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Synergistic effects of ethnomedicinal plants of Apocynaceae family and antibiotics against clinical isolates of *Acinetobacter baumannii*

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ABSTRACT

Objective: To investigate the efficacy of 17 ethnomedicinal plants belonging to Apocynaceae family used in combination with 16 conventional antibiotics against non-multidrug resistant-, multidrug resistant (MDR)-, and extensive drug resistant (XDR) *Acinetobacter baumannii* (*A. baumannii*). **Methods:** Antibacterial activity and resistance modifying ability of 272 combinations were determined by growth inhibition assays and further confirmed by time-kill assay. **Results:** Among the combinations of the antibiotics with Apocynaceae ethanol extracts on this pathogen, 15 (5%) had synergistic effects, 23 (8%) had partial synergistic effects and 234 (86%) had no effects. Synergistic activity was observed mostly when the Apocynaceae extracts were combined with rifampicin or cefazolin. Interestingly, 10 out of 17 combinations between the extracts and rifampicin displayed synergistic or partial synergistic behaviors. *Holarrhena antidysenterica* extract was additionally tested to restore rifampicin activity against clinical isolates of MDR and XDR *A. baumannii*. With respect to total or partial synergy, 70% was XDR *A. baumannii* isolates and 66% was MDR *A. baumannii* isolates. **Conclusions:** *Holarrhena antidysenterica* extract clearly demonstrated the ability to restore rifampicin activity against both *A. baumannii* ATCC19606 and clinically isolated *A. baumannii*. Additional studies examining its active principles as well as mechanisms of actions such as the effects on efflux pumps and outer membrane permeability alterations are recommended.

1. Introduction

Increasing prevalence of multidrug resistant (MDR) bacteria and limited treatment options have necessitated the discovery of new antibacterial and resistance modifying agents. Resistance modifying agents (RMAs) are compounds which potentiate the activity of an antibiotic against a resistant strain and may also target and inhibit MDR mechanisms[1]. An application of a RMA with a conventional antibiotic is well accepted. Augmentin® is an important example which uses a combination of amoxicillin and a microbial-derived beta-lactamase inhibitor as a RMA (clavulanate)[2]. Recent experiments have additionally demonstrated that molecules capable of blocking the action of efflux pumps have the potential to circumvent antimicrobial resistance[3]. Stermitz *et al* reported for the first time the synergistic effect of a plant-derived ineffective antibacterial agent, berberine and a multidrug resistance pump inhibitor, 5'-methoxyhydnocarpin produced by *Berberis* species against *S. aureus*[4]. Furthermore, several plant-derived alkaloids and polyphenols such as reserpine, quinine, harmaline, piperine, epigallocatechin gallate, tellimagrandin I, and rugosin B have been demonstrated to act as efflux pump inhibitors for Gram positive pathogen[5]. Recently, we have demonstrated that *Holarrhena antidysenterica* (Linn) Wall. (Apocynaceae) possessed a

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remarkable RMA ability in combination with novobiocin against *Acinetobacter baumannii* (*A. baumannii*) ATCC 19606[6].

To our knowledge, there is no report on the RMA activity of other ethnomedicinal plants from the family Apocynaceae as well as relatively few studies have been carried out to evaluate RMA activities of plant-derived compounds on *A. baumannii*. Therefore, this study was aimed to investigate the RMA activity of medicinal plants belonging to the family Apocynaceae in combination with conventional antibiotics against *A. baumannii* ATCC 19606 and a collection of clinical *A. baumannii* isolates.

2. Materials and methods

2.1. Bacterial strains and culture condition

Clinically isolated *A. baumannii* isolates were obtained from Songklanakarin Hospital from pus (n=1), blood (n=2), sputum (n=5), body fluid (n=4), and urine (n=7) samples of infected patients. *A. baumannii* ATCC 19606 was employed in this study as a quality control strain. The strains were cultured on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI) and incubated at 37 ℃ overnight. Colonies from the plates were grown in Mueller Hinton broth (MHB) (Difco Laboratories, Detroit, MI) at 37 ℃ for 18-24 h and adjusted to McFarland standards No. 0.5. The suspensions were further diluted with MHB to obtain inocula containing 1×10^6 CFU/mL.

