Study on Antibacterial Compounds from *Artocarpus heterophyllus* Heartwoods

Abdi Wira Septama

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

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Thesis Title	Study on antibacterial compounds from *Artocarpus heterophyllus* heartwoods
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Abstract

On the basis of antibacterial assay-guided isolation from the ethyl acetate extract of *Artocarpus heterophyllus* heartwoods, six antibacterial compounds, namely cycloartocarpin, artocarpin, artocarpanone, cyanomaclurin, dihydromorin and norartocarpetin were successfully purified. Among these compounds, artocarpin showed the strongest activity against gram-positive bacteria, including *Streptococcus mutans*, *S. pyogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, and *S. epidermidis* with MIC values of 4.4, 4.4, 17.8, 8.9, and 8.9 µM, respectively, and MBC values of 8.9, 8.9, 17.8, 8.9 and 8.9 µM, respectively. Artocarpin also exhibited antibacterial activity against *Pseudomonas aeruginosa*, a gram-negative bacterium with MIC value of 286.4 µM. While artocarpanone showed the strongest activity against *Escherichia coli* with MIC and MBC values of 12.9 and 25.8 µM, respectively. Artocarpin and artocarpanone were then selected to determine the synergistic effect with commercial antibiotics, including ampicillin, tetracycline, and norfloxacin against methicillin-resistant *S. aureus* (MRSA) as well as gram-negative bacteria *P. aeruginosa* and *E. coli*, using checkerboard method and time-kill assay. The interaction of artocarpin with all tested antibiotics produced synergistic effects against MRSA with fractional inhibitory concentration index (FICI) of 0.15-0.37. A combination of artocarpin and norfloxacin showed synergistic effect against *E. coli* with an FICI value of 0.37. The combinations of artocarpin and tetracycline as well as artocarpin and norfloxacin showed synergy against *P. aeruginosa* with FICI values of 0.24 and 0.37, respectively. Time-kill assays confirmed that artocarpin enhanced the potency of antibiotics against tested bacteria. While the interaction between artocarpanone and

norfloxacin exhibited a synergistic effect against MRSA with an FICI of 0.28. This combination altered the membrane permeability of MRSA. The antibacterial flavonoids, artocarpanone, artocarpin and cycloartocarpin, were then used as indicative marker for establishment of quantitative HPLC analysis of the extracts from *A. heterophyllus* heartwoods. Separation was achieved using a TSK-gel ODS-80Tm column (5 µm, 4.6 × 150 mm) at 25ºC with a gradient elution system of methanol and water as follows: 0-8 min, 60:40; 8-27 min, 80:20; 27-35 min, 60:40, v/v, at a flow rate of 1 mL/min, and a quantitative UV detection at 285 nm. The parameters i.e. linearity, specificity, sensitivity, accuracy, repeatability, and reproducibility were investigated for validating method. The high degree of specificity and sensitivity were achieved. The calibration curves of three flavonoid compounds exhibited good linearity with coefficient of determination (R^2) $\geq$ 0.9995. The percent recoveries of the method were 98-104%, and good reproducibility and repeatability (RSD less than 2%) were also obtained. Ethyl acetate could produce contents of artocarpanone, artocarpin, and cycloartocarpin in higher concentration. Therefore, this solvent was the suitable solvent for extraction.

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Abdi Wira Septama

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LIST OF ABBREVIATIONS AND SYMBOLS

BHI	brain-heart infusion
ºC	degree of Celsius
CFU	colony-forming unit
cm	centimeter
FIC	fractional inhibitory concentration
FICI	fractional inhibitory concentration index
g	gram
h	hour
J	coupling constant
HPLC	high performance liquid chromatography
IC_{50}	50% inhibitory concentration
MBC	minimum bactericidal concentration
mg	milligram
MHz	mega hertz
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
min	minute
mL	milliliter
m/z	mass-over-charge ratio
NMR	nuclear magnetic resonance
OD	optical density
s, d, t, m	singlet, doublet, triplet, multiplet
TLC	thin layer chromatography
UV-Vis	ultraviolet-visible
v/v	volume by volume
µg	microgram
µL	microliter
µM	micromolar
%	percentage

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Paper I Septama, AW., Panichayupakaranant, P. 2015. Antibacterial assay-guided isolation of active compounds from *Artocarpus heterophyllus* heartwoods. *Phamaceutical Biology* 53(11): 1608-1613.
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Paper III Septama, AW., Panichayupakaranant, P. Synergistic effect of artocarpanone on antibacterial activity of some antibiotics against methicillin-resistant *Staphylococcus aureus* and their effect on membrane permeability. Submit to *Journal of Natural Medicine* (Manuscript).

Paper IV Septama, AW., Panichayupakaranant, P. Simultaneous HPLC analysis of three flavonoids in the extracts of *Artocarpus heterophyllus* heartwoods. *Natural Product Science*. Manuscript accepted, 24 December 2015.

Proceeding Septama, AW., Panichayupakaranant, P. 2014. Antibacterial activities of some combinations of cyanomaclurin and artocarpin isolated from *Artocarpus heterophyllus* heartwoods. The 3rd Current Drug Development International Conference, May 1-3, 2014, Krabi, Thailand.

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GENERAL INTRODUCTION

Resistance to an antimicrobial had become a major public health problem in worldwide, because it can cause many negative impacts, such as increased of treatment failure and cost of treatment, as well as it may increase the risk of infection in hospitals. The resistant of antibiotics can be viewed as two aspects such as clinical and microbiological. In clinical aspect, the resistance is due to by the frequency of antibiotic use, combined with the level of compliance with infections control measures to prevent spread resistant bacteria. The widespread of the antibiotics is largely unregulated, selling antibiotics without prescription and even without involvement of pharmacist (Laxminarayan *et al*., 2013). On the other hand, microbiological resistance might be because the life cycle of bacteria that enables to adapt quickly with its environment. Bacteria will continue to develop their resistance that led the treatment failure (Rao *et al*., 2014). They use genetic mechanism to increase the rate of adaption, and it also spread through several mechanisms, such as alteration by mutation of antibacterial target, changes in permeability and transfer of resistance genes (Rodriguez *et al*., 2013).

The resistant of antibiotics are not only expensive in term of humans sustain but also in term of monetary. According to previous study, it has been reported that 25,000 people in recent year in European Union (EU) due to the infections that cannot be treated by antibiotics, and more than 1.5 billion Euro have been spend per year (Leung *et al*., 2011). Moreover, in the United State of America, infectious disease caused pathogens resistant towards antibiotic cost the health-care system in excess of 20 billion Dollars per year (Roberts *et al*., 2009). On the other hand, increasing rate of antibiotic resistance has also been reported in developing countries, such as India, China, Pakistan, Indonesia and Thailand. In the low-income and middle-income countries, where infectious diseases are common and the burden is high, antibiotic uses are increasing with rising income, high rate of hospitalization, and high prevalence of hospital infections (Laxminarayan *et al*., 2013).

Many of the bacterial pathogens associated with epidemic diseases and the use of antibiotics in large scale, such as *Staphylococcus aureus, S. epidermidis,*

Salmonela spp., *Streptococcus pneumonia* and *Bacillus subtilis*. Methicillin-resistant *S. aureus* (MRSA) is one of the resistant pathogens to numerous of antibiotics, in particular β-lactam antibiotics. The main resistant mechanisms of this bacterium are the over expression of β-lactamase that enable to hydrolyses β-lactam, large expression of efflux pump to reduce the accumulations of antibiotics by extruding antibiotics from pathogen, and acquisition of *mecA* gene that is responsible to reduce the activity of β-lactam antibiotics (Qin *et al*., 2013). In addition, the Gram-negative bacteria, such as *Pseudomonas aeruginosa* also leads to several clinical problems. Natural resistance of this bacterium, such as the low permeability of its membrane cell wall, mutation in its chromosome, expressions of gene resistance as well as the ability to gain resistance from other microorganism enable to provoke its resistant towards commercial antibiotics (Lambert, 2002).

There are three mechanisms of antibiotic resistant (**Figure 1**), such as; (a) altering the active target which antibiotic have to occupy inside the cell, and it may reduce the affinity of antibiotics binding. Introducing the mutations in receptor alters it, causing to reduction in the activity of the antibiotics against the microbe, (b) producing several enzymes that can destruct antibiotic through several action such as hydrolysis mechanism as well as reaction of reduction oxidation, and (c) expression of efflux pump and decreasing of membrane cell to reduce the antibiotics inside the cell (Hemaiswarya *et al*., 2008).

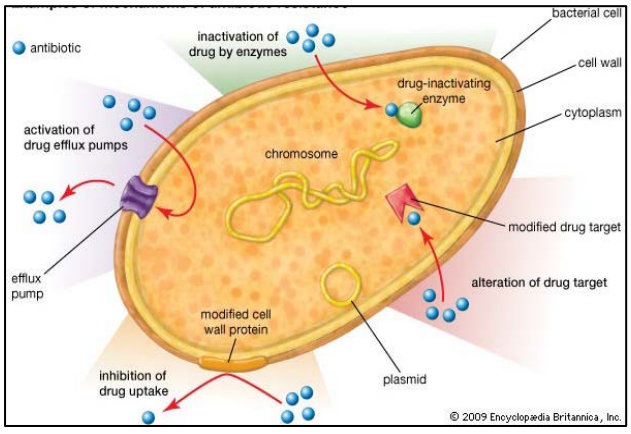

Figure 1. Mechanism of antibiotics resistant (adapted from Encyclopedia-Britannica, 2009)

According to the fact, it needs to find some strategies to overcome that problem. One approach is development of new antibacterial agents from natural resources including natural plants (Cushnie and Lamb, 2011). Another approach is the use of combinations of two or more antibacterial in order to enhance their activities against resistant pathogen. The synergistic effects of combination commercial antibiotics and antibacterial compounds can be achieved by some mechanisms such as multi-targets effect due to each agent may regulate the different site of actions, enhancing the bioavailability and inhibiting antibiotics resistant through the interaction of agents with resistance mechanisms of bacteria (Yang *et al.,* 2014; Wagner and Ulrich-Merzenich, 2009).

Natural products are a major source of chemical diversity and have provided important therapeutic agents for many bacterial diseases (Payne *et al*., 2007). Most of antibacterial agents and synthetic agents derived from microbe. However, plant-derived antibacterial compounds have been attracting much interest as natural alternative, this is in part attributed to the fact the plants can be rationally selected for antibacterial testing based on their ethno medicinal uses (Cos *et al*., 2006). Human has used plants to treat common infectious diseases and World Health Organization (WHO) has reported that more than 70% people in the world still use herbal medicine in order to get several drugs (WHO, 2010).

Natural products with their second metabolites have been a source of the most of active ingredients of medicines, and they also play an important role in the development of drugs for the treatment of human diseases (Rios and Recio, 2005; Harvey, 2003; Tietze *et al.,* 2003). It has been claimed that 75% of drugs, in particular for infectious diseases derived from nature and plant-derived compounds (McChesney *et al*., 2007). It has been reported that medicinal plants confer considerable antibacterial activity against several microorganisms, including bacteria. One of those plants is *Artocarpus heterophyllus* Lam. (Moraceae), which has been recognized in South-East Asia, South Asia and several countries in Africa to treat and prevent a range of ailments. The heartwood of this plant has also been used in folk medicine several countries for the treatment of inflammation, malarial fever and prevention of bacterial and fungal infections (Jagtap and Bapat, 2010). In Indonesia, it has been

commonly used in traditional folk medicine named “jamu” to treat inflammation, malaria fever and the like (Arung *et al*., 2010a).

According to previous scientific studies using disc diffusion method, the butanol fraction of *A. heterophyllus* bark extract possessed inhibitory effect against pathogenic bacteria with the zone of inhibition ranged from 14-18 mm (Khan *et al*., 2003). The plants are also known to produce a variety of flavonoid compounds as well as other secondary metabolites, such as carotenoids, volatile acid sterols and tannin (Arung *et al*., 2010b; Chandrika *et al*., 2004; Wong *et al*., 1992).

Flavonoids have been reported to possess number of biological activities for human health due to their interactions with number cellular targets. Artocarpanone isolated from *A. heterophyllus* heartwoods exhibited significantly effect by inhibiting the release of β-glucuronidase and enzyme lysozyme from the fMLP neutrophils-stimulated with IC_{50} values of 30 and 46.5 µM, respectively (Wei *et al*., 2005). Three compounds, including cycloheterophyllin, artonins A and B isolated from this plant inhibited iron-induced lipid peroxidation in rat brain homogenate with IC_{50} values of 0.96, 0.24 and 0.71 µM, respectively (Ko *et al*., 1998). It has been reported that steppogenin, norartocarpetin, artocarpesin, and 3-prenyl luteolin also possess tyrosinase inhibitory activity with IC_{50} values of 0.57, 0.46, 0.52, and 76.3 µM, respectively (Zheng *et al*., 2008; Arung *et al*., 2010c). In addition, Sato *et al*. (1996) have reported that artocarpin and artocarpesin exhibit antibacterial activities against various cariogenic bacteria with minimum inhibitory concentration (MIC) ranged from 3.13-12.5 µg/mL. Due to the beneficial properties from this plant, it has been attracting the attention of researcher to discover the antibacterial substances to overcome resistant problem.

Although some isolated compounds from *A. heterophyllus* heartwoods have been reported earlier, but little information is available for their antibacterial activity, mechanisms of action against pathogenic bacteria and synergistic effect of antibacterial compounds and antibiotics, as well as a method for quantitative determination of the antibacterial compounds in *A. heterophyllus* heartwood extracts. Therefore, the aims of this study focus on isolation and identification of antibacterial compounds, determination of synergistic effect of antibacterial compounds and its

mechanism of action, as well as the establishment of an HPLC analysis for the antibacterial compounds in *A. heterophyllus* heartwood extract.

The results of this study may provide some insights on the antibacterial activity of active compounds isolated from *A. heterophyllus* as well as their ability in enhancing the potency of commercial antibiotics against bacterial resistant, including the Gram-negative bacteria. In addition, the finding, in particular HPLC quantitative analysis may also provide the information regarding the method used for quality control of herbal medicine.

OBJECTIVES

1. To isolate and identify antibacterial compounds from *A. heterophyllus* heartwoods
2. To determine synergistic effect of the isolated antibacterial compounds
3. To determine some mechanisms of action of the antibacterial compounds
4. To establish an HPLC quantitative analysis for the antibacterial compounds in *A. heterophyllus* heartwood extracts.

SIGNIFICANT RESULTS AND DISCUSSION

1. Isolation and identification antibacterial compounds

On the basis of antibacterial assay-guided isolation (**Figure 2**), four flavonoid compounds have been purified from the ethyl acetate fraction of *A. heterophyllus*. According to their ^{1}H and ^{13}C NMR spectral data and by comparing with the spectral data from some literatures (Likhitwitayawuid *et al*., 2000; Wang *et al*., 2004; Zheng *et al*., 2008), the compounds were identified as cycloartocarpin (1), artocarpin (2), artocarpanone (3), and cyanomaclurin (4) (**Figure 3**). The result showed that artocarpin demonstarted the strongest antibacterial activities against Gram-positive bacteria, such as *Streptococcus mutans*, *S. pyogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, and *S. epidermidis* with minimum inhibitory concentrations (MICs) value in range 4.4-17.8 µM and minimum bactericidal concentrations (MBCs) value in range 8.9-17.8 µM. This compound also exhibited inhibitory activity against gram-negative bacteria, i.e. *Pseudomonas aeruginosa* with an MIC of 286.4 µM. On the other hand, compound 2 (artocarpanone) displayed the strongest antibacterial activity against gram-negative bacteria, *Escherichia coli* with MIC value of 12.9 and MBC value of 25.8 µM (**Paper I**).

Structure activity relationship indicated that substitutions of some groups, such as isoprenyl group (C-3), a 3-methyl-1-butenyl group (C-6), as well as a hydroxyl group (C-2') are probably important for the activity of artocarpin against gram-positive bacteria (Tsuchiya *et al*., 1996). The cyclization of the hydroxyl group (C-2') with the isoprenyl group (C-3) may decrease the antibacterial activity of cylcoartocarpin against tested bacteria, in particular gram-positive bacteria. Furthermore, saturation at the position of C-2 as well as C-3 of artocarpanone will increase the potency of compound 3 (artocarpanone) against gram-negative bacteria, i.e. *E. coli*, but decrease its activity against gram-positive bacteria. In case of cyanomaclurin, integration of ring C and B via heterocyclic saturation and hydroxyl group, and a substitution of hydroxyl group (C-14) may enhance the antibacterial activity against *S. mutans*, *S. aureus* and *S.epidermidis* (**Paper I**).

