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Original Research

A synergistic effect of artocarpanone from *Artocarpus heterophyllus* L. (Moraceae) on the antibacterial activity of selected antibiotics and cell membrane permeability

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ABSTRACT

Aim/Backgrounds: Artocarpanone isolated from *Artocarpus heterophyllus* L. (Moraceae) exhibits antibacterial activity. The present study investigated synergistic activity between artocarpanone and tetracycline, ampicillin, and norfloxacin, respectively, against methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Escherichia coli*. **Materials and Methods:** A broth microdilution method was used for evaluating antibacterial susceptibility. Synergistic effects were identified using a checkerboard method, and a bacterial cell membrane disruption was investigated by assay of released 260 nm absorbing materials following bacteriolysis. **Results and Discussion:** Artocarpanone exhibited weak antibacterial activity against MRSA and *P. aeruginosa* with minimum inhibitory concentrations values of 125 and 500 μg/mL, respectively. However, the compound showed strong antibacterial activity against *E. coli* (7.8 μg/mL). The interaction between artocarpanone and all tested antibiotics revealed indifference and additive effects against *P. aeruginosa* and *E. coli* (fractional inhibitory concentration index [FICI] values of 0.75-1.25). The combination of artocarpanone (31.2 μg/mL) and norfloxacin (3.9 μg/mL) resulted in synergistic antibacterial activity against MRSA, with an FICI of 0.28, while the interaction between artocarpanone and tetracycline, and ampicillin showed an additive effect, with an FICI value of 0.5. A time-kill assay also indicated that artocarpanone had a synergistic effect on the antibacterial activity of norfloxacin. In addition, the combination of artocarpanone and norfloxacin altered the membrane permeability of MRSA. **Conclusion:** These findings suggest that artocarpanone may be used to enhance the antibacterial activity of norfloxacin against MRSA.

KEY WORDS: Ampicillin, artocarpanone, norfloxacin, synergistic, tetracycline

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INTRODUCTION

The emergence of multidrug-resistant bacteria has led to global concerns about failure to cure certain highly dangerous infectious diseases. *Staphylococcus aureus* is an opportunistic Gram-positive bacterium that may cause dangerous infections, due to its ability to carry the resistance genes for many antibiotics [1]. Currently, the most prevalent resistant bacterium, particularly in a hospital environment is the methicillin-resistant *S. aureus* (MRSA). Acquisition of the *mec*A gene and its ability to over-express efflux pumps as well as to produce a β-lactamase enzyme are the underlining causes for the resistance of MRSA toward many antibiotics, especially β-lactam antibiotics [2]. A high prevalence of nosocomial infections caused by MRSA has been reported from many countries worldwide [3]. The increasing drug resistance of Gram-negative bacteria, including *Pseudomonas aeruginosa*, mainly due to mutation in target enzymes [4] has also raised concerns. Consequently, the identification and development of new antibiotics with new targets and modes of action are urgently needed, but major time

and cost factors are involved to ensure that the new compound is safe and effective and will not induce resistance when used clinically. The combination of conventional antibiotics with an agent that can enhance antibacterial activity has been suggested as an alternative strategy to overcome these problems [5].

Plant-derived compounds are recognized as an important source of new antibacterials. Many flavonoids have been reported to have antimicrobial activity [6], and some have been demonstrated to exert a synergistic effect on the activity of commercial products against resistant bacteria including MRSA [7,8]. Artocarpanone (Figure 1) is a flavonoid isolated from *Artocarpus heterophyllus*, which exhibits a range of pharmacological properties including antibacterial, anti-tyrosinase, and cytotoxic activity [9-11]. However, we have found no reports describing the synergistic effect of artocarpanone on the activity of antibiotics. The aim of the present study was to determine whether artocarpanone could enhance the antibacterial activity of the conventional antibiotics tetracycline, ampicillin, and norfloxacin that are normally used against *S. aureus* but are not effective against MRSA. The study as also extended to encompass the Gram-negative bacteria, *P. aeruginosa* and *Escherichia coli*. We also investigated the ability of artocarpanone/antibiotic combinations to disrupt bacterial cell membranes in a synergistic manner.

MATERIALS AND METHODS

Chemicals

Artocarpanone was purified from the crude ethyl acetate extract of A. *heterophyllus* heartwoods as described previously [9]. The antibiotics ampicillin, tetracycline, and norfloxacin were purchased from Sigma (Sigma-Aldrich, UK). Crystal violet was obtained from LabChem Inc. (Laboratory Chemical, Australia). Brain–heart infusion (BHI) was from the Becton, Dickinson and Company (Franklin Lakes, New Jersey, USA).