Susceptibility test was performed by the disk diffusion method according to Clinical and Laboratory Standard Institute (CLSI) recommendations[7]. MDR phenotypes were defined as isolates resistant to at least three different antimicrobial classes and the isolates resistant to all tested agents were classified as extensive drug resistant (XDR) phenotypes[8].

2.2. Medicinal plant materials and extraction

Seventeen selected plant species belonging to the Apocynaceae family were selected based on their potential use in folk medicine for treatments of diseases, or known to have antimicrobial activities as described in Table 1. The medicinal plants were purchased from medicinal herb retailers in Songkhla, Thailand and authenticated by a taxonomist, Dr. Katesarin Maneenoon and voucher specimens were deposited at the Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The samples were washed with distilled water and dried at 60 ℃ overnight. Ground plant material (100 g) was macerated with 95% (v/v) ethanol (500 mL) for 7 days at room temperature. After filtrations through a Whatman No. 1 paper, the filtrates were concentrated using a rotatory evaporator, and kept at 55 ℃ until they were completely dried. Yields (%; w/w) of each extracts were calculated as the ratio of the weight of the extract to the weight of the herb powder. A stock solution (200 mg/mL) was prepared by dissolving the dried extract in dimethylsulfoxide (DMSO) (Merck, Germany).

2.3. Resistant modifying ability of medicinal plant extracts

Intrinsic anti-*A. baumannii* ATCC19606 activities of the Apocynaceae extracts and a panel of selected antibiotics consisting of cell wall inhibitors (penicillin, oxacillin, ampicillin, imipenem, cefazolin, ceftazidime, and vancomycin), protein synthesis inhibitors (amikacin, gentamicin, streptomycin, fusidic acid, erythromycin, and tetracycline), DNA synthesis inhibitors (novobiocin and ciprofloxacin), and RNA synthesis inhibitors (rifampicin) were determined by growth inhibition assays as previously described[9]. Briefly, the culture, containing 1×10^6 CFU/mL (100 μL) was inoculated into a 96-well microtiter

Table 1
Medicinal properties and extraction yields of ethanol extracts of selected Apocynaceae ethnomedicinal plants.

Medicinal plants (Plant parts)	Medicinal properties	Extraction yield (%; w/w)
Adenium obesum (Forssk.) Roem. & Schult. (Leaves)	Anti-cancer activity[20]	1.85
Allamanda cathartica L. (Flowers)	Treating malaria and jaundice[21]	4.79
Alstonia macrophylla Wall. (Bark)	Body tonic and anti-fever agents[22]	2.76
Alstonia scholaris (L.) R.Br. (Bark)	Treating asthma and cardiac[23]	4.43
Alyxia reinwardtii BL. var. *lucida* Markgr. (Branch)	Antioxidant activity[24]	4.51
Carissa spinarum L. (Branch)	Wound healing activity[25]	3.50
Catharanthus roseus L. (Branch)	Used for treating cancers[26]	6.14
Cerbera manghas L. (Bark)	Anti-cancer activity[27]	12.20
Cerbera odollam Gaertn. (Bark)	Anti-cancer activity[28]	15.46
Holarrhena antidysenterica (L.) Wall. (Bark)	Antibacterial activity[29]	2.72
Holarrhena curtisii King & Gamble (Branch)	Leishmanicidal activities[30]	2.51
Nerium oleander L. (Branch)	Treating skin diseases[31]	4.52
Plumeria obtusa (Bark)	Treating skin diseases[32]	6.75
Plumeria rubra L. (Bark)	Antibacterial activity[33]	7.52
Rauvolfia serpentina (L.) Benth. ex Kurz (Root)	Antibacterial activity[34]	1.78
Thevetia peruviana (Pers.) K. (Bark)	Antidiarrhoeal and antimicrobial activities[35]	11.66
Wrightia tomentosa Roem. & Schult. (Branch)	Antibacterial activity[36]	2.75

plate containing 50 μL of the extract (1 000 μg/mL) or the antibiotic and 50 μL of MHB. The antibiotics were purchased from Becton Dickinson Microbiology Systems (Sparks, MD, USA), Difco (Detroit, MI, USA) or made using the laboratory collection of antibiotics.