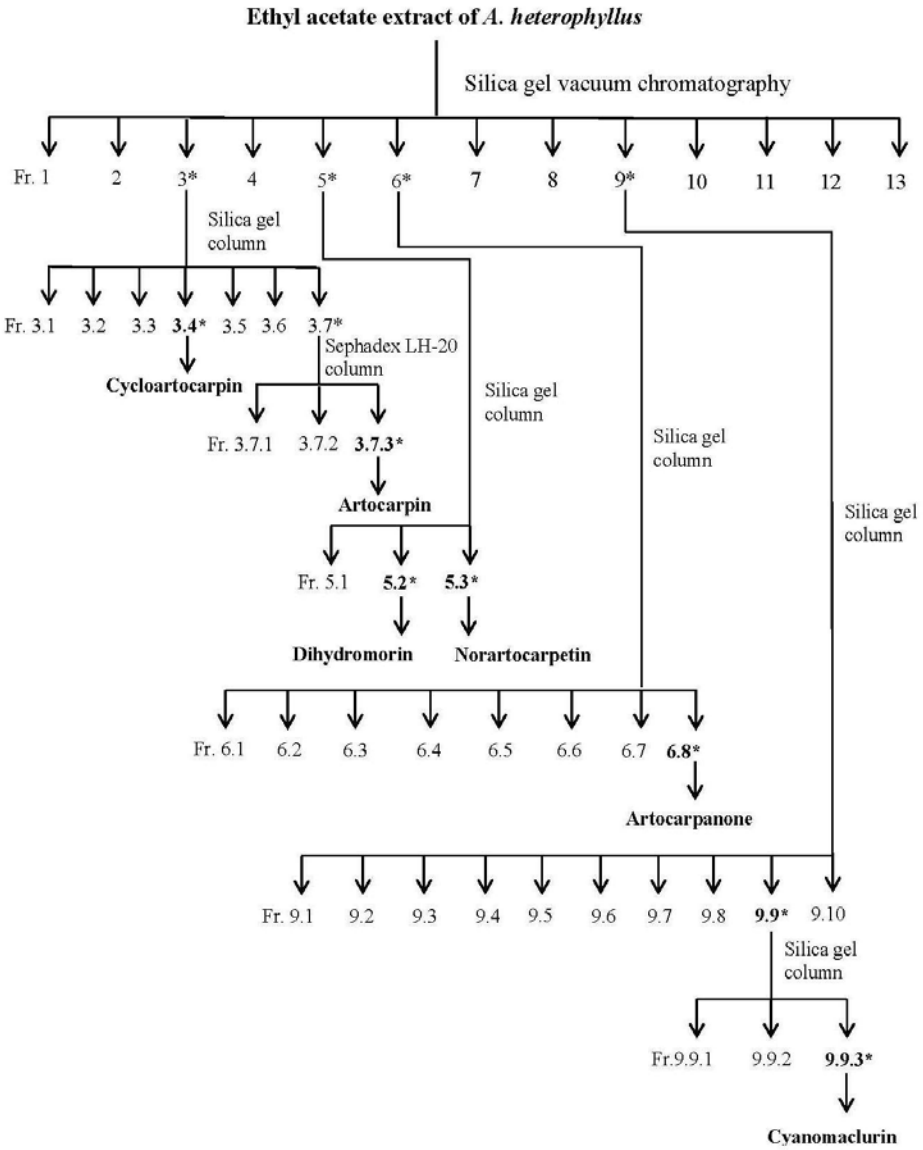

Figure 2. Antibacterial assay guided isolation process.

In addition, other antibacterial compounds have also been purified from the ethyl acetate extract of *A. heterophyllus*. Due to its interesting TLC pattern as well as the fraction showed the antibacterial activity, fraction 5 (102 mg) was then isolated using silica gel column chromatogarphy (4 cm in diameter × 50 cm in height) (1 g extract per 50 g silica gel) and eluted with a mixture of chloroform and methanol (95% v/v chloroform; 20 mL fractions were collected) to afford 3 pooled fractions (fraction 3.1-3.3). Yellow powder of compound 5 (18 mg) was obtained from fraction 3.2 and yellow powder of compound 6 (25 mg) was obtained from fraction 3.3 (**not published**). Both of compounds were structurally identified according to their spectral data of ^{1}H NMR and ^{13}C NMR and comparing with the spectral data from some literatures (Zheng *et al*., 2008; Lin *et al*., 1995).

Dihydromorin (5): Yellow powder, ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.21 (1H, d, *J* = 9.0 Hz, H-6'), 6.35 (1H, d, *J* = 3.0 Hz, H-5'), 6.34 (1H, d, *J* = 3.0 Hz, 3'), 5.90 (1H, d, *J* = 2.5 Hz, H-8), 5.86 (1H, d, *J* = 2.5 Hz, H-6), 5.38 (1H, d, *J* = 11.5 Hz, H-2), 4.77 (1H, d, *J* = 11.5 Hz, H-3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 198.9 (C-4), 168.5 (C-5), 165.3 (C-9), 164.9 (C-7), 160.1 (C-4'), 158.6 (C-2'), 130.8 (C-6'), 115.5 (C-1'), 107.9 (C-5'), 103.7 (C-3'), 101.9 (C-10), 97.1 (C-6), 96.2 (C-8), 79.9 (C-2), 72.5 (C-3).

Norartocarpetin (6): Yellow powder, ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.75 (1H, d, *J* = 8.5 Hz, H-6'), 7.12 (1H, s, H-3), 6.44 (1H, dd, *J* = 9.5, 2.5 Hz, H-5'), 6.42 (1H, d, *J* = 2 Hz, H-8), 6.41 (1H, d, *J* = 2.0 Hz, H-3'), 6.18 (1H, d, *J* = 2.5 Hz, H-6). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 184.4 (C-4), 165.8 (C-2), 164.2 (C-7), 163.3 (C-9), 163.1 (C-5), 160.3 (C-4'), 159.5 (C-2'), 130.9 (C-6'), 110.7 (C-1'), 109.1 (C-5'), 108.3 (C-3), 105.2 (C-10), 104.2 (C-3'), 99.8 (C-6), 94.8 (C-8).

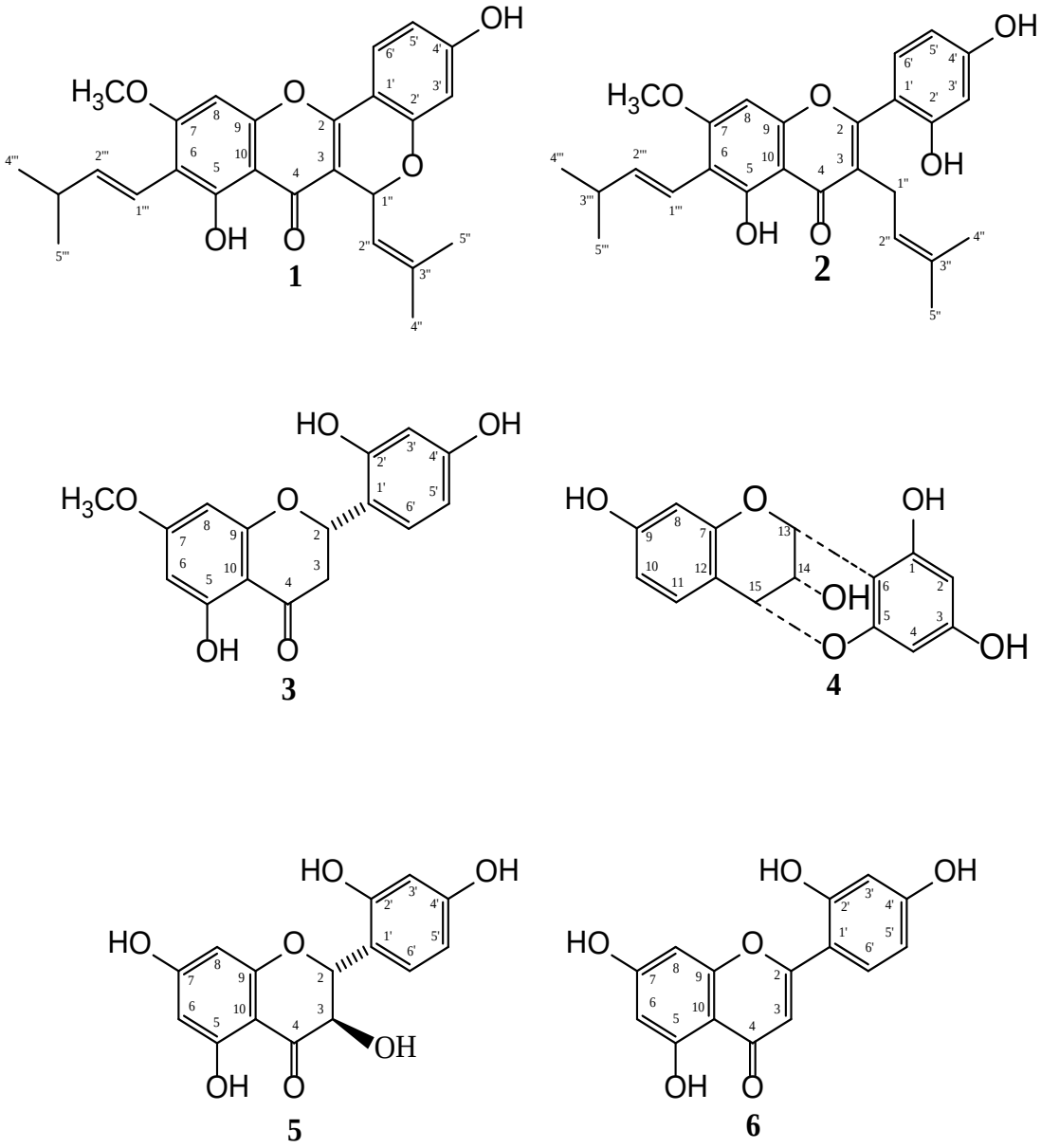

Figure 3. Chemical structure of flavonoid compounds, cycloartocarpin (1), artocarpin (2), artocarpanone (3), cyanomaclurin (4), dihydromorin (5), norartocarpetin (6)

Table 1. MIC and MBC values of dihydromorin, norartocarpetin and ampicillin.

Bacteria	Dihydromorin		Norartocarpetin		Ampicillin	
	MIC	MBC	MIC	MBC	MIC	MBC
	(µM)		(µM)		(µM)	
Streptococcus mutans	103.0	103.0	437.0	437.0	1.4	1.4
Streptococcus pyogenes	51.3	103.0	109.2	109.2	1.4	1.4
Bacillus subtillis	205.5	411.1	874.1	*na*	1.4	1.4
Staphylococcus aureus	205.5	411.1	437.0	874.1	1.4	1.4
Staphylococcus epidermidis	103.0	205.5	874.1	*na*	1.4	1.4
Pseudomonas aeruginosa	*na*	*na*	*na*	*na*	89.4	178.9
Escherichia coli	*na*	*na*	*na*	*na*	0.7	1.4

MIC (minimum inhibitory concentration), MBC (minimum bactericidal concentration), *na* (no activity).

The result (**Table 1**) showed that dihydromorin revealed strong antibacterial activity against *S. pyogenes* with MIC and MBC values of 51.3 and 103.0 µM, respectively. While only showed moderate antibacterial activity against *S. mutans*, *B. subtillis*, *S. aureus* and *S. epidermidis* with MIC values in the range of 103.0-205.5 µM, and MBC values in the range of 205.5-411.1 µM. Norartocarpetin only revealed moderate to weak activity against the gram-positive bacteria with MIC values in range of 51.3-205.5 µM, and MBC values in the range 103.0-411.1 µM. While, norartocarpetin only showed moderate antibacterial activity against *S. pyogenes* with MIC and MBC values 109.2 and 109.2 µM, respectively. However, both of compounds did not have any activities against the gram-negative bacteria including *P. aeruginosa* and *E. coli*.

2. Synergistic effect of antibacterial compounds and antibiotics

Bacterial resistance to antibiotics has become a great medical problem throughout the world, and it can cause in high morbidity and mortality. An alternative approach to overcome this problem is the use of combination two or more antibacterial agents in order to enhance their antibacterial activity against resistant pathogen. It have been known that the successful strategy to overcome the resistant problem to penicillinase is the use of combination of clavulinic acid with sublactam and tazobactam, however it can lead to the resistant bacterial strain when it is frequently applied in clinical (Hemaiswarya *et al*., 2008).

Plant-derived compounds are good sources for combination therapy. Flavonoid is a group of phytochemical possesses many biological activities, such as antimicrobial and synergistic effect with some antibiotics (Cushnie and Lamb, 2011). For this study, artocarpin and artocarpanone which are showed strong antibacterial activities were selected for further investigation to determine the synergistic effect for ampicillin, tetracycline and norfloxacin against methicillin-resistant *S. aureus* (MRSA) and the gram-negative bacteria including *P. aeruginosa* and *E. coli* (**Paper II and Paper III**).

The interaction of two antibacterial compounds was assessed using checkerboard method. The results of this method were interpreted by quantifying fractional inhibitory concentration index (FICI) for the two antibacterial compounds (Hsieh *et al*., 1993). The results were considered as synergistic when FICI is equal or less than 0.5. A FICI between 0.5 and 1 was considered as additive, when FICI falls between 1 to 4 is an indifferent, while a value is above 4 is indicated antagonism between two antibacterial (Odds, 2003). In this study, artocarpin in combination with all tested antibiotics ampicillin, tetracycline and norfloxacin showed the synergistic effect against MRSA with the FICI values of 0.15-0.37. This compound also exhibited synergism in combination with tetracycline and norfloxacin against *P. aeruginosa* (FICI values of 0.24 and 0.37, respectively). While, it only showed the synergistic effect against *E. coli* when combined with norfloxacin (**Paper II**).

Time-kill assay was performed in order to confirm any synergistic activity in inhibiting the growth of bacteria in different concentrations at different time points (Langeveld *et al*., 2014). The results of time-kill assays indicated that artocarpin enhanced the antimicrobial activity of tetracycline, ampicillin and norfloxacin against gram-positive bacteria, i.e. MRSA, as well as gram-negative bacteria, i.e. *P. aeruginosa* and *E. coli* (**Paper II**).

In the case of artocarpanone, this compound only showed synergistic effect in combination with norfloxacin against MRSA (**Paper III**). The time-kill result is also in agreement with checkerboard result. Further experiments were conducted to know the underlying mechanism of synergistic of this combination on membrane permeability using bacteriolysis and loss of 260 nm assay. Over expression of efflux pump in on MRSA is one of the mechanism of antibiotic resistant that may decrease accumulation of antibiotics inside the bacteria. It has been proposed that efflux pump can be inhibited through membrane disruption and inhibition of metabolic pathway (Gibbon, 2008). From the results of this study (**Paper III**), it can be concluded that artocarpanone have the synergistic effect with norfloxacin against MRSA trough different mechanism of actions. It has been known that the incorporation of flavanone with the lipophilic side of cell membrane can cause reduction of membrane fluidity (Hendrich, 2006). Therefore, the alteration may allow norfloxacin to enter to the cells and occupying its site of action for inhibiting DNA gyrase, which then interfering the cell division and induce the cells death (Crumplin *et al*., 1984).

Investigation of this synergistic activity between antibacterial compounds and commercial antibiotics may provide opportunities for understanding their mechanism of actions against MRSA and give new prospect for discovery alternative strategy to overcome resistant problems.

3. Quantitative HPLC determination and extraction of active compounds isolated from *A. heterophyllus* heartwoods

Recently, quality control of phytomedicine has been a great concern for the people. The herbal medicine contains many of chemical constituent, however less number of active constituent. Therefore, it is important to determine all the active components in the herbal medicine in order to guarantee its effect. The main objectives of quantitative analysis are separation and identification the bioactive constituents in herbal medicine and furthermore use that compounds as indicator, or standard, to evaluate their quality.

In order to obtain a high and consistent quality of the heartwood extracts of *A. heterophyllus*, a simple quantitative analysis method for the active markers of the extracts should be developed. Recently, the reversed-phase HPLC method has been popularly used for a standardization of herbal extracts due to its high sensitivity, selectivity and accuracy (Shabir, 2003). The present study was therefore focus on the establishment of a validated HPLC quantitative method for simultaneous determination of artocarpanone, artocarpin and cycloartocarpin and the method for sample preparation. In this study (**Paper IV**), gradient reversed-phased HPLC was used for quantitative determination of artocarpanon, artocarpin, and cycloartocarpin. The mixture of methanol and water in gradient system was optimized as mobile phase. All compounds can be detected at 285 nm and all the compounds were eluted within 35 min with good resolution. On the basis of HPLC analysis, artocarpin was detected as a major compound.

According to the international conference on harmonization (ICH), validation parameters are limit of detection (LOD), limit of quantification (LOQ), linearity, specificity, accuracy and precision (ICH, 2005). In this study (**Paper IV**), artocarpanone, artocarpin and cycloartocarpin exhibited good linearity with a coefficient of determination (R^2) $\geq$ 0.9995. The results indicated that the method has a high degree of specificity and sensitivity which enabled to detect the compounds at the lower concentration. The accuracy of analytical method was obtained with the percent recoveries in range 98-104%, and good reproducibility and repeatability (RSD

< 2%) was also achieved. Furthermore, ethyl acetate was claimed as a suitable solvent for extraction of artocarpanone, artocarpin and cycloartocarpin in the heartwood of *A. heterophyllus*.