Bacterial Strains

MRSA (DMST 20654), *P. aeruginosa* (DMST 15442), and *E. coli* (ATCC 25922) were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand.

Determination of Minimum Inhibitory Concentrations (MICs)

A microdilution assay was used to determine the MIC of each antibiotic system against the selected bacteria strains [12]. Two-fold dilutions of each sample in BHI were prepared in a sterile 96-well plate. Bacterial suspensions were prepared in 0.85% NaCl, and the turbidity was adjusted to 0.5 McFarland standard (equivalent to 1×10^8 CFU/mL). The suspension was diluted with normal saline to contain 1×10^6 CFU/mL and added into each well. The final cell concentration was 5×10^5 CFU/mL. The plate was incubated at 37°C for 24 h, and the MIC was recorded as the lowest concentration of the sample that produced suppression of visible growth.

Checkerboard Assay for Antibacterial Activity

The antibacterial activity of combination antibiotics was evaluated against the selected bacteria as described by Chang *et al.*, with a slight modification [13]. The assay was performed using artocarpanone in combination with ampicillin, tetracycline, and norfloxacin, respectively, in 96-well plates. Two-fold dilutions of artocarpanone were prepared in BHI along the X-axis, while 2-fold dilutions of the antibiotics were prepared along the Y-axis. Subsequently, each well was inoculated with bacteria suspension of 1×10^6 CFU/mL and the plates were incubated at 37°C for 24 h. The fractional inhibitory concentration index (FICI) was quantified as the FIC for artocarpanone and the FIC for antibiotic, where the FIC for artocarpanone was the MIC of artocarpanone in combination divided by MIC for artocarpanone alone, while the FIC for antibiotic was the MIC of antibiotic in combination divided by the MIC of antibiotic alone.

FICI = FIC of artocarpanone + FIC of the antibiotics

$$\text{FIC} = \frac{\text{MIC of artocarpanone or antibiotics in combination}}{\text{MIC of artocarpin or antibiotics alone}}$$

The results were interpreted as synergistic (FICI ≤ 0.5), additive (0.5 ≤ FICI ≤ 1), indifferent (1 ≤ FICI ≤ 4), or antagonistic (FICI > 4) [14].

Time-kill Assay

Bacterial suspension containing 1×10^6 CFU/mL was added to BHI broth containing various combinations of antibiotics to reach a final cell concentration of 5×10^5 CFU/mL, then incubated at 37°C. A time-kill assay was performed at eight time intervals (0, 1, 2, 4, 6, 8, 12, and 24 h). Aliquots (50 μL) of the cultures were diluted (1:10) with 450 μL of normal saline, and 20 μL of each dilution was cultured on BHI agar. The numbers of viable colonies were recorded after a 24-h incubation [15].

Bacteriolysis Assay

The alteration of cell membrane permeability was investigated by measuring uptake of crystal violet [16]. Briefly, a suspension of MRSA in normal saline was prepared from an overnight culture on BHI agar. A single dose of artocarpanone and norfloxacin as well as artocarpanone in combination with norfloxacin was added to the cell suspension and incubated at 37°C for 1 h. The final cell concentration was 5×10^7 CFU/mL. Untreated suspensions of MRSA were used as a negative control. The cells were harvested at 13,400 × g for 5 min and resuspended in a crystal violet solution (10 μg/mL in normal saline). The cells were incubated at 37°C for 10 min and harvested by centrifugation at 25°C for 15 min. The optical density (OD of the supernatant) at 590 nm was measured using an ultraviolet-visible (UV-Vis) spectrophotometer (Genesis-6, Becthai, Bangkok). The OD reading of the crystal violet solution used for the assay was considered to represent the value of 100%.

The percentage crystal violet uptake was calculated as the OD value of the sample supernatant/OD value of the crystal violet solution ×100. This experiment was performed in triplicate.

Loss of 260 nm Absorbing Material

The concentration of released UV-absorbing material from bacteria exposed to antibiotics is a measure of metabolites, nucleic acid, and ion that were detected at 260 nm [16-18]. Overnight cultures of MRSA were washed with normal saline and resuspended in normal saline. Artocarpanone and norfloxacin alone as well as combinations of artocarpanone and norfloxacin were added to the cell suspensions to give a final cell concentration of 5×10^7 CFU/mL. Untreated cell suspensions were used as the control. Test samples were incubated at 37°C for 1 h and then centrifuged at 25°C at 13,400 × g for 15 min. The OD_{260} of the supernatant was measured using a UV-Vis spectrophotometer to determine the quantity of intracellular UV-absorbing material released by the cells. The assay was performed in triplicate.