The intrinsic antibacterial activity was exhibited as the percentage of growth inhibition (GI) after incubation at 37 ℃ for 18 h and calculated from the following equation:

$$GI\ (\%) = (OD_{control} - OD_{test})/OD_{control} \times 100. \quad (1)$$

where, $OD_{control}$ is optical density (OD) 620 nm of bacteria culture in MHB supplemented with 1% (v/v) DMSO as a positive control and OD_{test} is OD 620 nm of the bacterial culture in MHB supplemented with the tested agent. The OD_{test} of respective blanks having only the extract was subtracted to give the final OD_{test}. GI_A and GI_E represent the percentage inhibition of bacterial growth of the antibiotic and extract, respectively.

Resistance modifying ability of each extract was observed by adding of 50 μL of the tested extract into the tested plate supplemented with the antibiotic instead of MHB. This biological activity was exhibited as the percentage of growth inhibition as well but calculated from the following equation:

$$\%\text{Growth inhibition of the combination } (GI_C) = OD_{control} - OD_{test}/OD_{control} \times 100. \quad (2)$$

where, $OD_{control}$ is OD 620 nm of the positive control culture and OD_{test} is OD 620 nm of the bacterial culture in MHB supplemented with the extract in combination with the antibiotic.

The interpretation of the combination was classified as synergism when GI_C/GI_A and GI_C/GI_E ratios were ≥2.0, partial synergism when 1.5≤the ratios<2.0, and no effect when the ratios <1.5. Ellagic acid at 40 μmol/L was included as a positive control RMA in combination with erythromycin, novobiocin, and rifampicin against *A. baumannii* ATCC19606.

The efficacy of combination therapy of the promising medicinal plants in combination with the antibiotics was additionally determined against 19 clinically isolated *A. baumannii* isolates using the growth inhibition assay as described above and further confirmed by a time-kill assay.

3. Results

In this present investigation, the growth inhibition assay was employed to develop another approach for combating *A. baumannii* infections using medicinal plants to potentiate the activity of antibiotics. Independently, 15 out of 17 tested ethanol extracts at concentration of 1 000 μg/mL displayed low inherent anti-*A. baumannii* activity (% of bacterial growth inhibition was less than 75%) (Table 2). Only *Alstonia macrophylla* and *Carissa spinarum* which completely inhibited the bacterial growth at this concentration possessed moderate antibacterial activity.

From 272 combinations tested between 17 medicinal plants and 16 antibiotics, 15 (5%) showed synergism, 23 (8%) had partial synergistic interaction, and 234 (86%) had no effect. Synergistic activity was observed mostly when the Apocynaceae extracts were combined with rifampicin or cefazolin against *A. baumannii* ATCC19606. Synergistic behaviors were displayed in cefazolin in combination with *Alstonia scholaris*, *Cerbera odollam*, *Holarrhena antidysenterica*, *Nerium oleander*, or *Thevetia peruviana* or rifampicin in combination with *Adenium obesum*, *Holarrhena antidysenterica*, *Plumeria obtuse*, *Thevetia peruviana*, or *Wrightia pubescens* (Table 3).

The ability of a representative effective resistance modifier, *Holarrhena antidysenterica* to potentiate the antibacterial activity of rifampicin against clinically isolated *A. baumannii* was additionally evaluated to explore the potential of developing a promising RMA (Table 4). The interaction between the ethanol extract and rifampicin was synergistic and partially synergistic in 8 (42.1%) and 3 (15.8%) isolates of *A. baumannii* tested, respectively. With respect to total or partial synergy, 70%, 66%, and 33% of the isolates were XDR *A. baumannii*, MDR *A. baumannii*, and non-MRD *A. baumannii*, respectively.

The synergistic effect of this combination was further confirmed by time-kill assay as illustrated in Figure 1. At the tested concentration, the extract exhibited no antibacterial potencies, but it was shown to be a powerful RMA in combination with rifampicin against *A. baumannii* ATCC 19606, non-MDR *A. baumannii*, and XDR *A. baumannii*.