CONCLUSIONS

From this research works the following conclusions can be drawn:

1. Three flavonoid compounds, namely artocarpanone, artocarpin, and cycloartocarpin have been purified as the antibacterial compounds. Artocarpin exhibited the strongest antibacterial activities against gram-positive bacteria, while artocarpanone revealed the strongest antibacterial activity against gram-negative bacteria i.e. *Escherichia coli*. These compounds might be used as the standard markers for the standardization of *A. heterophyllus* heartwood extracts.

2. Artocarpin and artocarpanone exhibited the synergistic effects with some antibiotics, such as ampicillin, tetracycline, and norfloxacin against resistant bacteria i.e. MRSA. These compounds might be considered as the adjuvants in order to enhance the antibacterial activities of antibiotics against resistant bacteria.

3. Artocarpanone can enhance the antibacterial activity of norfloxacin by altering membrane permeability of MRSA. This finding might provide an insight for understanding the mechanism of action of this combination and give a prospect for discovery an alternative approach to overcome the resistant problem.

4. The simple, specific, precise, accurate, rapid and reproducible HPLC method has been established. This study has also purposed that ethyl acetate is a suitable solvent for extraction of artocarpanone, artocarpin, and cycloartocarpin. Moreover, the sample preparation and HPLC method can be used in the routine standardized process of the extract from *A. heterophyllus* heartwoods.

Nevertheless, further experiments are required to obtain more knowledge of the mechanism of actions that underlie the synergism, not only on membrane disruption but also on efflux pump, receptor modification and enzymatic degradation.

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APPENDICES

Appendix 1 Reprint of papers, manuscripts and proceeding.

Appendix 2 Vitae

PAPER 1

Antibacterial assay-guided isolation of active compounds from *Artocarpus heterophyllus* heartwoods
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ORIGINAL ARTICLE

Antibacterial assay-guided isolation of active compounds from *Artocarpus heterophyllus* heartwoods

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Abstract

Context: Preparations from *Artocarpus heterophyllus* Lam. (Moraceae) heartwoods are used in the traditional folk medicine for the treatment of inflammation, malarial fever, and to prevent bacterial and fungal infections.

Objective: The objective of this study was to isolate pure antibacterial compounds from *A. heterophyllus* heartwoods.

Materials and methods: The dried and powdered *A. heterophyllus* heartwoods were successively extracted with the following solvents: hexane, ethyl acetate, and methanol. Each of the extracts was screened for their antibacterial activities using a disc diffusion method (10 mg/disc). Their minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined using a broth microdilution method. The extract that showed the strongest antibacterial activities was fractionated to isolate the active compounds by an antibacterial assay-guided isolation process.

Results and discussion: The ethyl acetate extract exhibited the strongest antibacterial activities against *Streptococcus mutans*, *S. pyogenes*, and *Bacillus subtilis* with MIC values of 78, 39, and 9.8 μg/mL, respectively. Based on an antibacterial assay-guided isolation, four antibacterial compounds: cycloartocarpin (**1**), artocarpin (**2**), artocarpanone (**3**), and cyanomaclurin (**4**) were purified. Among these isolated compounds, artocarpin exhibited the strongest antibacterial activity against Gram-positive bacteria, including *S. mutans*, *S. pyogenes*, *B. subtilis*, *Staphylococcus aureus*, and *S. epidermidis* with MICs of 4.4, 4.4, 17.8, 8.9, and 8.9 μM, respectively, and MBCs of 8.9, 8.9, 17.8, 8.9, and 8.9 μM, respectively, while artocarpanone showed the strongest activity against *Escherichia coli*, a Gram-negative bacteria with MIC and MBC values of 12.9 and 25.8 μM, respectively. Only artocarpin showed inhibitory activity against *Pseudomonas aeruginosa* with an MIC of 286.4 μM.

Keywords

Artocarpanone, artocarpin, cycloartocarpin, cyanomaclurin

History

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Introduction

Natural products have been used as sources of substances to alleviate diseases because they often contain specific compounds with potential therapeutic values. According to the World Health Organization (WHO), about 70% of the world population relies on these herbal products to obtain a variety of drugs (WHO, 2010). Infectious diseases are a major problem in the world, particularly in most of the developing countries. The pharmaceutical industries have produced many commercial antibiotics in order to decrease diseases. However, in recent times, the numbers of multi drug-resistant micro-organisms have greatly increased. Because of this, there have been many incentives for research workers to seek out and identify new naturally derived antimicrobial substances. Antibacterial compounds derived from plants can have enormous therapeutic potential, and many have been proved to be effective for treatment with fewer side effects than synthetically derived chemicals (Christudas et al., 2012).

Artocarpus heterophyllus Lam. (Moraceae), popularly known as jackfruit, is widely found in tropical and subtropical countries. It has been well documented as one of the ingredients of ancient Ayurvedic and Yunani medicines (Saxena et al., 2009). The heartwoods of this plant have been traditionally used in folk medicine in Thailand and Indonesia for treatment of inflammation, malarial fever, and prevention of bacterial and fungal infections (Salguero, 2003). A variety of flavonoids and prenylflavonoids have been isolated from this plant (Chung et al., 1995; Lin et al., 1995). Their biological effects have also been reported, including anti-inflammatory (Wei et al., 2005), inhibition of tyrosinase

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250 mL of solvent with the aid of a vacuum pump. The column was eluted using a step-gradient elution starting from mixtures of hexane and ethyl acetate (100, 70, 50, and 30% v/v hexane) followed by mixtures of ethyl acetate and methanol (100, 70, and 50% v/v ethyl acetate). As a result of the TLC chromatograms obtained from each fraction, 13 pooled fractions (fractions 1–13) were obtained. The fractions were then subjected to antibacterial assay. Only fractions 3, 6, and 9 showed antibacterial activity.

The fraction 3 (800 mg) was further purified by a silica gel column (4 × 50 cm) (1 g extract per 50 g silica gel) and eluted with a mixture of hexane and ethyl acetate (80% v/v hexane; 20 mL fractions were collected) to afford seven pooled fractions (fractions 3.1–3.7). A pale yellow powder of compound **1** (20 mg) was obtained from the fraction 3.4. The antibacterial fraction 3.7 (160 mg) was also purified by a Sephadex LH-20 column (2.5 × 70 cm) using a mixture of methanol and chloroform (70% v/v methanol; 10 mL fractions were collected) and a yellow powder of compound **2** (105 mg) was obtained from the fraction 3.7.3 (Figure 1).

The fraction 6 (1 g) was purified by a silica gel column (3 × 50 cm) using chloroform as the eluent (25 mL fractions were collected). A white powder of compound **3** (25 mg) was obtained from the fraction 6.8 (Figure 1).

The fraction 9 (1.8 g) was further purified by a silica gel column (4 × 50 cm), eluted with a mixture of hexane and ethyl acetate (60% v/v hexane; 25 mL fractions were collected) to give 10 pooled fractions (fractions 9.1–9.10). The antibacterial fraction 9.9 (250 mg) was further purified by a silica gel column (3 × 50 cm) using a mixture of chloroform and methanol (95% v/v chloroform; 15 mL fractions were collected) as the eluent to afford a colorless powder of compound **4** (104 mg) from fraction 9.9.3 (Figure 1).

Identification of the purified compounds

Compounds **1–4** were structurally identified based on their spectral analyses including ^{1}H NMR and ^{13}C NMR. The spectral data were compared with the data in the literature (Likhitwitayawuid et al., 2000; Wang et al., 2004; Zheng et al., 2008).

Cycloartocarpin (**1**): pale yellow powder, ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 13.41 (1H, OH), 7.65 (1H, d, J = 8.5 Hz, H-6′), 6.69 (1H, dd, J = 16.1, 7.5 Hz, H-2‴), 6.56 (1H, d, J = 16.2 Hz, H-1‴), 6.52 (1H, dd, J = 8.5, 2.5 Hz, H-5′), 6.44 (1H, s, H-8), 6.40 (1H, d, J = 2.5 Hz, H-3′), 6.24 (1H, d, J = 9.2 Hz, H-1″), 5.41 (1H, d, J = 9.2 Hz, H-2″), 3.92 (3H, s, OCH_3), 2.46 (1H, m, H-3‴), 1.95 (3H, s, H-4″), 1.67 (3H, s, H-5″), 1.08 (3H, d, J = 6.5 Hz, H-4‴), 1.08 (3H, d, J = 6.5 Hz, H-5‴); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 55.9 (OCH_3), 178.5 (C-4), 162.5 (C-4′), 160.8 (C-7) 159.0 (C-5), 157.9 (C-2′), 155.2 (C-2), 142.7 (C-2‴), 139.3 (C-3″), 125.3 (C-6′), 120.9 (C-2″), 115.6 (C-1‴), 110.0 (C-5′), 109.9 (C-1′), 109.5 (C-6), 108.8 (C-3), 105.6 (C-10), 104.5 (C-3′), 89.9 (C-8), 69.9 (C-1″), 33.1 (C-3‴), 25.9 (C-5″), 22.7 (C-5‴), 22.6 (C-4‴), 18.7 (C-4″).

Artocarpin (**2**): Yellow powder, ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 13.47 (1H, OH), 7.18 (1H, d, J = 8.8 Hz, H-6′), 6.68 (1H, dd, J = 16.35, 7.08 Hz, H-2‴), 6.53 (1H, br d, J = 1.63, H-1‴), 6.49 (1H, dd, J = 9.0, 2.2 Hz, H-5′), 6.48 (1H, d, J = 2.1 Hz, H-3′), 6.38 (1H, s, H-8), 5.12 (1H, br t, J = 6.71 Hz, H-2″), 3.84 (1H, s, OCH_3), 3.09 (1H, d, J = 7.0 Hz, H-1″), 2.44 (1H, m, H-3‴), 1.60 (3H, s, H-4″), 1.42 (3H, s, H-5″), 1.08 (6H, d, J = 7.0 Hz, H-4‴, H-5‴); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 55.9 (OCH_3), 182.2 (C-4), 162.8 (C-7), 159.3 (C-2), 158.9 (C-4′), 158.6 (C-5), 155.1 (C-2′), 155.0 (C-9), 142.6 (C-2‴), 133.3 (C-3″), 131.5 (C-6′), 121.5 (C-2″), 120.8 (C-3), 115.6 (C-1‴), 112.5 (C-1′), 109.7 (C-6), 108.6 (C-5′), 104.9 (C-10), 103.8 (C-3′), 89.4 (C-8), 33.0 (C-3‴), 25.7 (C-5″), 24.4 (C-1″), 22.7 (C-5‴), 22.3 (C-4‴), 17.7 (C-4″).

Artocarpanone (**3**): White powder, ^{1}H NMR (CD_3OD, 500 MHz) δ: 7.22 (1H, d, J = 8.0 Hz, H-6′), 6.33 (1H, dd, J = 8.0, 2.3 Hz, H-5′), 6.32 (1H, d, J = 2.3 Hz, H-3′), 6.04 (1H, d, J = 2.3 Hz, H-8), 6.02 (1H, d, J = 2.3 Hz, H-6), 5.62 (1H, dd, J = 13.2, 3.0 Hz, H-2), 3.80 (3H, s, OCH_3), 3.09 (1H, dd, J = 17.2, 13.2 Hz, H-3a), 2.72 (1H, dd, J = 17.2, 3.0 Hz, H-3b); ^{13}C NMR (CD_3OD, 125 MHz) δ: 199.1 (C-4), 169.5 (C-7), 165.4 (C-4′), 165.3 (C-9), 160.0 (C-5), 157.0 (C-2′), 129.1 (C-6′), 117.0 (C-1′), 107.9 (C-5′), 104.2 (C-10), 103.6 (C-3′), 95.8 (C-8), 94.9 (C-6), 76.2 (C-2), 56.4 (OCH_3), 43.3 (C-3a; C-3b).

Cyanomaclurin (**4**): Colorless powder, ^{1}H NMR (CD_3OD, 500 MHz) δ: 7.10 (1H, d, J = 8.4 Hz, H-11), 6.34 (1H, dd, J = 8.4 Hz, 2.4 Hz, H-10), 6.18 (1H, d, J = 2.3 Hz, H-8), 5.89 (1H, d, J = 2.1 Hz, H-2), 5.74 (1H, d, J = 2.1 Hz, H-4), 5.23 (1H, d, J = 3.4 Hz, 1.9 Hz, H-13), 4.99 (1H, t, J = 2.2 Hz, H-14), 4.18 (1H, t, J = 3.1 Hz, H-15); ^{13}C NMR (CD_3OD, 125 MHz) δ: 160.7 (C-3), 160.5 (C-1), 159.9 (C-9), 155.9 (C-5), 155.9 (C-7), 133.2 (C-11), 114.1 (C-12), 109.8 (C-10), 103.8 (C-8), 100.4 (C-6), 96.5 (C-2), 95.5 (C-4), 73.1 (C-13), 67.0 (C-15), 64.3 (C-14).

Results and discussion

Three solvents including hexane, ethyl acetate, and methanol were used to prepare the heartwood extracts of *A. heterophyllus* containing compounds with different polarities. Among these, the ethyl acetate extract showed the strongest antibacterial activity against *S. mutans*, *S. pyogenes*, and *B. subtilis* with MIC values of 78, 39, and 9.8 μg/mL, and MBCs of 78, 39, and 39 μg/mL, respectively. This result indicated that the most active compounds had partial non-polar properties and dissolved completely in ethyl acetate. In addition, hexane and methanol may be less efficient in extracting the more active compounds or were extracting different compounds with less activity.

In order to identify the antibacterial compounds, the ethyl acetate extract was subjected to antibacterial assay-guided isolation. Four known flavonoids, cycloartocarpin (**1**), artocarpin (**2**), artocarpanone (**3**) and cyanomaclurin (**4**), were identified (Figure 2). All these pure compounds showed good antibacterial activity against all tested Gram-positive bacteria. Among these compounds, artocarpin (**2**) exhibited the strongest antibacterial activities against *S. mutans*, *S. pyogenes*, *B. subtilis*, *S. aureus*, and *S. epidermidis* with MICs and MBCs of 4.4–17.8 and 8.9–17.8 μM, respectively (Table 1). These results supported previous report that artocarpin isolated from *A. heterophyllus* completely inhibited the growth of primary cariogenic bacteria at 7.1–28.6 μM (Sato et al., 1996). Also artocarpin isolated from *Artocarpus integer* (Thunb.)

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(Zheng et al., 2008), cytotoxicity (Arung et al., 2010), antiplatelet (Lin et al., 1996), and antibacterial activities (Sato et al., 1996).

In this report, we describe the antibacterial activities of extracts from *A. heterophyllus* heartwoods and have used chromatographic methods to purify compounds that were active against several pathogenic bacteria.

Materials and methods

Plant material

Artocarpus heterophyllus heartwoods were collected from the Hat Yai District, Songkhla Province, Thailand, in June 2012. The plant was identified by one of the authors (PP), and the voucher specimen (SKP 117 01 08 01) was deposited at the Herbarium of the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The plant material was dried at 50 °C for 24 h in a hot air oven, and reduced to a powder using a grinder, and the powders were passed through a sieve No. 45.

Preparation of plant extract

The dried plant powder (80 g) was extracted successively with *n*-hexane (500 mL × 3), ethyl acetate (500 mL × 3), and methanol (500 mL × 3), under reflux conditions for 1 h. The three extracts of each solvent were combined and concentrated under reduced pressure to produce the *n*-hexane (0.58 g), ethyl acetate (0.66 g), and methanol (2.17 g) extracts.

Microorganisms and media

Streptococcus mutans (DMST 26095), *S. pyogenes* (DMST 17020), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 14990), *Pseuodomonas aeruginosa* (DMST 15442), and *Escherichia coli* (ATCC 25922) were from the Department of Medical Sciences, Thailand. *Bacillus subtilis* was from the Faculty of Science, Prince of Songkla University. Brain Heart infusion (BHI), Muller-Hinton broth (MHB), and agar (MHA) were from the Becton, Dickinson and Company (Franklin Lakes, NJ).

Antibacterial susceptibility testing

This test was performed by the disc diffusion method (NCCLS, 2008) with a slight modification. *Streptococcus mutans*, *S. pyogenes*, *B. subtilis*, *S. aureus*, and *S. epidermidis* were incubated with BHI at 37 °C for 24 h. *Escherichia coli* and *P. aeruginosa* were incubated with MHA at 37 °C for 24 h. The inocula were prepared by mixing a few bacterial colonies in the sterile Ringer solution (0.85% NaCl) and the turbidity of the suspension was adjusted to that of the standard 0.5 McFarland solution, which was equivalent to 10^8 CFU/mL. The prepared inocula were streaked over the surface of the media with a cotton swab. A sterile paper disc (6 mm diameter) was impregnated with 10 µL of plant extract (1000 mg/mL) and the disc was placed on the agar. The concentration of each plant extract was 10 mg/disc. DMSO was used as a negative control, while a standard ampicillin disc (10 µg/disc) was used as a positive control. Plates were then incubated at 37 °C for 24 h. All disc diffusion tests were performed in triplicate and the antibacterial activities were expressed as the means of the diameters of the inhibition zone (mm).