Statistical Analysis

All experiments were carried out in triplicate with the average value and standard deviations reported. The data were analyzed using ANOVA followed by the Tukey's honestly significant difference *post-hoc* test to identify significant difference between group means. Statistical significance was accepted at the level $P < 0.01$.

RESULTS

MICs

Artocarpanone exhibited strong antibacterial activity against *E. coli* with an MIC of 7.8 μg/mL, but it had a weak antibacterial activity against *P. aeruginosa* and MRSA with MICs of 500 and 125 μg/mL, respectively [Table 1]. Norfloxacin showed the strongest antibacterial activity against *E. coli* and *P. aeruginosa* with MICs of 1.9 μg/mL while tetracycline and ampicillin also demonstrated strong antibacterial activity with MIC values of 7.81-15.62 μg/mL. However, all tested antibiotics only revealed moderate-weak antibacterial activity against MRSA (MIC of 62.5-125 μg/mL).

Checkerboard Analysis

Interaction between artocarpanone (125 μg/mL) and norfloxacin (0.9 μg/mL) showed an additive effect against *P. aeruginosa* (FICI of 0.75), while combination of artocarpanone (250 μg/mL) and tetracycline (7.8 μg/mL) as well as artocarpanone (125 μg/mL) and ampicillin (15.6 μg/mL) gave the indifference effects with FICIs of 1 and 1.25, respectively [Table 2]. On the one hand, artocarpanone (3.9 μg/mL) exhibited an additive effect on the antibacterial activity of tetracycline (1.9 μg/mL) against *E. coli* with FICI of 0.75 and showed indifference effect in combination artocarpanone (0.9 μg/mL) and ampicillin (15.6 μg/mL) as well as artocarpanone (3.9 μg/mL) and norfloxacin (0.5 μg/mL) with FICIs of 1.1 and 1, respectively [Table 3]. In case of MRSA, artocarpanone (31.2 μg/mL) also performed additive effects when combined with tetracycline (31.2 μg/mL) and ampicillin (15.6 μg/mL) with FICIs of 0.5. Interestingly, in combination with norfloxacin, artocarpanone (31.2 μg/mL) enhanced the antibacterial activity of norfloxacin (3.9 μg/mL) with a synergistic effect (FICI value of 0.28) [Table 4].

Time-kill Assay

The combination of 31.2 μg/mL artocarpanone and 3.9 μg/mL norfloxacin completely inhibited bacterial growth at the limit of quantification (10^2) within 12 h, while artocarpanone and norfloxacin alone at the concentration of 62.5 μg/mL did not completely inhibit bacterial growth until 24 h [Figure 2].

Bacteriolysis

The percentage of uptake of crystal violet indicated the bacteriolytic activity of the compounds against MRSA

Table 1: Antibacterial activity of artocarpanone and antibiotics against three tested bacteria

Bacteria	MIC (μg/mL)			
	Artocarpanone	Tetracycline	Ampicillin	Norfloxacin
E. coli	7.8	7.8	15.6	1.9
P. aeruginosa	500	15.6	15.6	1.9
MRSA	125	125	62.5	125

MRSA (methicillin-resistant *Staphylococcus aureus*)

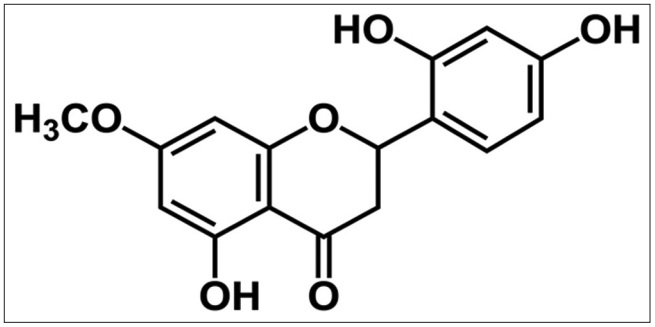

Figure 1: Chemical structure of artocarpanone

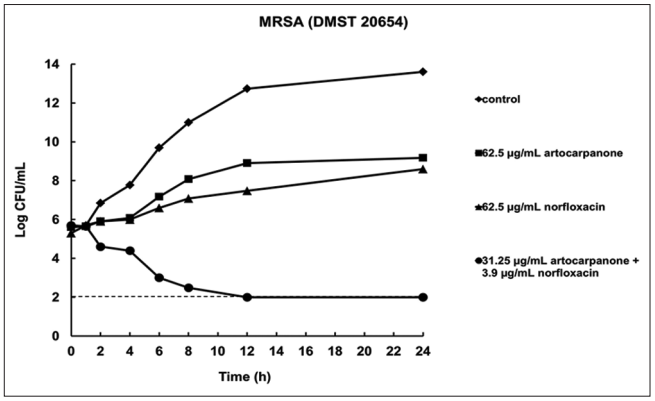

Figure 2: Time-kill curves of artocarpanone, norfloxacin, and their combination against methicillin-resistant *Staphylococcus aureus*