Table 2

Intrinsic anti-*Acinetobacter* activity[a] of Apocynaceae ethnomedicinal plants.

Bacterial growth inhibition (%)[b]	No. of Apocynaceae (Plant species)
0-25	3 (*Allamanda cathartica*; *Cerbera manghus*; *Thevetia peruviana*)
26-49	8 (*Adenium obesum*; *Catharanthus roseus*; *Holarrhena antidysenterica*; *Holarrhena curtisii*; *Nerium oleander*; *Plumeria obtusa*; *Plumeria rubra*; *Wrightia pubescens*)
50-75	4 (*Alstonia scholaris*; *Alyxia reinwardtii*; *Cerbera odollam*; *Rauvolfia serpentine*)
76-100	2 (*Alstonia macrophylla*; *Carissa spinarum*)

[a]An antibacterial activity of phytochemicals is considered to be significant if MIC values are below 100 μg/mL for crude extract and 10 μg/mL for pure compounds[37].

[b]The percentage inhibition of bacterial growth was calculated by using the equation:

Bacterial growth inhibition (%) = $(OD_{control} - OD_{test})/OD_{control} \times 100$. Where, $OD_{control}$ represents the optical density at 620 nm of the control culture in MHB containing 1% (v/v) DMSO, OD_{test} represents the optical density at 620 nm of the culture in MHB containing 1 mg/mL of the ethanol extract. The OD_{test} of respective blanks having only the extract was subtracted to give the final OD_{test}.

Table 3

Resistance modifying ability of Apocynaceae ethanol extracts in combination with selected antibiotics against *Acinetobacter baumannii* ATCC 19606.

Plant species	Number of synergy or partial synergy interactions [Antibiotics[a]; (GI_C:GI_A/GI_C:GI_E)[b]]			
	Synergy[c]		Partial synergy[c]	
Adenium obesum	1	RIF (2.8/2.0)	3	MEM (1.8/4.6), GEN (1.5/1.7), ERY (1.5/4.9)
Allamanda cathartica	0	–	1	FUS (1.7/3.3)
Alstonia macrophylla	0	–	0	–
Alstonia scholaris	1	KZ (21.6/7.9)	0	–
Alyxia reinwardtii	0	–	2	KZ (17.8/1.5), RIF (2.6/1.5)
Catharanthus roseus	0	–	1	STR (1.8/22.0)
Carissa spinarum	0	–	0	–
Cerbera manghus	3	MEM (3.5/4.7), GEN (4.2/2.6), ERY (2.4/4.3)	2	AMP (1.7/3.6), RIF (4.7/1.8)
Cerbera odollam	1	KZ (21.4/3.5)	5	PEN(2.4/1.9), OXA (2.2/1.9), AMP (2.3/1.7), VAN (2.7/1.5), RIF (2.0/1.9)
Holarrhena antidysenterica	3	KZ (21.4/3.5), STR (3.9/9.5), RIF (2.7/3.0)	0	–
Holarrhena curtisii	0	–	1	KZ (11.9/1.6)
Nerium oleander	1	KZ (15.7/2.7)	1	RIF (1.7/2.0)
Plumeria obtusa	1	RIF (2.0/4.2)	0	–
Plumeria rubra	0	–	2	GEN (1.6/2.3), RIF (1.7/1.5)
Rauvolfia serpentin	0	–	0	–
Spirolobium cambodianum	0	–	0	–
Thevetia peruviana	3	KZ (21.1/18.4), FUS (2.2/2.3), RIF (2.1/6.2)	0	–
Wrightia pubescens	1	RIF (2.0/2.1)	5	MEM (1.7/6.4), KZ (1.8/35.3), GEN (1.8/3.1), STR (1.9/24.0), ERY (1.9/9.0),

[a]PEN=Penicillin G; OXA=Oxacillin; AMP=Ampicillin; MEM=Meropenem; KZ=Cephazolin; VAN=Vancomycin; GEN=Gentamicin; STR=Streptomycin; FUS=Fusidic acid; ERY=Erythromycin; RIF=Rifampicin.

[b]GI_C represents the percentage inhibition of bacterial growth of tested antibiotics in combination with the extracts. GI_A and GI_E represent the percentage inhibition of bacterial growth of the antibiotics and the extracts, respectively.