Determination of minimum inhibitory and bactericidal concentration

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the broth microdilution assay (NCCLS, 2008) with a slight modification. Ampicillin and DMSO were used as positive and negative controls, respectively. The MIC was defined as the lowest concentration of the compound required to inhibit the growth of the test microorganisms and the MBC was defined as the lowest concentration of the compound required to kill the microorganisms.

Bioassay-guided isolation

A dried heartwood powder of *A. heterophyllus* (1 kg) was extracted with ethyl acetate (3 L × 3), under reflux conditions for 1 h. The extracts were combined and concentrated under reduced pressure to produce an ethyl acetate crude extract. The marcs were subsequently extracted with methanol and concentrated under the same conditions to produce a methanol extract. The methanol extract was then partitioned between ethyl acetate and water (50:50, v/v, 500 mL each) to obtain the ethyl acetate fraction.

These ethyl acetate fractions were combined with the ethyl acetate crude extract, and subjected to fractionation using silica gel vacuum chromatography (Figure 1). The dried ethyl acetate extract (25 g) was pre-adsorbed on silica gel and applied to the top of a silica gel column (13 cm in diameter and 6 cm in height). The column was subsequently eluted with

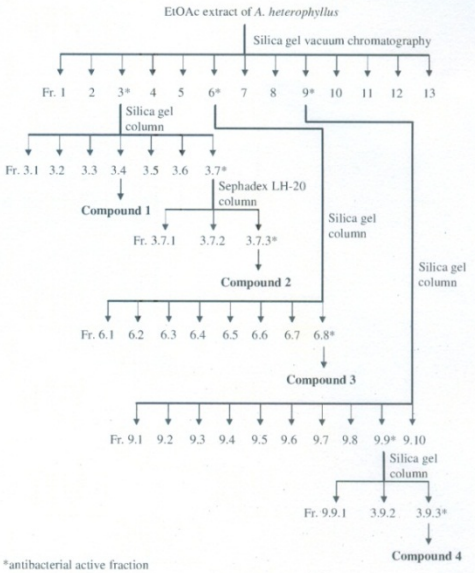

Figure 1. Antibacterial assay guided isolation process.

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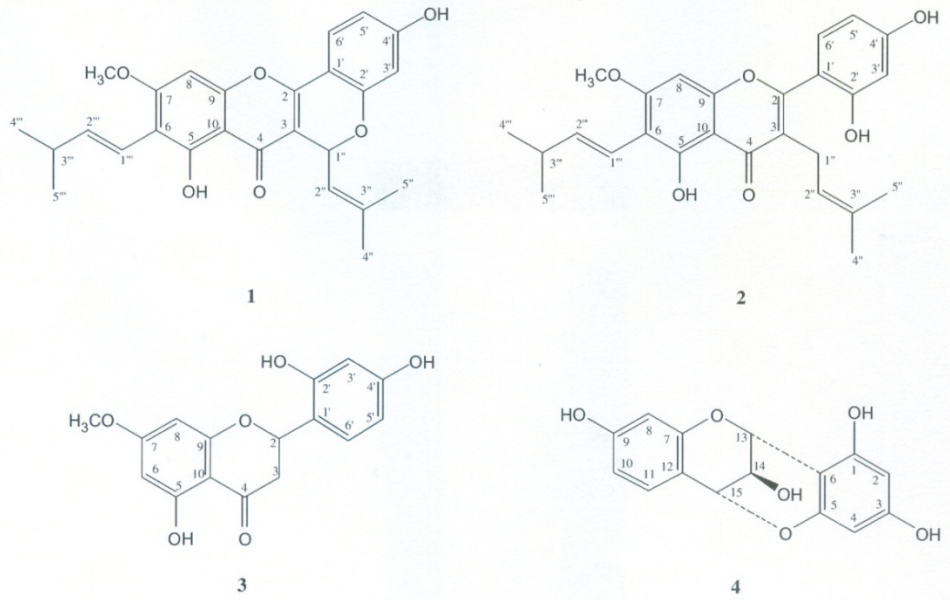

Figure 2. Chemical structures of the compounds isolated from *A. heterophyllus*.

Table 1. MIC and MBC values of the purified compounds and standard drug.

	Cycloartocarpin (1)		Artocarpin (2)		Artocarpanone (3)		Cyanomaclurin (4)		Ampicillin	
Bacteria	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)
S. mutans	35.9	35.9	4.4	8.9	25.8	51.6	6.8	54.4	1.4	1.4
S. pyogenes	71.8	71.8	4.4	8.9	25.8	51.6	54.4	435.2	1.4	1.4
B. subtillis	35.9	71.8	17.8	17.8	25.8	103.2	217.6	435.2	1.4	1.4
S. aureus	71.8	143.6	8.9	8.9	413.5	827.0	217.6	217.6	1.4	1.4
S. epidermidis	35.9	71.8	4.4	8.9	413.5	413.5	54.4	217.6	1.4	1.4
E. coli	143.6	143.6	71.6	143.2	12.9	25.8	27.2	54.4	0.7	1.4
P. aeruginosa	NA	NA	286.4	>1145.6	NA	NA	NA	NA	89.4	178.9

Merr. (Moraceae) roots showed antibacterial activities against *S. aureus*, *S. epidermidis*, and *Propionibacterium acnes* with MIC values of 4.6–9.2 μM (Dej-adisai et al., 2013).

For the Gram-negative bacteria, only artocarpin (**2**) showed some antibacterial activity against *P. aeruginosa* with an MIC value of 286.4 μM (Table 1). This bacterium is a difficult bacterium to control with a wide range of antibiotics. Its resistance is due to its unique characteristics such as the low permeability of its cell membrane, ability to express genetic resistance mechanisms, ability to regulate resistance genes through mutation in its chromosome and it can also modulate the activities of itself and other organisms via transfer of plasmids, transposons and bacteriophages to produce additional resistance genes (Lambert, 2002). In addition, artocarpin (**2**) had a satisfactory antibacterial activity against *E. coli* with an MIC value of 71.6 μM.

Artocarpanone (**3**) showed strong antibacterial activity against *S. mutans*, *S. pyogenes*, and *B. subtilis* with MIC values of 25.8 μM, and MBCs of 51.6–103.2 μM (Table 1). It was of interest that this compound also showed strong activity against *E. coli* with MIC and MBC values of 12.9 and 25.8 μM, respectively. Cyanomaclurin (**4**) which has a different flavonoid skeleton had a specific strong antibacterial activity against *S. mutans* and *E. coli* with MIC values of 6.8 and 27.2 μM, and MBCs of 54.4 and 54.4 μM, respectively. Cycloartocarpin (**1**) showed moderate antibacterial activities with MIC and MBC values of 35.9–143.6 μM.

The results of the antibacterial activities also indicated something about the structure–activity relationships of these four flavonoids. Substitutions of an isoprenyl group on C-3, a 3-methyl-1-butenyl group on C-6, and a hydroxy group on C-2′ for cycloartocarpin (**1**) and artocarpin (**2**) might be required for their antibacterial activity

(Dej-adisai et al., 2013; Tsuchiya et al., 1996). Cyclization of the hydroxy group on C-2′ with the isoprenyl group on C-3 for cycloartocarpin (**1**) resulted in a decrease of antibacterial activity. In addition, a 4′-hydroxylation on ring B was also important for antibacterial activity. It has been reported that the ring B should be moderately hydrophilic with an essential 2′-hydroxy group and an additional hydroxy group at the meta position (Tsuchiya et al., 1996).

Saturation of the C-2 and C-3 may decrease the antibacterial activity of artocarpanone (**3**) and cycloartocarpin (**4**) against Gram-positive bacteria, but increased the antibacterial activity against *E. coli*. In addition, isoprenyl and 3-methyl-1-butenyl substitutions may not be essential for their antibacterial activity against *E. coli*. Investigation of the antibacterial activity of the two isolated flavones from *A. heterophyllus*, artocarpin (**1**) and artocarpesin indicated that a substitution of a methoxy group on the C-7 of artocarpin (**1**) may be important to increase its antibacterial activity against *S. mutans* (Sato et al., 1996). Although cyanomaclurin (**4**) had no methoxy group substituted on ring A, an incorporation between the ring C and B through the saturated heterocyclic and hydroxy group, as well as a substituted hydroxy group on C-14 could increase the antibacterial activity against *S. mutans, S. aureus,* and *S. epidermidis*.

Previous reports have shown that artocarpanone (**3**) possessed cytotoxic and apoptotic effects against cancer cells (Lee et al., 2006), an inhibitory effect on melanin biosynthesis (Arung et al., 2006), as well as antioxidant activities (Rajendran et al., 2004). The compounds artocarpin (**2**) and cycloartocarpin (**1**) were also capable of inducing apoptosis of various cancer cells, such as SMMC-7721 and SGC-7901 (Yang et al., 2010). In addition, artocarpin (**2**) also showed antioxidant activities by reducing the level of reactive oxygen species and lipid peroxidation, and exhibited anti-inflammatory activity by decreasing the levels of pro-inflammatory cytokines, such as tumor necrosis factor α and interleukin 1β (Lee et al., 2013) and inhibited lipopolysaccharide-induced nitric oxide (NO) production (Han et al., 2006). In addition, it also possessed some antiplatelet activity (Lin et al., 1996). Cyanomaclurin (**4**) inhibited mushroom tyrosinase activity with an IC_{50} value of 68.7 μM (Zheng et al., 2008). Thus, some of these bioactive flavonoids may be recommended for use as markers for standardization of the extracts of *A. heterophyllus* heartwoods in a quality control process that might be involved in developing preparations suitable for use as herbal medicines.

Conclusions

The results obtained from this study provide sufficient information to confirm the potential for the use of such extracts from *A. heterophyllus* heartwoods for the treatment of bacterial infections, in particular those caused by Gram-positive bacteria. This study is the first to report the antibacterial activities of artocarpin (**2**) against Gram-negative bacteria, *E. coli* and *P. aeruginosa*, as well as for the antibacterial activities of artocarpanone (**3**), cycloartocarpin (**1**), and cyanomaclurin (**4**), especially the strong antibacterial activity of artocarpanone (**3**) and cyanomaclurin (**4**) against *E. coli*.

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Declaration of interest

The authors report that there are no declarations of interest. The authors alone are responsible for the content and writing of the article. The authors wish to thank the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission as well as Directorate General of Higher Education (DGHE), Ministry of National Education and Culture, Indonesia for support in the form of a research grant

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PAPER 2

Synergistic effect of artocarpin on antibacterial activity of some antibiotics against methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*

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ORIGINAL ARTICLE

Synergistic effect of artocarpin on antibacterial activity of some antibiotics against methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*

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Abstract

Context: Antibacterial resistance has dramatically increased and resulted in serious health problems worldwide. One appealing strategy to overcome this resistance problem is the use of combinations of antibacterial compounds to increase their potency.
Objective: The objective of this study is to determine the synergistic effects of artocarpin for ampicillin, norfloxacin, and tetracycline against methicillin-resistant *Staphylococcus aureus* (MRSA) as well as the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*.
Materials and methods: A broth microdilution method (1.95–250 μg/mL) was used to determine the minimum inhibitory concentration (MIC) of artocarpin and the antibiotics. Any synergistic effects were evaluated at their own MIC using the checkerboard method and a time-kill assay at 37 °C for 24 h.
Results and discussion: Artocarpin showed antibacterial activity against MRSA and *E. coli* with an MIC value of 62.5 μg/mL, and against *P. aeruginosa* with an MIC value of 250 μg/mL. The interaction of artocarpin with all tested antibiotics produced synergistic effects against MRSA with a fractional inhibitory concentration index (FICI) of 0.15–0.37. In addition, a combination of artocarpin and norfloxacin showed a synergistic effect against *E. coli* with an FICI value of 0.37, while the combinations of artocarpin and tetracycline as well as artocarpin and norfloxacin exhibited synergy interactions against *P. aeruginosa* with FICI values of 0.24 and 0.37, respectively. Time-kill assays indicated that artocarpin enhanced the antimicrobial activities of tetracycline, ampicillin, and norfloxacin against MRSA as well as Gram-negative bacteria.

Keywords

Ampicillin, *Artocapus heterophyllus*, norfloxacin, MRSA, tetracycline

History

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Introduction

During the last decade, bacterial resistance to antibiotics has become a great public health problem throughout the world, and can result in high morbidity and mortality. Antibacterial resistance could be due to the abuse and misuse of antibiotics in humans as well as animals. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the resistant pathogens to numerous commercial antibiotics, in particular the β-lactam antibiotics. The resistance mechanisms of this pathogen are largely due to the expression of a β-lactamase that hydrolyzes β-lactams, after acquisition of the *mecA* resistance gene as well as to the over-expression of an efflux pump that reduces the accumulation of antibiotics (Qin et al., 2013). In addition, the Gram-negative bacteria, such as *Pseudomonas aeruginosa*, often leads to clinical problems. This bacterium is difficult to control using commercial antibiotics due to the natural resistance mechanisms, such as the low permeability of its cell wall, expressions of gene resistance, mutations in its chromosome and its ability to gain resistance from other microorganisms (Lambert, 2002).

Some strategies have been adopted to overcome bacterial resistance. Developing new antibacterial agents is one common strategy. However, finding out novel antibacterial agents can be difficult and new resistance mechanisms will occur when the compound is used in clinical applications. A new approach to overcome the resistance is the use of combinations of two or more antibacterial agents in order to enhance their antibacterial activities against the resistant pathogen. Plant-derived compounds are potential sources for these combinations. Flavonoids are known to be one source of therapeutic compounds for many infectious diseases, including antimicrobial agents. These compounds in combination with antibiotics have been reported to possess antibacterial action against bacterial resistance (Shibata et al., 2003; Wagner & Ulrich-Merzenich, 2009). For example, diosmetin

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enhanced the antibacterial activity of erythromycin against MRSA (Chan et al., 2013), while combinations of apigenin and amoxicillin also exhibited a synergistic effect against a resistant *E. coli* (Eumkeb et al., 2012).

Artocarpin (Figure 1) is a polyphenolic flavonoid isolated from *Artocarpus* spp. This compound has been found to possess many pharmacological activities, including antimicrobial (Septama & Panichayupakaranant, 2015), anticariogenic (Sato et al., 1996), antioxidant (Lee et al., 2013), and anti-apoptosis (Yang et al., 2010). However, there is no information about the possible synergistic effects of artocarpin on the antibacterial activities of any antibiotics, especially against MRSA, *P. aeruginosa* and *E. coli*. This study was therefore conducted to assess the possibility of interactions of artocarpin isolated from *Artocarpus heterophyllus* Lam. (Moraceae) in combination with antibiotics, including ampicillin, norfloxacin, and tetracycline against MRSA and two Gram-negative bacteria, *P. aeruginosa* and *E. coli*. The broth microdilution method was used to determine the MIC values of each drug, while the checkerboard method was used to determine the interaction between these combinations of drugs.

Materials and methods

Chemicals

Artocarpin was purified from the ethyl acetate extract of *A. heterophyllus* heartwoods using the method previously described (Septama & Panichayupakaranant, 2015). The antibiotics ampicillin, norfloxacin, and tetracycline were obtained from Sigma (Sigma-Aldrich, Gillingham, UK). Brain heart infusion (BHI) and agar were from the Becton, Dickinson and Company (Franklin Lakes, NJ).

Bacterial strains

Methicillin-resistant *Staphylococcus aureus* (MRSA) (DMST 20654), *Pseudomonas aeruginosa* (DMST 15442), and *Escherichia coli* (ATCC 25922) were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand.

Determination of minimum inhibitory concentrations

All tested bacteria were incubated on BHI agar at 37 °C for 24 h. The inocula were prepared by mixing a few bacterial colonies in normal saline solution (NaCl 0.85%) and the turbidity of the suspension was adjusted to that of the standard 0.5 McFarland solution, which was claimed to be equivalent to 1×10^8 CFU/mL. The suspension was diluted (1:100) with sterile normal saline solution to contain approximately 1×10^6 CFU/mL.

The MIC values were determined by the broth microdilution assay (NCCLS, 2008) with slight modification. Each sample was initially dissolved in DMSO, and subsequently diluted with BHI broth to reach the desired final concentration. Two-fold dilutions were prepared in a 96-well plate. The bacterial suspensions (1×10^6 CFU/mL) were added into each well and then incubated at 37 °C for 24 h. The MIC value was determined as the lowest concentration of sample that inhibited the bacterial growth.

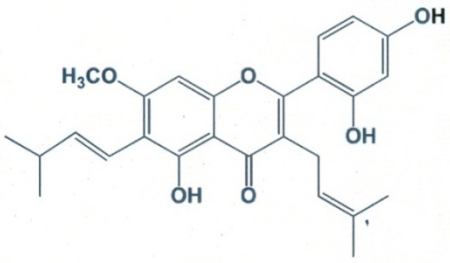

Figure 1. Chemical structure of artocarpin.