[Figure 3]. Artocarpanone significantly increased the uptake of crystal violet when compared to the control ($P < 0.01$), while norfloxacin did not have any significant effect. It was of interest that the crystal violet uptake of artocarpanone in combination with norfloxacin was significantly higher than the other groups, including the control as well as a single dose of artocarpanone and norfloxacin ($P < 0.01$).

Loss of 260 nm Absorbing Material

The result indicated that the absorbance of the combined artocarpanone and norfloxacin was significantly higher than for the control group as well as those of the single compounds, artocarpanone and norfloxacin ($P < 0.01$) [Figure 4].

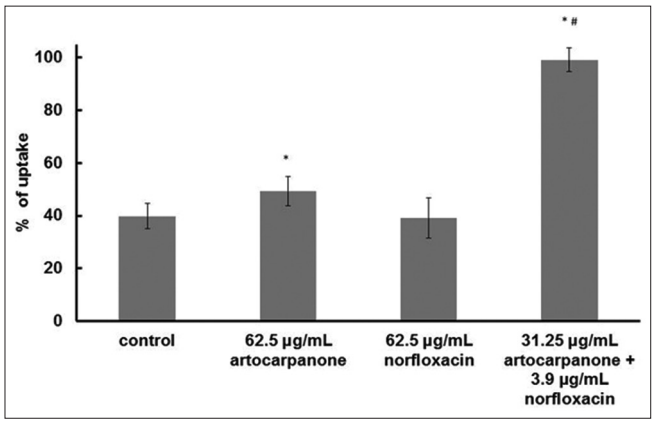

Figure 3: Crystal violet uptake of artocarpanone, norfloxacin, and their combination treated methicillin-resistant *Staphylococcus aureus*. The mean ± standard deviation for three replicates is illustrated. *Samples demonstrate significant differences compared to control ($P < 0.01$), #combination of artocarpanone and norfloxacin demonstrates significant difference compared to drugs alone ($P < 0.01$)

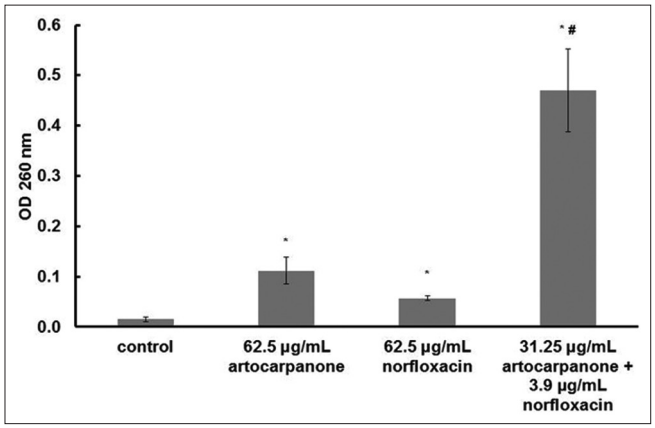

Figure 4: Presence of 260 nm absorbing material in the supernatant of methicillin-resistant *Staphylococcus aureus* treated with artocarpanone, norfloxacin, and their combination. The mean ± standard deviation for three replicates is illustrated. *Samples demonstrate significant differences compared to control ($P < 0.01$), #combination of artocarpanone and norfloxacin demonstrates significant difference compared to drugs alone ($P < 0.01$)

Table 2: Effect of artocarpanone on the antibacterial activity of antibiotics against *P. aeruginosa*

	MIC[a] (µg/mL)	MIC[c] (µg/mL)	FIC	FICI	Interaction
Artocarpanone-Tetracycline					
Artocarpanone	500	250	0.5	1	Indifference
Tetracycline	15.6	7.8	0.5		
Artocarpanone-Ampicillin					
Artocarpanone	500	125	0.25	1.25	Indifference
Ampicillin	15.6	15.6	1		
Artocarpanon-Norfloxacin					
Artocarpanone	500	125	0.25	0.75	Additive
Norfloxacin	1.9	0.9	0.5		

[a]MIC of one sample alone, [c]MIC of samples in combination)
FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index)

Table 3: Effect of artocarpanone on the antibacterial activity of antibiotics against *E. coli*