[c]Combinations were classified as synergistic effects if both GI_C:GI_A and GI_C:GI_E ratios were $\geqslant$2.0, partially synergetic effects if 1.5$\leqslant$the ratios<2.0, indifferent effects if the ratios were <1.5.

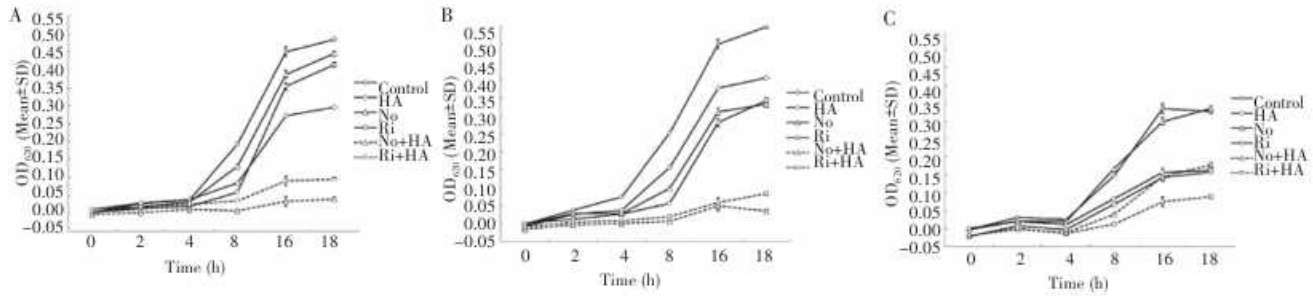

Figure 1. Time-kill activities of *Holarrhena antidysenterica* (HA; 125 μg/mL), rifampicin (Ri; 1/4 MIC), novobiocin (No; 1/4 MIC), the combination of *Holarrhena antidysenterica* and rifampicin (Ri+HA), and the combination of *Holarrhena antidysenterica* and novobiocin (No+HA) againt *Acinetobacter baumannii* ATCC 19606 (A), non-MDR *A. baumannii* (B), and XDR *A. baumannii* (C). Minimun inhibitory concentrations of Ri and No against *A. baumannii* ATCC 19606, non-MDR *A. baumannii*, and XDR *A. baumannii* were 0.63 and 6.25, 5.00 and 25.00, and 1.25 and 6.25 μg/mL, respectively.

4. Discussion

Uses of rifampicin in combination with colistin have been studied for the treatment of MDR *A. baumannii* infections. Both *in vitro* studies and clinical studies were employed to recommend the safety and clinical effectiveness of rifampicin in combination with colistin against this pathogen[10-13]. It was suggested that colistin probably causes rapid permeabilization of the outer membrane, which enhances penetration and activity of rifampicin. Similarly, plant-derived compounds that act as permeabilizers such as coriander oil (*Coriandrum sativum*)[14], geraniol (*Helichrysum italicum*)[15], and [6]-dehydrogingerdione and [10]-gingerol (*Zingiber officinale*)[16] have been shown to reduce the resistance of *A. baumannii* to other antibiotics. Even though antibacterial activity of *Holarrhena antidysenterica* and its constituents have been reported, there is to our knowledge no published scientific literature of RMA activity on rifampicin of this plant or its constituents.

Rifampicin resistance in *A. baumannii* is related to the synergistic interaction between modifications of antibiotic permeability, enzymatic modification by rifampicin

Table 4

Effects of *Holarrhena antidysenterica* ethanol extract (HA; 125 μg/mL) as a resistant modifying agent for rifampicin (Ri; 0.5 μg/mL) against clinically isolated *A. baumannii*.