Checkerboard dilution

The interactions of two different combinations of samples were assessed using the checkerboard method (Chang et al., 1995) with slight modifications. This assay was performed with artocarpin in combination with ampicillin, norfloxacin, and tetracycline. The combinations in the 96-well plates were performed as follows: artocarpin was diluted by two-fold dilutions along the *x*-axis of the plates, while antibiotics were diluted by two-fold dilutions along the *y*-axis. Subsequently, each well was inoculated with the tested bacterial suspension (1×10^6 CFU/mL). The plates were then incubated at 37 °C for 24 h. The MIC value was considered as the lowest concentration of the compounds, alone or in combination, required to inhibit the growth of the test microorganisms. The interaction between artocarpin and the antibiotics was determined by quantifying the fractional inhibitory concentration index (FICI) using the following formula:

$$\mathrm{FICI} = \mathrm{FIC\ of\ artocarpin} + \mathrm{FIC\ of\ the\ antibiotics}$$

$$\mathrm{FIC(fractional\ inhibitory\ concentration)} = \frac{\mathrm{MIC\ of\ artocarpin\ or\ antibiotics\ in\ combination}}{\mathrm{MIC\ of\ artocarpin\ or\ antibiotics\ alone}}$$

The results were considered as synergistic ($\mathrm{FICI} \leq 0.5$), additive ($0.5 \leq \mathrm{FICI} \leq 1$), indifferent ($1 \leq \mathrm{FICI} \leq 4$), and antagonistic ($\mathrm{FICI} > 4$) (Milne & Gould, 2012).

Time-kill assay

In order to confirm any synergistic effect on the inhibition of bacterial growth of the samples in different concentration, a time kill assay was performed. The bacterial cultures incubated in BHI at 37 °C for 24 h were diluted with normal saline solution to contain the bacterial suspensions (1×10^6 CFU/mL). The bacterial suspension was added into BHI broth containing the mixture of samples with different concentrations, and then incubated at 37 °C. The final bacterial concentration was 5×10^5 CFU/mL. DMSO was used as a negative control. Aliquots (50 µL) of the cultures were collected at eight time intervals (0, 1, 2, 4, 6, 8, 12, and 24 h), diluted (1:10) with 450 µL of normal saline, and 10-fold

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serial dilutions were prepared in normal saline. Then, 20 µL of each dilution was cultured on BHI agar and the numbers of viable colonies were calculated after 24 h incubation. Antimicrobial agents are considered as bactericidal when they are able to reduce colony formatting unit per mL (CFU/mL) to less than $3 \times \log_{10}$. The synergistic effect of antimicrobial in combination is considered when the CFU/mL is reduced to >2 $\log_{10}$ (Hamoud et al., 2014).

Results and discussion

Artocarpin showed moderate antibacterial activity against MRSA and *E. coli* with an MIC value of 62.5 µg/mL, but it had a weak antibacterial activity against *P. aeruginosa* with an MIC value of 250 µg/mL (Table 1). Ampicillin exhibited a moderate antibacterial activity against MRSA (an MIC value of 62.5 µg/mL), while tetracycline and norfloxacin had a weak antibacterial activity against MRSA (an MIC value of 125 µg/mL). However, norfloxacin showed a strong antibacterial activity against *P. aeruginosa* and *E. coli* with an MIC value of 1.95 µg/mL, while tetracycline and ampicillin exhibited strong-moderate antibacterial activity with MIC values of 7.81–15.62 µg/mL.

The checkerboard method was used to determine the interaction between artocarpin and the combination of antibiotics. The interactions were interpreted using their FICI values. Artocarpin showed a synergistic effect on the antibacterial activity of all tested antibiotics against MRSA with the FICI values of 0.15–0.37. The concentrations of artocarpin and each antibiotic used for this synergistic effect varied, but were much lower than their own MICs (Table 2). To confirm their synergistic effect against MRSA, a time-kill assay was conducted. The obtained results agreed with those from the checkerboard method. Combinations of artocarpin with the antibiotics inhibited bacterial growth, whereas artocarpin or the antibiotic alone did not (Figures 2–4). The combination of 15.62 µg/mL artocarpin and 7.81 µg/mL ampicillin was able to reduce the number of colony counts after 8 h incubation, while the combinations of 7.81 µg/mL artocarpin and 15.62 µg/mL tetracycline as well as 1.95 µg/mL artocarpin and 15.62 µg/mL norfloxacin completely inhibited bacterial growth within 12 h. This finding indicated that artocarpin may overcome the problems associated with some multidrug-resistant pathogen, e.g., MRSA, when used in a combination with the commonly used antibiotics i.e., ampicillin, norfloxacin, and tetracycline.

In addition, artocarpin (31.25 µg/mL) enhanced the antibacterial activities of tetracycline (1.95 µg/mL) and norfloxacin (0.5 µg/mL) against *P. aeruginosa* with a synergistic effect (FICI values of 0.24 and 0.37, respectively), but it produced an additive effect with ampicillin (7.81 µg/mL) with an FICI value of 0.62 (Table 3). The time-kill assays also confirmed the synergistic effect of these combinations, as shown in Figures 5 and 6. The combinations of 31.25 µg/mL artocarpin and 1.95 µg/mL tetracycline as well as 31.25 µg/mL artocarpin and 0.5 µg/mL norfloxacin demonstrated the synergistic effect by completely inhibiting bacterial growth after 6 and 4 h incubation, respectively. In the case of *E. coli*, artocarpin (7.81 µg/mL) showed a synergistic effect only when combined with norfloxacin (0.5 µg/mL), and gave an FICI value of 0.37, and showed an additive effect for tetracycline (3.9 µg/mL) and ampicillin (7.81 µg/mL) with an FICI value of 0.52 (Table 4). The time-kill assays also confirmed the synergistic effect of a combination of artocarpin and norfloxacin against *E. coli* (Figure 7). The combination of 7.81 µg/mL artocarpin and 0.5 µg/mL norfloxacin completely inhibited bacterial growth within 6 h. The results indicated that artocarpin may be used to enhance the antibacterial activities of these antibiotics against Gram-negative pathogenic bacteria, especially when used in combination with norfloxacin.

Mechanisms of enhanced activities of combination of antibiotics against Gram-negative bacteria may be explained by assuming that artocarpin is a flavonoid compound. Flavonoids are well known to possess antibacterial activity with many possible mechanisms of action, e.g., reduction of membrane fluidity due to interactions of flavonoids with the lipophilic side of cell membrane (Cushnie & Lamb, 2011). Alterations to membrane cell fluidity may allow norfloxacin to enter to its site of action for inhibiting DNA gyrase (Crumplin et al., 1984). Furthermore, the loss of membrane integrity may also help tetracycline to traverse the outer membrane and to occupy its site of action through inhibition of protein synthesis by blocking the incorporation of aminoacyl-tRNA with the bacterial ribosomes (Chopra & Roberts, 2001). The synergistic effect may also be due to more than one mechanism of action that occurred at different target sites, and by regulating the same response in the cell. In contrast, the different agents may also give a synergistic effect of their antibacterial activity by regulating the same target site (Yang et al., 2014).

The synergistic effect of plant-derived antibacterial compounds to the antibiotics against drugs-resistant bacteria may

Table 1. Antibacterial activity of artocarpin and antibiotics against three tested bacteria.

	MIC (µg/mL)			
Bacteria	Artocarpin	Tetracycline	Ampicillin	Norfloxacin
E. coli	62.50	7.81	15.62	1.95
P. aeruginosa	250	15.62	15.62	1.95
MRSA	62.50	125	62.50	125

MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 2. Effects of artocarpin on antibacterial activity of antibiotics against MRSA.

	MIC*a* (µg/mL)	MIC*c* (µg/mL)	FIC	FICI	Interaction
Artocarpin–ampicillin					
Artocarpin	62.50	15.62	0.25	0.37	Synergistic
Ampicillin	62.50	7.81	0.12		
Artocarpin–tetracycline					
Artocarpin	62.50	7.81	0.12	0.24	Synergistic
Tetracycline	125	15.62	0.12		
Artocarpin–norfloxacin					
Artocarpin	62.50	1.95	0.03	0.15	Synergistic
Norfloxacin	125	15.62	0.12		

MIC*a*, MIC of one sample alone; MIC*c*, MIC of samples in combination; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index.

provide the prospect that the use of plant-derived antibacterial compounds and antibiotics in combination may be a promising route to overcome some of the antibiotic-resistance problems. Nevertheless, further studies are required to define the mechanisms of action that underlie this synergistic effect.

Conclusions

Artocapin enhanced the antibacterial activities of all tested antibiotics. It produced a synergistic effect with norfloxacin against MRSA, *P. aeruginosa*, and *E. coli*, and a synergistic effect with tetracycline against MRSA and *P. aeruginosa*, and a synergistic effect with ampicillin

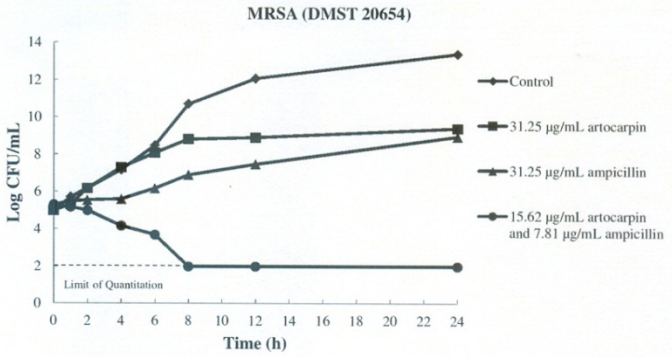

Figure 2. Time–kill curves of artocarpin, ampicillin, and their combination against MRSA.

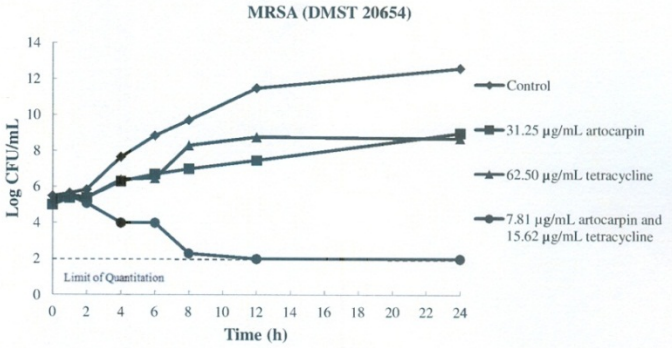

Figure 3. Time–kill curves of artocarpin, tetracycline, and their combination against MRSA.

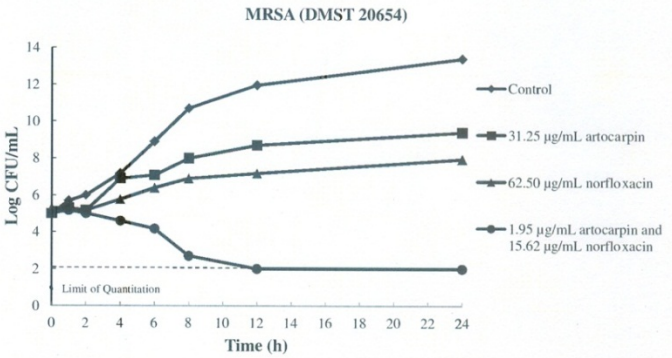

Figure 4. Time–kill curves of artocarpin, norfloxacin, and their combination against MRSA.

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Table 3. Effects of artocarpin on antibacterial activity of antibiotics against *P. aeruginosa*.

	MIC*a* (μg/mL)	MIC*c* (μg/mL)	FIC	FICI	Interaction
Artocarpin–ampicillin					
Artocarpin	250	31.25	0.12	0.62	Additive
Ampicillin	15.62	7.81	0.50		
Artocarpin–tetracycline					
Artocarpin	250	31.25	0.12	0.24	Synergistic
Tetracycline	15.62	1.95	0.12		
Artocarpin–norfloxacin					
Artocarpin	250	31.25	0.12	0.37	Synergistic
Norfloxacin	1.95	0.50	0.25		

MIC*a*, MIC of one sample alone; MIC*c*, MIC of samples in combination; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index.

Table 4. Effects of artocarpin on antibacterial activity of antibiotics against *E. coli*.

	MIC*a* (μg/mL)	MIC*c* (μg/mL)	FIC	FICI	Interaction
Artocarpin–ampicillin					
Artocarpin	62.50	7.81	0.12	0.62	Additive
Ampicillin	15.62	7.81	0.50		
Artocarpin–tetracycline					
Artocarpin	62.50	7.81	0.12	0.62	Additive
Tetracycline	7.81	3.90	0.50		
Artocarpin–norfloxacin					
Artocarpin	62.50	7.81	0.12	0.37	Synergistic
Norfloxacin	1.95	0.50	0.25		

MIC*a*, MIC of one sample alone; MIC*c*, MIC of samples in combination; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index.

Figure 5. Time–kill curves of artocarpin, tetracycline, and their combination against *P. aeruginosa*.

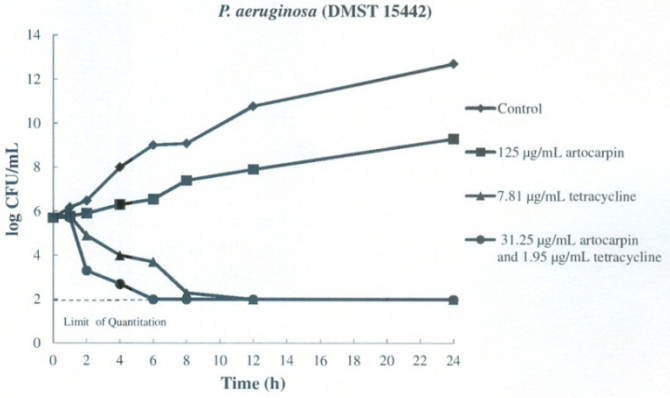
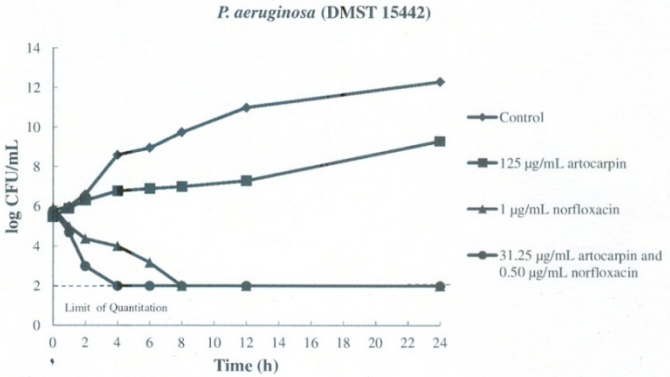


Figure 6. Time–kill curves of artocarpin, norfloxacin, and their combination against *P. aeruginosa*.



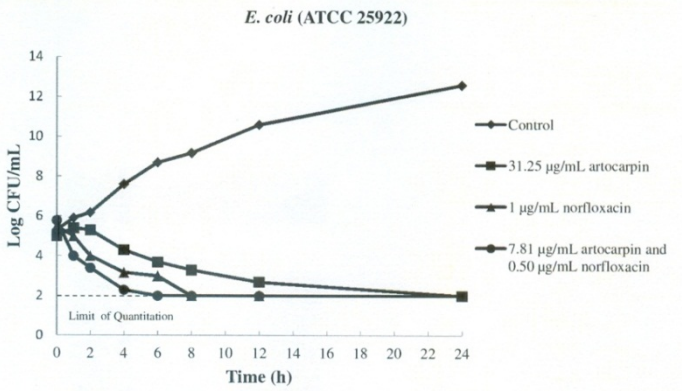


Figure 7. Time–kill curves of artocarpin, norfloxacin, and their combination against *E. coli*.

against MRSA. Antagonistic effect was not found for all drug combinations.

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Declaration of interest

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PAPER 3

Synergistic effect of artocarpanone on antibacterial activity of some antibiotics against methicillin-resistant *Staphylococcus aureus* and their effect on membrane permeability

(Submitted to Journal of Natural Medicine)

A synergistic effect of artocarpanone on the antibacterial activity of norfloxacin against methicillin-resistant *Staphylococcus aureus* and their effect on membrane permeability

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a human pathogen that causes numerous nosocomial infections that are difficult to control. Artocarpanone isolated from *Artocarpus heterophyllus* Lam. possesses antibacterial activity. The present study was focused on investigating any interaction between artocarpanone and some antibiotics including tetracycline, ampicillin and norfloxacin against MRSA as well as determining any disruptive effect on bacterial membranes. A broth microdilution method was used for the susceptibility assay. Any synergistic effect was determined using a checkerboard method, and any membrane disruption effect was investigated using a bacteriolysis assay and a measurement of the released 260 nm absorbing materials. Artocarpanone and the antibiotics showed weak antibacterial activities against MRSA with MIC values of 125 µg/ml, except for ampicillin that had a moderate antibacterial activity (MIC of 62.5 µg/ml). However, the interaction between artocarpanone (31.2 µg/ml) and norfloxacin (3.9 µg/ml) exhibited a synergistic antibacterial activity against MRSA, with a fractional inhibitory concentration index (FICI) of 0.28, while the interaction between artocarpanone (31.2 µg/ml) and tetracycline (31.2 µg/ml), and ampicillin (15.6 µg/ml) 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, a combination of artocarpanone and norfloxacin altered the membrane permeability of MRSA. These findings suggested that artocarpanone may be considered as an adjuvant to enhance the antibacterial activity of norfloxacin against MRSA.