	MIC[a] (µg/mL)	MIC[c] (µg/mL)	FIC	FICI	Interaction
Artocarpanone-Tetracycline					
Artocarpanone	7.8	3.9	0.5	0.75	Additive
Tetracycline	7.8	1.9	0.25		
Artocarpanone-Ampicillin					
Artocarpanone	7.8	0.9	0.1	1.1	Indifference
Ampicillin	15.6	15.6	1.0		
Artocarpanone-Norfloxacin					
Artocarpanone	7.8	3.9	0.5	1.0	Indifference
Norfloxacin	1.9	0.5	0.5		

[a]MIC of one sample alone, [c]MIC of samples in combination)
FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index)

Table 4: Effect of artocarpanone on the antibacterial activity of antibiotics against MRSA

	MIC[a] (µg/mL)	MIC[c] (µg/mL)	FIC	FICI	Interaction
Artocarpanone-Tetracycline					
Artocarpanone	125	31.2	0.25	0.5	Additive
Tetracycline	125	31.2	0.25		
Artocarpanone-Ampicillin					
Artocarpanone	125	31.2	0.25	0.5	Additive
Ampicillin	62.5	15.6	0.25		
Artocarpanone-Norfloxacin					
Artocarpanone	125	31.2	0.25	0.28	Synergistic
Norfloxacin	125	3.9	0.03		

[a]MIC of one sample alone, [c]MIC of samples in combination)
FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index)

DISCUSSION

On the basis of the broth microdilution method, artocarpanone has demonstrated variable antibacterial activity against tested bacteria. Against Gram-negative bacteria, norfloxacin was the strongest agent. However, all tested antibiotics as well as artocarpanone only showed a weak antibacterial activity against MRSA. It has been known that many antibiotics in sublethal

concentration cannot significantly exhibit any activities against MRSA due to its resistant mechanism. One appealing strategy to overcome resistant problem is the use of drug in combination. This strategy may increase their biological activities due to the interaction of each compound. Checkerboard method was used to determine the interaction of combination between artocarpanone and antibiotics. Interestingly, against resistant bacteria, artocarpanone only had a synergistic interaction when combined with norfloxacin. By this combination, artocarpanone could decrease the dose of norfloxacin by 32-fold. The time-kill assay was conducted to confirm the synergistic effect of artocarpanone on the anti-MRSA activity of norfloxacin. These results indicated that artocarpanone may overcome the problems associated with MRSA when used in combination with the conventional antibiotic, i.e., norfloxacin.

A use of drug in combination may increase their biological activities due to the interaction of each compound. Different compounds may have different target sites and influence each site to achieve the same response that leads to enhanced biological activities in the cells. On the other hand, the different compounds might affect the same target site and that could result in an agonistic activity [19]. Over-expression of the efflux pump is one of the resistance mechanisms of MRSA toward antibiotics. It has been suggested that the efflux pump can be inhibited by altering the membrane permeability as well as by inhibiting the metabolic pathway [20]. Cell membrane disruption is one of the antibacterial mechanisms of flavonoids [6,21]. This study therefore also focused on investigation any cell membrane disruption by artocarpanone, norfloxacin, and their synergistic mixtures. Based on the bacteriolysis assay, artocarpanone in combination with norfloxacin had a bacteriolytic activity by increasing the uptake of crystal violet. A further study was performed to determine the release of UV-absorbing material at 260 nm that indicated the leakage of the intracellular components of MRSA as an indicator for membrane damage [22]. This result corresponded well with the synergistic bacteriolytic effect of the mixture of artocarpanone and norfloxacin. It implied that the mixture of artocarpanone and norfloxacin enabled the alteration of the membrane permeability and caused a release of intracellular components.

This finding indicated that the synergistic activity of artocarpanone and norfloxacin against MRSA may be operated through different targets sites. It has been shown that the incorporation of flavonoids, especially a flavanone at the lipophilic side of the cell membrane, can cause a reduction of membrane fluidity [23]. For example, sophoraflavanone G isolated from *Sophora exigua* exhibited antibacterial activity against MRSA by reducing the fluidity of the cellular membrane as well as by reducing the cytoplasmic contents [20,24]. Therefore, such membrane alteration may allow norfloxacin to enter the cells more easily and occupy its site of action for inhibiting the DNA gyrase that resulted in interfering with cell division and induced the cells death [25]. Investigation of this synergistic activity between artocarpanone and norfloxacin may provide opportunities for understanding their mechanism of actions against MRSA and provide a new prospect for the discovery an alternative strategy to overcome resistance problems. Nevertheless, further experiments are required to elucidate other mechanisms of action including any inhibitory activity on the efflux pumps.

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