	**Clinical 2**	**Clinical 3**	**Clinical 4**	**Clinical 5**
C. lancealatus	0.25 (S)	0.25 (S)	≤ 0.125-0.25 (S)	0.5 (S)	0.5 (S)	1 (I)
E. citridora	0.25 (S)	0.25 (S)	0.25 (S)	0.5 (S)	0.5 (S)	0.5 (S)
R. tomentasa	0.25 (S)	0.25 (S)	≤ 0.125 (S)	0.5 (S)	0.5 (S)	1 (I)
S. cumini	0.5 (S)	0.25 (S)	0.25 (S)	0.5 (S)	1 (I)	0.25 (S)
X. chrysanthus	0.25 (S)	≤ 0.125 (S)	≤ 0.125 (S)	≤ 0.25 (S)	0.5 (S)	0.5 (S)

FICI: < 1; Synergistic (S), 1 - 4; Indifference (I), > 4; Antagonistic (A)

Table 4 Effects of a combination of meropenem (1/4×MIC) and *X. chrysanthus* extract on the growth of *A. baumannii* ATCC 19606.

Combination	Number of bacteria ($\times 10^4$ cfu/mL)	*p*-value
1/4×MIC meropenem	37888 ± 2524	-
1/4×MIC meropenem + 31.25 µg/mL *X. chrysanthus*	4600 ± 624	0.0011
1/4×MIC meropenem + 62.5 µg/mL *X. chrysanthus*	4244 ± 568	0.0012
1/4×MIC meropenem + 125 µg/mL *X. chrysanthus*	3111 ± 192	0.0016
1/4×MIC meropenem + 250 µg/mL *X. chrysanthus*	2400 ± 458	0.0021
1/4×MIC meropenem + 500 µg/mL *X. chrysanthus*	28 ± 4	0.0015
1/4×MIC meropenem + 1000 µg/mL *X. chrysanthus*	0	0.0015

[a]Values were means ± standard deviations.

[b]*p*-values compared combination therapy and meropenem alone.

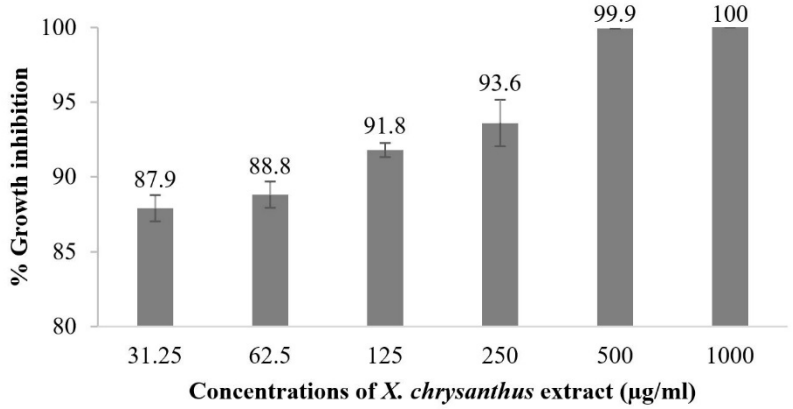

Figure 1 Bacterial growth inhibition of a combination of meropenem (1/4×MIC) and *X. chrysanthus* extract against *A. baumannii* ATCC 19606.

Discussion

Acinetobacter baumannii is an opportunistic bacterial pathogen generally associated with severe nosocomial infections. Increasing multidrug resistance among *Acinetobacter* isolates to most of the currently available antibiotic agents has been documented [1,2,4]. The carbapenem class of β-lactam antibiotics, including meropenem, has been used as the last-line treatment for infections with multidrug-resistant *A. baumannii* [14]. However, during the last few years, a significant increase in the prevalence of carbapenem-resistant *A. baumannii* strains has been reported [15,16]. The incidence has dramatically become a serious problem that limits the therapeutic options for *A. baumannii* infection [15-17]. Considering the bacteria resistance to currently available antimicrobial agents, the development or discovery of new substances, as well as drug combinations, against multidrug-resistant *A. baumannii* infection are essential.