Keywords: artocarpanone; *Artocapus heterophyllus*; norfloxacin; MRSA; membrane disruption

Introduction

The emergence of multi-drug resistance to antibiotics has rapidly increased and has led to a failure to cure some health problems, including 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). Its acquisition of the *mecA* gene and ability to over express the efflux pumps as well as to produce a β-lactamase hydrolysis enzyme are the underlining causes for the resistance of MRSA towards many antibiotics, especially β-lactam antibiotics [2]. A high prevalence of nosocomial infections caused by MRSA has been reported from many countries worldwide [3]. Consequently the identification and development of new antibiotics with new targets and modes of action is urgently needed. However, it is a big challenge to find and produce new antibiotics, because of the time and costs required to ensure that new is safe and effective and will not induce new resistance mechanism when frequently used clinically. The use of a combination of conventional antibiotics and some additional agents that can enhance their antibacterial activity has been suggested to be an alternative strategy to overcome the resistance problem [4].

Plant-derived compounds are often a source of new antibacterial agents. Many flavonoids have been reported to have antimicrobial activity [5], and some have been

demonstrated to have a synergistic effect on the antibacterial activity of some commercial antibiotics against resistant bacteria, including MRSA [6,7]. Artocarpanone is a flavonoid isolated from an *Artocarpus* sp., and possesses many pharmacological activities such as having antibacterial, anti-tyrosinase and cytotoxic effects [8-10]. However, there has been no report on any synergistic effects of artocarpanone on the antibacterial activity of any antibiotic. The aim of the present study was to evaluate any synergistic effect of artocarpanone on the antibacterial activity of some conventional antibiotics, including tetracycline, ampicillin, and norfloxacin that are normally used against *S. aureus* but are not effective against MRSA. In addition, its effect on its ability to disrupt cell membranes when used as a synergistic mixture was evaluated.

Materials and methods

Chemicals

Artocarpanone was purified from the crude ethyl acetate extract of *A. heterophyllus* heartwoods using a method previously described by Septama and Panichayupakaranant [8]. The antibiotics, ampicillin, tetracycline, and norfloxacin were from Sigma (Sigma-Aldrich, UK). Crystal violet was from Lab-Chem (Laboratory Chemical, Australia). Brain heart infusion (BHI) and agar were from the Becton, Dickinson and Company (Franklin Lakes, New Jersey, USA).

Bacterial strains

Methicillin-resistant *Staphylococcus aureus* (DMST 20654) was obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand.

Determination of minimum inhibitory concentrations

The microdilution assay [11] with a slight modification was used to determine the MICs. Two-fold dilutions of each sample in BHI were prepared in a sterile 96-well plate. The bacteria suspensions were prepared in 0.85% NaCl, and the turbidity of the suspension was adjusted to the 0.5 McFarland standards (equivalent to 1×10^8 CFU/ml). This suspension was diluted with normal saline to contain 1×10^6 CFU/ml, and it was then added into each well. The final cell concentration was 5×10^5 CFU/ml. The plate was incubated at 37ºC for 24 h. The MIC was considered to be the lowest concentration of the sample that produced suppression of visible growth.

Checkerboard assay

This method was conducted to evaluate the interactions of two different combinations of samples against the tested bacteria as described by Chang et al., with a slight modification [12]. This assay was performed with artocarpanone in combination with antibiotics in 96-well plates. Two-fold dilutions of artocarpanone were prepared in BHI along the x-axis, while two-fold dilution of the antibiotics was diluted along the y-axis. Subsequently, each well was inoculated with a bacteria suspension of 1×10^6 CFU/ml. The plates were then incubated at 37ºC for 24 h. The fractional inhibitory concentration index (FICI) was quantified as the fractional inhibitory concentration (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) [13].

Time-kill curve

Each time-kill assay was performed eight times (0, 1, 2, 4, 6, 8, 12 and 24 h). The bacterial suspension contained 1×10^6 CFU/ml was added to BHI broth containing the mixture of samples to reach the final cell concentration of 5×10^5 CFU/ml, then incubated at 37ºC. Aliquots (50 µl) of the cultures were removed at eight time intervals, diluted (1:10) with 450 µl of normal saline, and ten-fold serial dilutions were prepared in normal saline. Then, 20 µl of each dilution was cultured on BHI agar and the numbers of viable colonies were calculated after a 24 h incubation [14].

Bacteriolysis assay

This method was used to detect the alteration of membrane permeability by calculating the percentage uptake of crystal violet [15]. Briefly, a suspension of

MRSA in normal saline was prepared from the overnight culture on BHI agar. The cell was then suspended in normal saline. A single dose of artocarpanone and norfloxacin, as well as artocarpanone in combination with norfloxacin were added to the cell suspension, the sample without treatment was used as a negative control, and incubated at 37ºC for 1 h. The final cell concentration was 5×10^7 CFU/ml. The cells were harvested at 9,300 × g for 5 min. After that, the cells were resuspended in a crystal violet solution (10 µg/ml in normal saline). The cells were then incubated at 37ºC for 10 min. Next, the cells were centrifuged at 13,400 × g for 15 min. The optical density (OD) 590 nm of the supernatant was measured using a UV-Vis spectrophotometer. The OD value of the crystal violet solution, which was originally used in this assay, was taken and this was considered to be 100%. The percentage of crystal violet uptake was calculated as the OD value of the sample/OD value of the crystal violet solution × 100. This experiment was done in triplicate.

Loss of 260 nm absorbing material method

The concentration of released UV-absorbing material were measured using a UV-Vis spectrophotometer [15,16]. Overnight cultures of MRSA were washed with normal saline and suspended in normal saline. Artocarpanone and norfloxacin alone as well as the mixture of artocarpanone and norfloxacin were added to the cell suspension. The cell without treatment was used as the control. The final cell concentration was 5×10^7 CFU/ml. All the samples were incubated at 37ºC for 1 h. This assay was performed in triplicate. After treatment, the samples were centrifuged at 13,400 × g for 15 min. The OD_{260} of the supernatant was then measured by a UV-Vis

spectrophotometer. The value of the supernatant was considered to be a percentage of the intracellular UV-absorbing material released by the cells.

Statistical analysis

All experiments were carried out in triplicate with the average value and standard deviations reported. The data were analyzed using ANOVA to know significant differences between these groups. Followed by the Tukey's HSD post hoc test denoted by the presence of a statistically significant difference and it was considered to be significant at the level $p<0.01$.

Results and discussion

On the basis of the broth microdilution assay, artocarpanone, tetracycline and norfloxacin showed only weak antibacterial activity against MRSA with MIC values of 125 µg/ml, while ampicillin had a moderate antibacterial activity with an MIC value of 62.5 µg/ml. Further investigations on the interaction of artocarpanone on the anti-MRSA activity of these antibiotics using the checkerboard method revealed that artocapanone (31.2 µg/ml) enhanced the antibacterial activity of norfloxacin (3.9 µg/ml) with a synergistic effect (FICI value of 0.28) (Table 1). By this combination, artocarpanone could decrease the dose of norfloxacin by 32-fold. In contrast, the combinations of artocarpanone (31.2 µg/ml) and tetracycline (31.2 µg/ml) or ampicillin (15.6 µg/ml) exhibited only an additive effect against MRSA with an FICI value of 0.5. The time-kill assay was conducted to confirm the synergistic effect of artocapanone on the anti-MRSA activity of norfloxacin. 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 artocapanone and norfloxacin alone at the concentration of 62.5 µg/ml did not completely inhibit bacterial growth until 24 h (Fig. 2). These results indicated that artocarpanone may overcome the problems associated with MRSA when used in a 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 lead 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 [17]. 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 [18]. Cell membrane disruption is one of the antibacterial mechanisms of flavonoids [5,19]. This study therefore also focused on investigation any cell membrane disruption by artocapanone, norfloxacin and their synergistic mixtures. Based on the bacteriolytic assay, the percentage of uptake of crystal violet indicated the bacteriolytic activity of the compounds against MRSA (Fig. 3). Artocapanone 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 significantly was higher than the other groups, including the control as well as a single dose of artocarpanone and

norfloxacin (p<0.01). 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 [20]. 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) (Fig. 4). 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 [21]. For example, sophoraflavanone G isolated from *Sophora exigua* exhibited an antibacterial activity against MRSA by reducing the fluidity of the cellular membrane as well as by reducing the cytoplasmic contents [19,22]. 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 [23]. 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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Table 1. 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		

MIC*a* (MIC of one sample alone), MIC*c* (MIC of samples in combination)

FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index)

Figure legends

Figure 1 Chemical structure of artocarpanone.

Figure 2 Time-kill curves of artocarpanone, norfloxacin, and their combination against MRSA.

Figure 3 Crystal violet uptake of artocarpanone, norfloxacin and their combination treated MRSA. The mean ± SD for three replicates are illustrated. *: samples demonstrates 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 MRSA treated with artocarpanone, norfloxacin and their combination. The mean ± SD for three replicates are illustrated. *: samples demonstrates significant differences compared to control (p<0.01), #: combination of artocarpanone and norfloxacin demonstrates significant difference compared to drugs alone (p<0.01).

Figure1

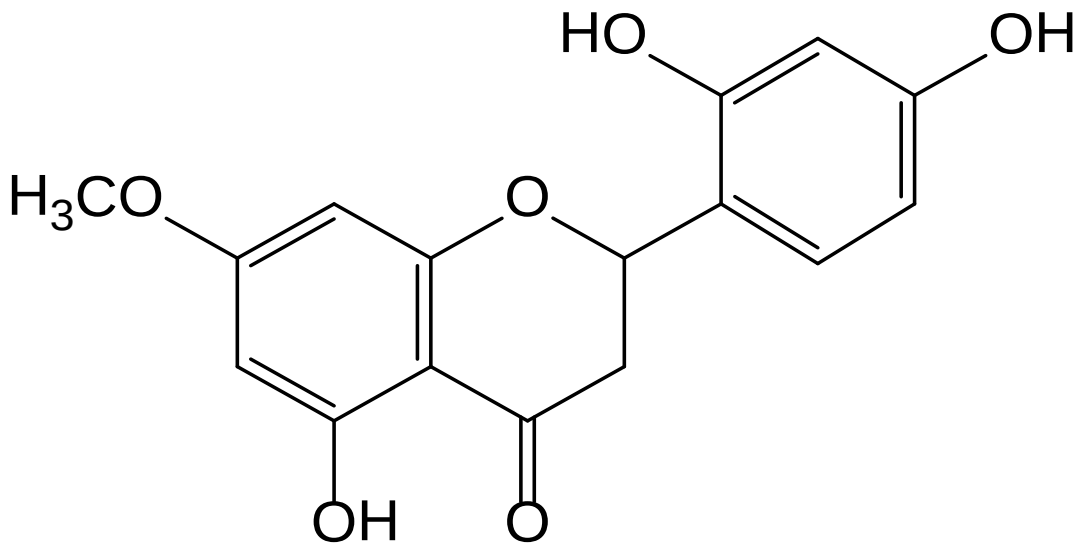


Figure 2

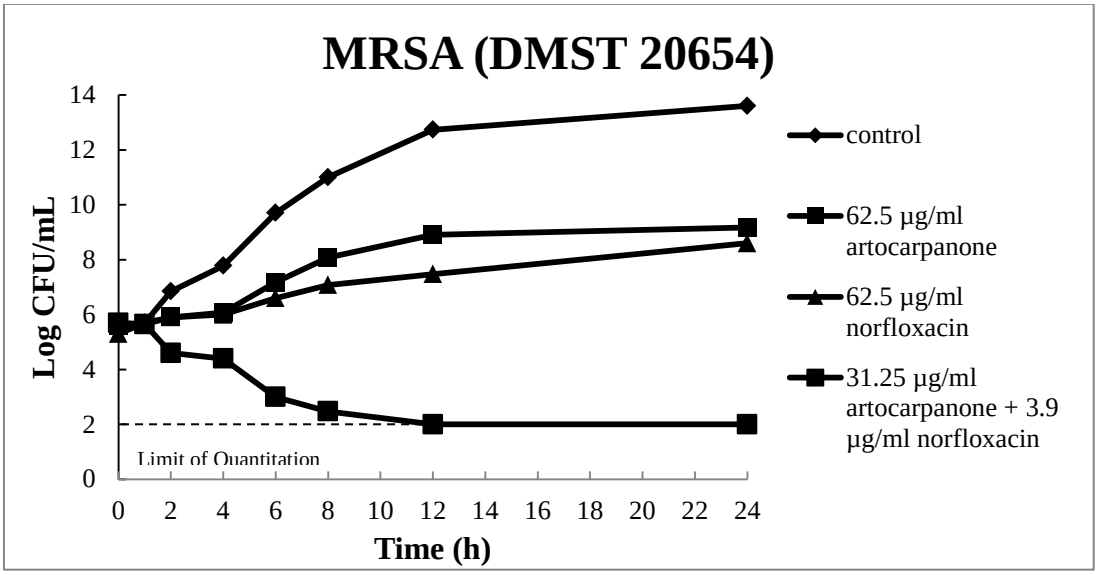

MRSA (DMST 20654)
Log CFU/mL
14
12
10
8
6
4
2
0
Limit of Quantitation
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)
control
62.5 µg/ml artocarpanone
62.5 µg/ml norfloxacin
31.25 µg/ml artocarpanone + 3.9 µg/ml norfloxacin

Figure 3

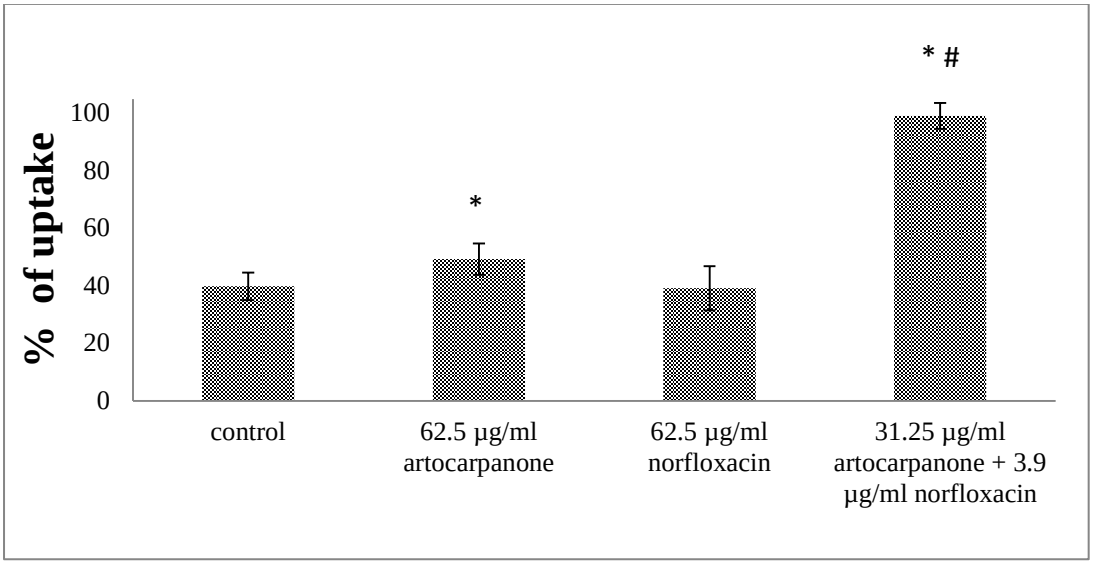
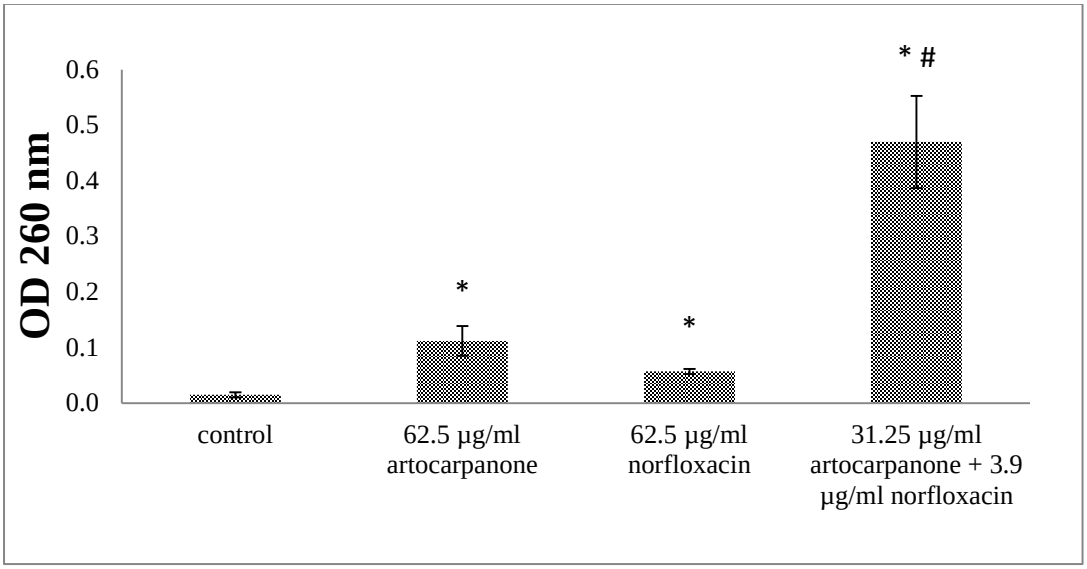