MDR- *A. baumannii* isolates (Sources)[a]	Antibiogram profile[b]	GI (%)[c] in the presence of		GI_C:GI_A/GI_C:GI_E[d]
		Ri (0.5 μg/mL)	HA (125 μg/mL)	
NPRC AB002 (BF)	SRISRSSSSS	13.2±1.3	13.6±1.1	1.0/1.0
NPRC AB003 (S)	RRRRRRRRSR	40.8±1.4	35.7±1.0	2.5/2.9
NPRC AB004 (U)	SRISISSSSS	89.2±1.2	2.0±1.7	1.1/7.0
NPRC AB005 (U)	RRRRR-RSIS	19.2±0.6	-21.0±1.5	1.8/1.7
NPRC AB010 (B)	RRRRRRRRSR	30.7±1.8	35.5±3.7	1.6/1.4
NPRC AB011 (U)	RRRRRRRRIR	37.2±0.5	37.3±1.3	1.0/1.0
NPRC AB013 (B)	RRRRRRRRRR	17.8±2.6	37.9±2.9	4.8/2.3
NPRC AB014 (U)	SRRSR-SRIR	19.0±4.1	22.0±2.8	4.4/3.8
NPRC AB015 (U)	RRRRR-RRRR	25.3±0.7	23.0±1.4	2.1/2.3
NPRC AB016 (U)	SRRSRRSSSS	51.8±1.9	23.8±5.0	1.2/2.7
NPRC AB017 (BF)	RRRRRRRRRR	64.7±0.9	56.1±1.1	1.6/1.9
NPRC AB018 (S)	RRRRRRRRRR	32.8±2.4	34.8±2.0	2.0/1.9
NPRC AB019 (S)	RRRRRRRRRR	33.3±0.8	30.0±0.8	2.3/2.5
NPRC AB021 (S)	SRISR-SRSR	40.7±2.8	27.5±1.6	2.1/3.2
NPRC AB022 (U)	RRRRRRRRRR	18.1±1.6	39.2±1.1	2.1/0.4
NPRC AB024 (BF)	-RRR-R-R--	57.9±0.9	27.8±1.9	1.8/3.7
NPRC AB026 (S)	RRRRRRRRSR	34.4±0.7	37.3±0.8	4.7/3.0
NPRC AB028 (BF)	RRRRRRRRIR	35.9±1.5	28.8±1.0	2.1/2.6
NPRC AB029 (P)	SRSSSSSSSS	17.4±4.8	27.5±3.8	1.4/8.7

[a]Clinically isolated *A. baumannii* were obtained from pus (P), blood (B), sputum (S), body fluid (BF), and urine (U) samples of infected patients.
[b]The antibiogram profile is the susceptibility results for amikacin, ampicillin, cefotaxime, cefazolin, cefuroxime, ciprofloxacin, gentamicin, imipenem, cephoperazon/sulbactam, and meropenem.
[c]GI represents the percentage inhibition of bacterial growth of the tested compounds.
[d]Combinations were classified as synergistic effects (GI_C:GI_A and GI_C:GI_E ratios $\leqslant$2.0), partially synergetic effects (1.5$\leqslant$$GI_C$:$GI_A$ and GI_C:GI_E ratios<2.0), indifferent effects (GI_C:GI_A and GI_C:GI_E ratios <1.5). GI_A, GI_E, and GI_C represent the percentage inhibition of bacterial growth of Ri, HA, and the Ri+HA combination, respectively.

ADP-ribosyl-transferase (arr-2), or mutation in rpoB[17-19]. A recent finding by Giannouli *et al* proposed that the combined treatment with colistin/rifampicin versus colistin alone were evident only in *A. baumannii* strains with no chromosomal mutations in RNA polymerase β-subunit rpoB target gene[11]. Interestingly, phenylalanine arginine β-naphthylamide (PAβN), an efflux pump inhibitor, reduced the minimun inhibitory concentration of rifampicin at 256 μg/mL by approximately 30-fold in *A. baumannii* isolate that showed no mutation in the rpoB target gene[11].

The present results indicate that the ethanol extract of *Holarrhena antidysenterica* is a promising resistance modifying agent for rifampicin against *A. baumannii*, due to its synergistic effect in combination with rifampicin against both *A. baumannii* ATCC19606 and clinically isolated *A. baumannii*. The findings may lead to development of an effective alternative treatment in combating the antimicrobial resistance in *A. baumannii*. Therefore, the mechanisms of action of this combination as well as the active constituents of *Holarrhena antidysenterica* should be further investigated.

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Conflict of interest statement

We declare that we have no conflict of interest.

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