Several plants, such as extracts from *Anthocleista schweinfurthii, Nauclea pobeguiinii,* and *Zehneria scobra*, have been reported to have antibacterial activity and found to have antibiotic-resistance modifying activity against Gram-negative multi-drug resistant bacteria [18,19]. Five *Myrtaceae* plant extracts have been previously reported to have antibacterial activity, particularly Gram-positive bacteria [20]. This study demonstrated that all ethanol extracts had no antibacterial activity against XDR-*A. baumannii* Gram-negative bacteria. However, the crude extracts could enhance XDR-*A. baumannii* sensitivity to meropenem. Therefore, the extracts might have vital composition to interrupt the drug-resistant mechanisms of the bacteria. Numerous plant-derived compounds with antibiotic-modulation activity against drug-resistant bacteria have been noted to inhibit antibacterial resistance in a variety of mechanisms [21,22]. Guttiferone-A and 7-epiclusianone, isolated from the fruits of *Garcinia brasiliensis*, could reverse resistance to ß-lactams by inhibiting ß-lactamase [23], while tannic acid was found to be a drug-resistant modifying agent through the inhibition of carbapenemase [24].

XDR-*A. baumannii* has various drug-resistant mechanisms, including the production of β-lactamases, the modification of penicillin-binding proteins, and the decrease of porin permeability. Moreover, the presence of efflux pumps and MDR-proteins in the bacterial cells significantly contribute to both intrinsic and acquired resistance to antibiotics [25,26]. Plant-derived compounds have resulted in the inhibition of efflux pumps in *A. baumannii* [27] such as *trans*-cinnamaldehyde, eugenol [28], ellagic acid, tannic acid [21], and berberine [29]. In this study, *A. baumannii* ATCC 19606 was used as a meropenem-resistant reference strain [30]. The common antibiotic resistant mechanism of the strain is a multidrug efflux system using resistance-nodulation-division (RND) efflux pumps, specifically Gram-negative microorganisms [31]. In addition, the tested clinical isolates were representative of the absence of $bla_{\text{OXA-23}}$, the carbapenemase producing gene [32]. The strains might resist meropenem via efflux

pump systems. According to the prominent synergistic effect between *X. chrysanthus* extract and meropenem against the bacteria, the plant extract might play a role as an efflux pump inhibitor, leading to improvement in the activity of antibiotics. However, the mechanisms of *X. chrysanthus* extract on bacterial cells should be further investigated. Furthermore, individual compounds of the crude extract should be identified to find the major active compounds with greatest potency.

Conclusions

Myrtaceae plant extract, especially *X. chrysanthus* extract, could improve the activity of ineffective meropenem against drug-resistant pathogens. The drug resistance reversal potential of the extract has been reported for the first time. Therefore, the findings may serve as therapeutic options for XDR-*A. baumannii* infections in the future. In addition, the active constituents of the promising plant should be further investigated.

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Supplementary data

Table S1 Antibiotic sensitivity patterns of *Acinetobacter baumannii* isolates.

Antibiotic class	Antibiotic	*Acinetobacter baumannii*				
		clinical 1	**clinical 2**	**clinical 3**	**clinical 4**	**clinical 5**
aminoglycosides	amikacin	R	S	R	R	R
	gentamicin	R	R	R	R	S
	netilmicin	R	R	R	R	R
carbapenems	imipenem	R	R	R	R	R
	meropenem	R	R	R	R	R
	ertapenem	R	R	R	R	R
cephalosporins	cefotaxime	R	R	R	R	R
	cefoxitin	R	R	R	R	R
	ceftriaxone	R	R	R	R	R
	cefuroxime	R	R	R	R	R
	sulperazone	I	S	R	R	S
fluoroquinolones	ciprofloxacin	R	R	R	R	R
	norfloxacin	R	S	R	R	R
penicillin + β-lactamase inhibitor	ampicillin	R	R	R	R	R
	tazocin (piperacillin/ tazobactam)	R	R	R	R	R
polymyxins	colistin	S	S	R	S	S
sulfonamides	co-trimoxazole (trimethoprim/ sulfamethoxazole)	R	R	S	R	R
tetracycline	tigecycline	R	S	R	I	I