% of uptake
100
80
60
40
20
0
*
* #
control
62.5 µg/ml artocarpanone
62.5 µg/ml norfloxacin
31.25 µg/ml artocarpanone + 3.9 µg/ml norfloxacin

Figure 4


OD 260 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
* #
control
62.5 µg/ml artocarpanone
62.5 µg/ml norfloxacin
31.25 µg/ml artocarpanone + 3.9 µg/ml norfloxacin

PAPER 4

Simultaneous HPLC analysis of three flavonoids in the extracts of *Artocarpus heterophyllus* heartwoods

(Accepted in Natural Product Science)

Simultaneous HPLC Analysis of Three Flavonoids in the Extracts of *Artocarpus heterophyllus* Heartwoods

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Abstract – A reversed-phase high-performance liquid chromatographic method is described for the simultaneous determination of three antibacterial flavonoids, artocarpanone, artocarpin, and cycloartocarpin in ethyl acetate extracts from *Artocarpus heterophyllus* heartwoods. Separation was achieved using a TSK-gel ODS-80Tm column (5 µm, 4.6 × 150 mm) at 25°C with a gradient elution system of methanol and water as follows: 0-8 min, 60:40; 8-27 min, 80:20; 27-35 min, 60:40, v/v, at a flow rate of 1 mL/min, and a quantitative UV detection at 285 nm. The method was validated by measuring the key parameters, including specificity, linearity, sensitivity, accuracy, repeatability and reproducibility. A high degree of specificity and sensitivity was achieved. The calibration curves for all three flavonoids showed good linearity with a coefficient of determinations (R^2) of ≥ 0.9995. The recoveries of the method were from 98-104%, with good reproducibility and repeatability (RSD values of less than 2%) were also achieved. Ethyl acetate was the best solvent for extraction of these three flavonoids using the heat reflux conditions for 1 h. This optimized sample preparation and HPLC method can be practically used for a routine standardization process of the extracts from the *A. heterophyllus* heartwoods.

Keywords - *Artocarpus heterophyllus*; artocarpanone; artocarpin; cycloartocarpin; HPLC

Introduction

The heartwoods of *Artocarpus heterophyllus* Lam. (Moraceae) have been used as a popular folk medicine in several Asian countries for the treatment of bacterial and fungal infection as well as some other diseases associated with inflammation, malarial fever and diabetes.[1,2] The flavonoid compounds isolated from the heartwoods of this plant possess many biological activities including anticariogenic, antioxidant and tyrosine inhibitory activities.[3-5] Recently, we have isolated some flavonoid compounds with antibacterial activity, i.e. artocarpanone, artocarpin and cycloartocarpin (Fig. 1.) from the heartwoods of *A. heterophyllus*.[6] Artocarpanone also possessed anti-inflammatory activity and had an inhibitory effect on melanin biosynthesis[7,8], while artocarpin possessed anticancer activity and inhibited neuraminidase[9,10], while cycloartocarpin possessed antiplatelet activity.[11] These three flavonoids were therefore considered to be the best bioactive markers used for standardization of the heartwood extracts of *A. heterophyllus* before being used for medicinal purposes.

A standardized method for assessing the quality and effectiveness of herbal medicines is becoming a hot issue for many people including those in the herbal medicine industries. In order to obtain a high and consistent quality of the heartwood extracts of *A. heterophyllus*, a simple quantitative analytical method for the active compounds in the extracts should be developed. Recently, a reversed-phase HPLC method has been popularly used for standardization of herbal extracts due to its high sensitivity, selectivity and accuracy.[12] In this study there has been a focus on establishing a validated quantitative HPLC method for the simultaneous

determination of artocarpanone, artocarpin and cycloartocarpin as well as determining the most suitable solvent for sample preparation.

Experimental

Chemicals and reagents – Standard artocarpanone, artocarpin and cycloartocarpin had been previously purified.[6] Methanol (HPLC-grade) was from Merck (Bangkok, Thailand). Analytical-grade solvents (hexane, chloroform, ethyl acetate and methanol) were from Labscan limited (Songkhla, Thailand). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Plant material – *A. heterophyllus* heartwoods were collected from Songkla Province, Thailand, in June 2012. The voucher specimen (SKP 117 01 08 01) was deposited at the Herbarium of the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The plant material was washed and dried at 60ºC in a hot air oven for 24 h, then ground and passed through a No. 45 sieve.

Standard solution - Stock solutions of artocarpanone, artocarpin, and cycloartocarpin were prepared in methanol. The working solution of the combined standard compounds was subsequently prepared in methanol and diluted to give a series of standard solutions of 6.25, 12.5, 25, 50 and 100 µg/mL. Calibration curves were constructed for each of the target analytes.

Sample preparation – The *A. heterophyllus* heartwood powder (1 g) was extracted with ethyl acetate (250 mL) under reflux conditions for 1 h. The extract was concentrated and dried under reduced pressure. The dried extract (5 mg) was dissolved and adjusted to 10 mL with methanol, and then filtered through a 0.45 µm

membrane filter, and analyzed immediately after extraction in order to avoid possible chemical degradation.

HPLC analyses - HPLC analysis was carried out using the Waters 1500 series equipped with a Waters 2998 photodiode-array detector (PDA) and Waters 2707 auto sampler. For analysis of the data, Waters Chemstation for Empower software was used. Separation was achieved at 25ºC on a TSK-gel ODS-80Tm column (150 × 4.6 mm i.d.) (Tosho Bioscience, Japan). The mobile phase consisted of methanol and water, with a gradient elution system (0-8 min, 60:40; 8-27 min, 80:20; 27-35 min, 60:40, v/v) at a flow rate of 1 mL/min. The sample injection volume was 20 µL, and detection was by UV at a wavelength of 285 nm.

Validation of method - Validation of the analytical method was achieved by following the guidelines of the International Conference on the Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use.[13] The HPLC method was validated for linearity, accuracy, precision, specificity, limit of detection (LOD), and limit of quantification (LOQ) of the analytes.

Linearity - Calibration curves of the three standard flavonoids were constructed on three consecutive days by analysis of a mixture containing each of the standard compounds at five concentrations (6.25-100 µg/mL), and plotting peak areas against the concentrations of each reference standard. The standard curves were analyzed using the linear least-squares regression equation derived from the peak area. The coefficient of determination (R^2) of the regression line should be not less than 0.999.

Accuracy – Sample portions were fortified with known quantities of standards (6.25, 25, and 100 µg/mL) in order to assay the accuracy of the data. Prior to fortification of the analyte, the background levels of artocarpanone, artocarpin and cysloartocarpin in extracts of *A. heterophyllus* heartwood were determined so as to calculate actual recoveries. The amount of each analyte was determined in triplicate, and the percentage recoveries were then calculated.

Precision – Precision experiments were conducted for intraday and interday. A solution of sample was used to test for repeatability. The repeatability (intraday precision) data was obtained from six injections of one sample solution performed on the same day. The data were used to calculate the % RSD for intraday precision (less than 2%). The inter-day precision of the extraction procedure was validated by repeating the extraction procedure on the same sample of *A. heterophyllus* heartwood. This parameter was evaluated by repeating the extraction in triplicate on 3 different days with a freshly prepared mobile phase and samples. An aliquot of each extract was then injected and quantified. The data were used to calculate the % RSD. (less than 5%) for the interday precision.

Specificity - Peak identification was carried out using the authentic standards and by scanning the UV spectrum of each peak using a photodiode-array detector. The UV spectra were taken at various points of the peaks and at least three different points to check for the peak homogeneity.

LOD and LOQ - Serial dilutions of a sample solution were made with methanol and analyzed by the HPLC method. The LOD and LOQ were obtained as the ratio of the signal to noise ratio equal to 3 and 10, respectively.

Determination of solvent for extraction - To optimize the solvent for extraction, *A. heterophyllus* heartwood powder (1 g) was separately extracted with methanol, ethyl acetate, chloroform and hexane (250 mL) under reflux conditions for 1 h. The extracts were then filtered and the solvents were evaporated under reduced pressure. The dried extracts (5 mg) were dissolved in methanol, and volume adjusted to 10 mL, and subjected to HPLC analysis. This experiment was performed in triplicate.

Statistical analysis – Data are presented as mean values ± SD. The data were analyzed using a one-way analysis of variance (ANOVA) for multiple comparisons, followed by Tukey's HSD post hoc test that denoted the presence of a statistically significant difference and was considered to be significant at the $P < 0.05$ level.

Results and discussion

A reversed-phase gradient HPLC system was established for the simultaneous quantitative determination of three bioactive flavonoids: artocarpanone, artocarpin and cycloartocarpin in extracts from *A. heterpohyllus* heartwoods. These three flavonoids showed a high UV absorption at 285 nm therefore this wavelength was used for their quantitative determination. Mixtures of methanol and water were examined as the mobile phases, with different ratios as well as a gradient elution system were optimized. The most suitable gradient elution system was 0-8 min, 60:40; 8-27 min, 80:20; 27-35 min, 60:40, v/v. All three flavonoids were eluted within 35 min with a satisfactory resolution (Fig. 2.). Artocarpin was detected as the major flavonoid in the ethyl acetate extract, while artocarpanone and cycloartocarpin were minor components. This HPLC method is relatively simple and also slightly

faster than a previous HPLC method for determination of artocarpanone in a *A. heterophyllus* heartwood extract that has been described by Zheng et al. (2009).[14]

The HPLC method was validated by means of determination of the important parameters, including linearity, accuracy, precision, specificity, LOD and LOQ. Linearity was evaluated using the standard samples over five calibration points (6.25-100 µg/mL) with six measurements for each calibration points. Three calibration curves were obtained by plotting the peak areas against concentrations. Artocarpanone, artocarpin and cycloartocarpin all exhibited an excellent linearity with the coefficient of determinations (R^2) of 0.9997, 0.9998 and 0.9995, respectively (Table 1). The results of the LOD and LOQ indicated that this HPLC method was sensitive for determination of these flavonoids in the heartwood extract, even though at a very low concentration (Table 1). The specificity of the analytical method was determined using the UV absorption spectra at three points of each peak. When compared with authentic samples, the results showed that all peaks were homogenous without any impurity. The accuracy of the analytical method was studied by the spiking technique. The percentage recoveries in the ranges of 98-104% were obtained for all analytes (Table 2). This result indicated that the established HPLC method possessed a good accuracy. The precision of the HPLC method was evaluated by assessing the RSD values of intraday and interday analysis. Intra-day precision was performed using six injections in the same day, and the RSD values for all three flavonoids were less than 1% (Table 3). Analysis of three independently prepared samples on three different days estimated the inter-day precision. The RSD values for all three flavonoids were less than 2% (Table 3). These results indicated that the

HPLC method enabled the quantitative determinations of artocarpanone, artocarpin and cycloartocarpin in *A. heterophyllus* heartwood extracts with a high degree of precision.

Four solvents with different polarities were used to decide the best for extraction and sample preparation included hexane, chloroform, ethyl acetate and methanol. Among the three flavonoids, artocarpanone was the most polar compound, and was therefore sparingly extracted with hexane. An increased polarity of the solvent used for extraction resulted in an increase of the flavonoid content of the extracts. Although, methanol gave the highest yield of the crude extract, but the lowest contents of these three flavonoids due to its nonselective extractability (Fig. 3.). The most suitable solvent for extraction of all three flavonoids should have partial polarity. Although both ethyl acetate and chloroform produced extracts with higher levels of artocarpin and cycloartocarpin than the other two solvents, only ethyl acetate gave a high amount of artocapanone (Table 4). Therefore, ethyl acetate was considered to be the most suitable solvent for sample preparation of the flavonoid extract from *A. heterophyllus* heartwoods and was used for quantitative HPLC analysis.

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Table and Figure legends

Table 1. HPLC calibration data for artocarpanone, artocarpin, and cycloartocarpin

Table 2. Recovery data for the three flavonoids spiked into *A. heterophyllus* heartwood extracts

Table 3. Intraday and interday precision data for the quantitative determination of the three flavonoids

Table 4. Content of the three flavonoids in different solvents for extracting *A. heterophyllus* heartwood

Fig. 1. Chemical structures of artocarpanone (**1**), artocarpin (**2**), and cycloartocarpin (**3**)

Fig. 2. HPLC chromatograms of (A) authentic compounds and (B) heartwood extract of *A. heterophyllus.* (**1**: Artocarpanone, **2**: Artocarpin, **3**: Cycloartocarpin)

Fig. 3. HPLC chromatograms of hexane, chloroform, ethyl acetate, and methanol extracts from of *A. heterophyllus* heartwoods.

Table 1.

Compounds	Linear range (μg/mL)	t_R (min)	Equation[a]	Linearity (R^2)	LOD (μg/mL)	LOQ (μg/mL)
Artocarpanone	6.25-100	6.7	$Y = 52290X - 35882$	0.9997	0.06	0.2
Artocarpin	6.25-100	17.8	$Y = 47287X - 44018$	0.9998	0.04	0.2
Cycloartocarpin	6.25-100	23.1	$Y = 25630X - 37434$	0.9995	0.2	0.4

[a] $Y = aX + b$, where Y is a peak area and X is the concentration of the analyzed material

Table 2.

Compounds	Spiked level (µg/mL)	% Recovery (Mean ± SD)
Artocarpanone	100	101.9 ± 0.83
	25	98.2 ± 1.10
	6.25	103.4 ± 0.30
Artocarpin	100	103.5 ± 0.75
	25	101.7 ± 0.20
	6.25	101.6 ± 0.74
Cycloartocarpin	100	102.1 ± 0.63
	25	103.8 ± 0.73
	6.25	102.4 ± 0.55

Table 3.

Compounds	RSD (%)	
	intra-day (*n*=6)	inter-day (*n*=3)
Artocarpanone	0.76	1.23
Artocarpin	0.78	1.49
Cycloartocarpin	0.64	1.27

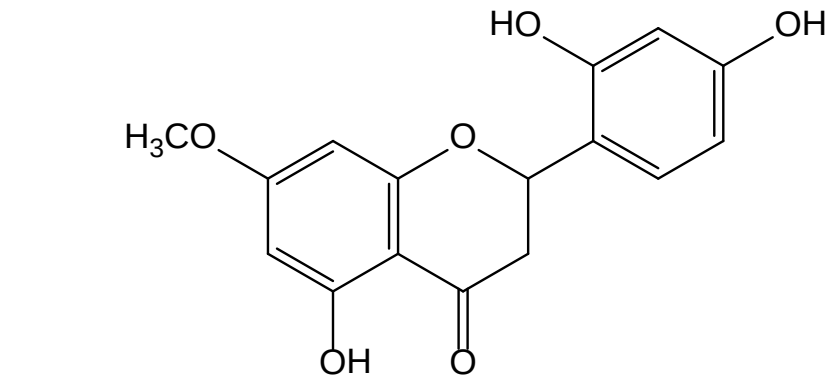
RSD = Relative standard deviation

Table 4.

Solvents	Yield of dried extracts (% w/w; mean ± SD)	Content (% w/w; mean ± SD)		
		Artocarpanone	Artocarpin	Cycloartocarpin
Hexane	2.2 ± 0.07*	n.a.	2.84 ± 0.16*	2.62 ± 0.02*
Chloroform	5.4 ± 0.15*	1.03 ± 0.03*	21.72 ± 0.57	5.51 ± 0.12
Ethyl acetate	7.2 ± 0.20	4.95 ± 0.01	21.84 ± 0.60	5.75 ± 0.20
Methanol	25.6 ± 1.47*	1.09 ± 0.05*	3.01 ± 0.10*	1.09 ± 0.03*

* Significant difference ($p < 0.05$) when compared with ethyl acetate in the same column. n.a. = not analyzed due to it being lower than the limit of quantification.

Fig. 1.



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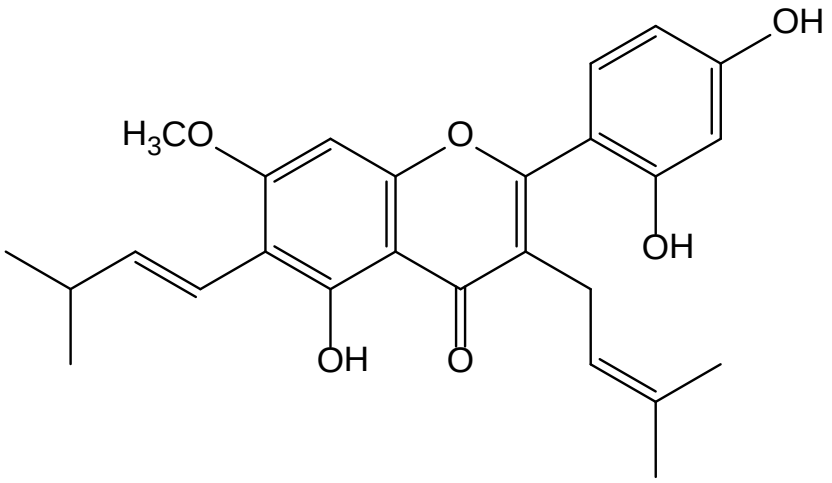

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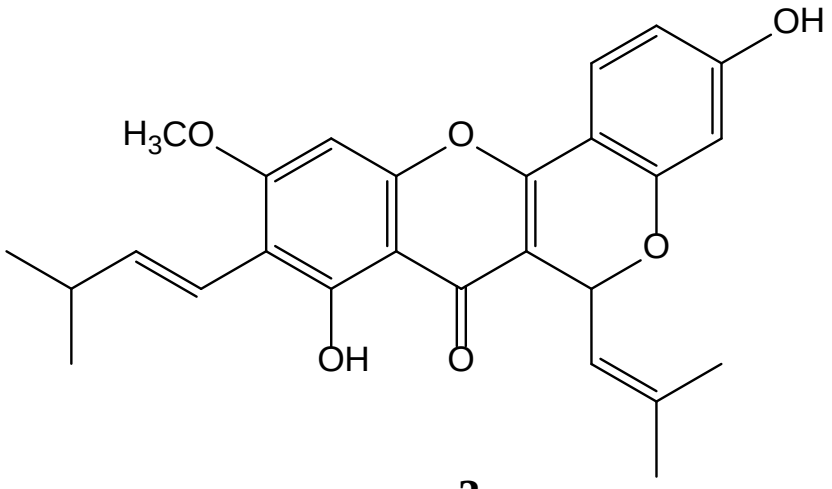

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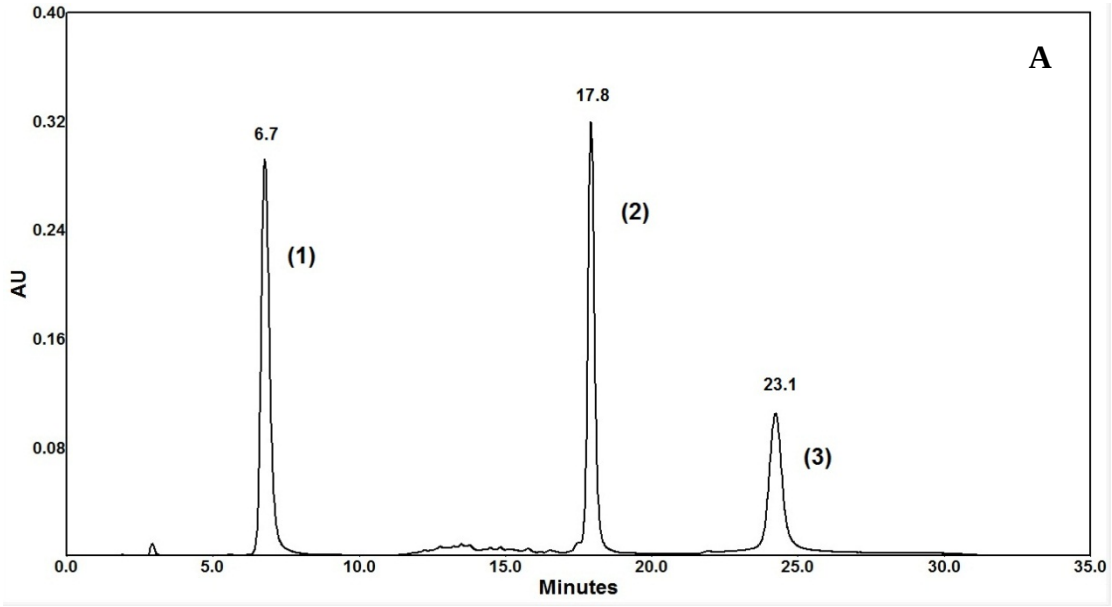
Fig. 2.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Minutes

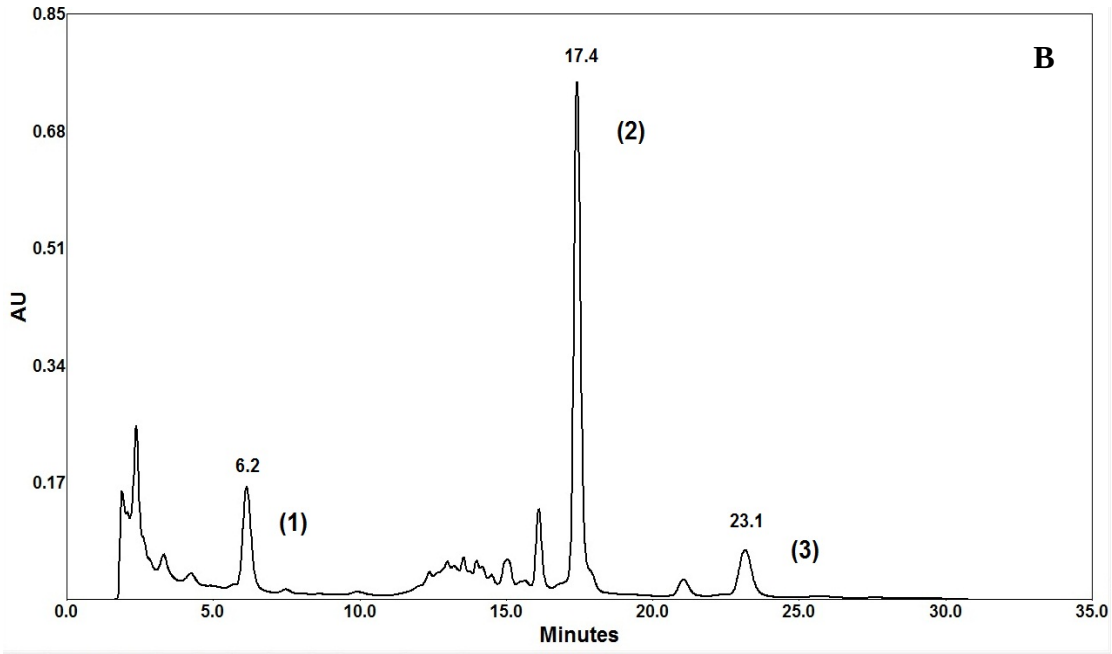
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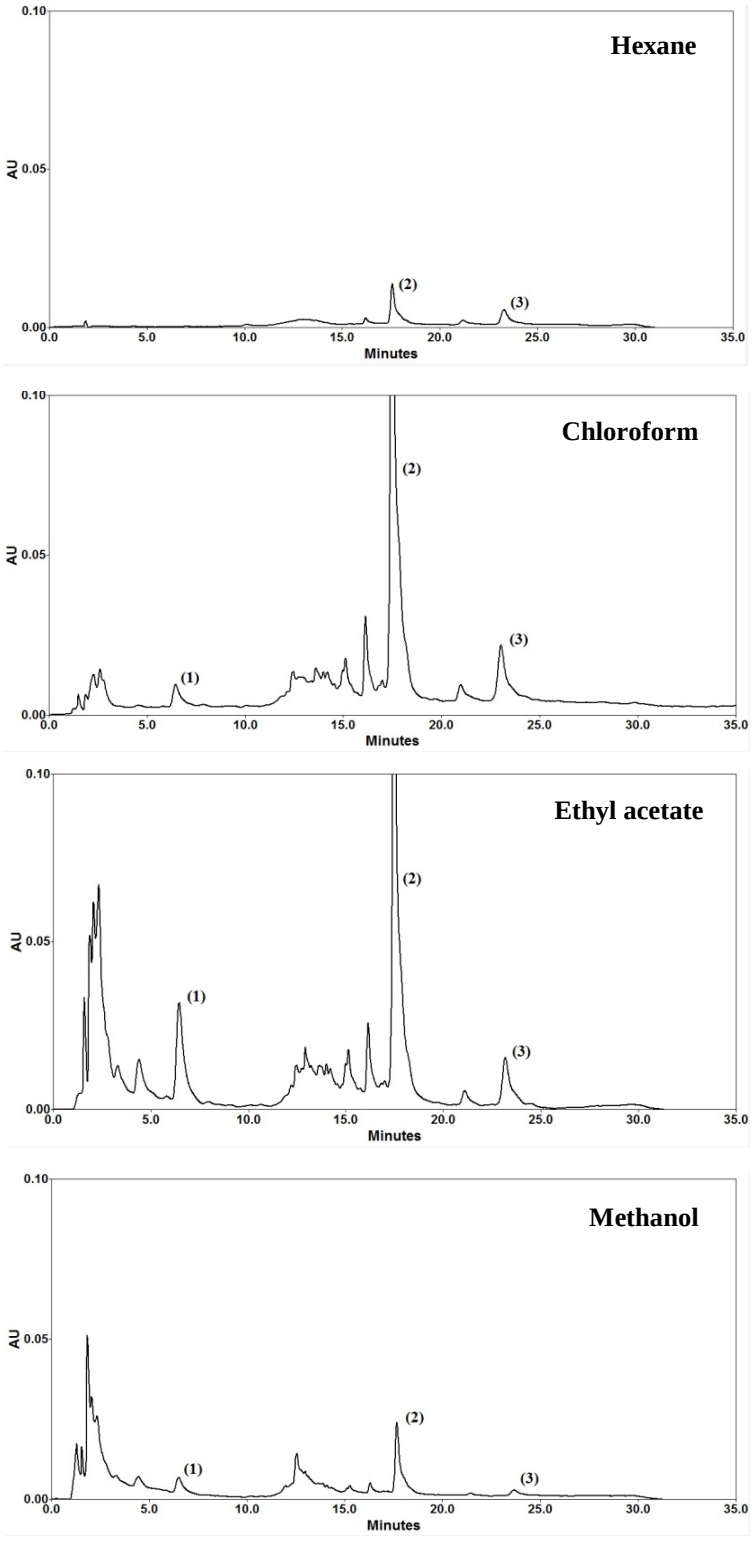
Fig. 3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Proceeding 1

Antibacterial activities of some combinations of cyanomaclurin and artocarpin isolated from *Artocarpus heterophyllus* heartwoods

(Manuscript submit to *the 3rd Current Drug development International Conference, May 1-3, 2014, Pavilion Queen's Bay, Krabi, Thailand*)

CDD 2014
PROCEEDINGS

Antibacterial activities of some combination of cyanomaclurin and artocarpin isolated from *Artocarpus heterophylus* heartwoods

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Abstract The antibacterial activity of combination of artocarpin and cyanomaclurin isolated from *Artocarpus heterophylus* heartwoods were evaluated against *Streptococcus pyogenes* and *Staphylococcus epidermidis* using broth microdilution methods. The mixture of compounds in several ratios tended to increase antibacterial activities of cyanomaclurin.

Introduction

Artocarpus heterophylus Lam. (Moraceae) heartwoods have been used in traditional medicine in several countries. The plant is known to produce a variety of flavonoids [1]. Flavonoids are ubiquitous compound that occurred widely in the plant. It has been reported to possess many pharmacological activities, including antibacterial [2]. Antibiotic resistance has become a great problem for infectious treatment. Combination of antibiotic has been applied to broaden the spectrum of antibiotic as well as to obtain synergistic effect. Therefore, this work aims to investigate possible synergistic effect of the two flavonoid compounds, cyanomaclurin and artocarpin.

Materials and methods

1) Plant material: A. heterophylus heartwoods were collected from the Hat Yai District, Songkhla Province, Thailand, in June 2012. The plant was dried at 50^0C for 24 h in a hot air oven, and reduced to powder using a grinder, and the powders were passed through sieve No. 45.

2) Bioassay guided isolation: Twenty five grams of ethyl acetate crude extract was fractioned by a vacuum liquid chromatography using a step gradient elution starting from hexane, ethyl acetate and methanol. The active fraction 9 was further isolated by silica gel column eluted with a mixture of ethyl acetate and hexane (40:60). The antibacterial fraction was further purified on a silica gel column using a mixture of chloroform and methanol to obtain **cyanomaclurin** (104 mg). Meanwhile, eight hundred gram of active fraction 3 was further fractioned on a silica gel column eluted with a mixture of ethyl acetate and hexane (80:20), and then the active fraction was further purified on a Sephadex LH-20 column to obtain **artocarpin** (105 mg).

3) Antibacterial activity of a combination of two flavonoids: Two flavonoids, cyanomaclurin and artocarpin were combined in the ratios of 3:1, 2:1, 1:1, 1:2, and 1:3, respectively. Antibacterial activity against *S. pyogenes* and *S. epidermidis* was evaluated by broth microdilution [3]. Interaction between two compounds in combination was monitored by calculating fractional inhibitory concentration (FIC) using the following formula:

$$FIC = \frac{MIC\ of\ compound\ in\ combintaion}{MIC\ of\ compound\ alone}$$

FIC<0.5 (synergy), 0.5≤FIC≤1 (addition), indifference (1<FIC≤4), antagonist (FIC>4) [4].

Results and discussion

The results of this study are shown in table 1. Artocarpin exhibited strong antibacterial activities against *S. pyogenes* and *S. epidermidis* with MICs of 1.9 µg/mL, while cyanomaclurin showed intermediate antibacterial activities against both of bacteria with MICs of 15.6 µg/mL. In all combinations of cyanomaclurin and artocarpin, artocarpin gave a synergistic effect to cyanomaclurin against both of tested bacteria with FICs of 0.25. In contrast, cyanomaclurin gave only an additional effect to artocarpin against both of tested bacteria at the combination ratios (cyanomaclurin:artocarpin) of 1:2 and 1:3, with FICs of 1. The combinations containing artocarpin less than 50% showed indifference effect. No antagonist effect was observed in any combinations of these two flavonoids. These results suggested that combination of cyanomalurin and artocarpin at the ratio 1:2 that gave additional antibacterial effect may be an alternative source of pure artocarpin.

Table 1 Fractional inhibitory concentration (FIC) of a combination of two compounds against *S. pyogenes* and *S. epidermidis*.

	S. pyogenes				*S. epidermidis*			
	MIC_a	**MIC_c**	**FIC**	**Type of interaction**	**MIC_a**	**MIC_c**	**FIC**	**Type of interaction**
Cyanomaclurin-Artocarpin (3:1)								
Cyanomaclurin	15.6	3.9	0.25	Synergy	15.6	3.9	0.25	Synergy
Artocarpin	1.9	3.9	2.05	Indifference	1.9	3.9	2.05	Indifference
Cyanomaclurin-Artocarpin (2:1)								
Cyanomaclurin	15.6	3.9	0.25	Synergy	15.6	3.9	0.25	Synergy
Artocarpin	1.9	3.9	2.05	Indifference	1.9	3.9	2.05	Indifference
Cyanomaclurin-Artocarpin (1:1)								
Cyanomaclurin	15.6	1.9	0.25	Synergy	15.6	3.9	0.25	Synergy
Artocarpin	1.9	1.9	1	Addition	1.9	3.9	2.05	Indifference
Cyanomaclurin-Artocarpin (1:2)								
Cyanomaclurin	15.6	1.9	0.25	Synergy	15.6	1.9	0.25	Synergy
Artocarpin	1.9	1.9	1	Addition	1.9	1.9	1	Addition
Cyanomaclurin-Artocarpin (1:3)								
Cyanomaclurin	15.6	1.9	0.25	Synergy	15.6	1.9	0.25	Synergy
Artocarpin	1.9	1.9	1	Addition	1.9	1.9	1	Addition

MIC_a : MIC of one compound alone
MIC_c : MIC of combination of two flavonoids

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2. The Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

List of Publications and Proceeding

Publication

1. **Septama, AW**., Panichayupakaranant, P. 2016. Synergistic effect of artocarpin on antibacterial activity of some antibiotics against methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. *Pharmaceutical Biology*.
   DOI: 10.3109/13880209.2015.1072566

2. **Septama, AW**., Panichayupakaranant, P. 2015. Antibacterial assay-guided isolation of active compounds from *Artocarpus heterophyllus* heartwoods. *Pharmaceutical Biology* 53(11): 1608-1613.

3. Harun, NH., **Septama, AW**., Jantan, I. 2015. Immunomodulatory effects of selected Malaysian plants on the CD18/11a expression and phagocytosis activities of leukocytes. *Asian Pacific Journal of Tropical Biomedicine* 5(1): 48-53.

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Proceeding

1. **Septama, AW.,** Panichayupakaranant, P. 2014. Antibacterial activities of some combinations of cyanomaclurin and artocarpin isolated from *Artocarpus heterophyllus* heartwoods. The 3rd Current Drug development International Conference, May 1-3, 2014, Pavilion Queen's Bay, Krabi, Thailand.