

# Antibacterial activity of artocarpalone isolated from *Artocarpus heterophyllus* heartwoods against diarrheal pathogens and its mechanism of action on membrane permeability

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## ABSTRACT

*Artocarpus heterophyllus* has been used as ingredient of folk medicine. Artocarpalone isolated from the heartwood of *A. heterophyllus* exhibited an antibacterial activity. The present study was undertaken to investigate its activity against diarrheal pathogenic bacteria including *Escherichia coli*, *Vibrio cholera*, *Shigella sonnei*, *Salmonella typhimurium*, and *S. typhi*. The effect of this compound on membrane permeability was also evaluated. Broth microdilution was used to determine antibacterial activity. Time-kill assay was selected to confirm any bactericidal activity. Bacteriolysis assay and loss of 260 nm absorbing material were used to assess the alteration on membrane cell. Artocarpalone displayed strong antibacterial activity against *E. coli* with MIC and MBC value of 3.9 and 7.8 µg/mL, respectively. Time-kill curve also confirmed the bactericidal activity of this compound. Artocarpalone only showed moderate antibacterial activity against *V. cholera* and did not perform any activity against *S. typhimurium*, and *S. typhi*. Supra-MIC (4 × MIC) of artocarpalone was able to decrease membrane permeability of *E. coli* after 24 h incubation and lead cell lysis. These results indicated that artocarpalone has potency as antibacterial against *E. coli* by altering membrane cell.

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## INTRODUCTION

Diarrhea is a still a major public health problem which lead to millions death worldwide. It has been reported that up to 80% of mortality occur in Africa and South Asia countries (Ibrahim and Sarhan, 2015). In microbiological aspect, the disease was caused by bacterial infection such as *Escherichia coli*, *Vibrio cholera*, and *Salmonella* sp (Huang *et al.*, 2002; Holland, 1990). *E. coli* is a Gram-negative pathogenic bacterium

which is a major cause of gastrointestinal tract infection (Ganapathy and Kapargam, 2016). Enterotoxigenic *E. coli* (ETEC) play an important role to cause diarrhea in human. Moreover, cholera toxin derived from *V. cholera* is also linked with the disease (Dubreuli, 2013). Antibiotic was commonly used as a principle management for diarrhea. However, the agent only kills suspected bacteria without inhibiting bacterial toxins. The use of antibiotics also leads to the increase of resistance toward antibiotics (Clatworthy, 2007). Thus, it needs to find the alternative strategy to overcome this problem.

Natural product is a potential source for the discovery of alternative medicine including antibacterial compounds. In case of diarrhea problems, world health organization (WHO) has encouraged several studies for the treatment and prevention of the disease using medicinal plants (WHO, 2005).

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It has been reported that several plants extract such as *Ocimum bacilissum*, *O. gratissimum*, *Hibiscus sabdarifa*, and *Adansania digitate* possessed antibacterial activities against diarrheal pathogens (Ibrahim and Sarhan, 2015; Djeusi *et al.*, 2013). *Artocarpus heterophyllus* Lam. has been used as a folk medicine in Asia for the treatment of ailments including bacterial infection (Saxena *et al.*, 2009). This plant was also known as an important source of flavonoid compounds (Arung *et al.*, 2006). Artocarpanone (Fig. 1) is an isolated flavonoid from the heartwoods of *A. heterophyllus*. This compound possessed many pharmacological properties such as anti-tyrosinase, antiinflammation, and antioxidant (Dej-Adisai *et al.*, 2014; Arung *et al.*, 2010). Previously, it has been reported that artocarpanone had antibacterial activity against several pathogenic bacteria (Septama and Panichayupakaranant, 2015). Little information is available about the effect of this compound against diarrheal pathogenic bacteria. Thus, the aims of this study are to determine the antibacterial activity of artocarpanone against several enteropathogenic bacteria as well as to investigate its mechanisms of action on membrane permeability.

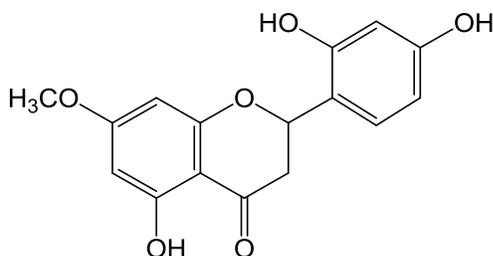


Fig. 1: Chemical structure of artocarpanone.

## MATERIALS AND METHODS

### Chemicals

Artocarpanone was isolated from the active fraction of *Artocarpus heterophyllus* heartwoods as previously described (Septama and Panichayupakaranant, 2015). Ampicillin was from Sigma (Sigma-Aldrich, UK). Brain heart infusion (BHI) and agar were from the Becton, Dickinson and Company (Franklin Lakes, NJ).

### Bacterial strains

*Escherichia coli* (ATCC 25922), *Vibrio cholera* (ATCC 14035), *Shigella sonnei* (ATCC 29930), *Salmonella typhimurium* (ATCC 29630), and *Salmonella typhi* (ATCC 19430) were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand.

### Determination of minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) was determined using the broth microdilution assay (NCCLS, 2008). The sample was initially dissolved in DMSO, and subsequently in media broth to reach a desired final concentration. Two fold dilutions were prepared in 96-well plate, 100  $\mu$ L of working solution was added

into well 1 of dilution series, each remaining plate were added 50  $\mu$ L broth. Further, 50  $\mu$ L solution from well 1 were transferred into well 2 and mix the solution. After that, 50  $\mu$ L solutions from well 2 were transferred into well 3, and this process was continued until the last final concentration was obtained. Well 12 as a growth control, receive no sample or antibacterial agent. Bacteria suspension was prepared in normal saline solution (NSS) adjusted to  $10^8$  CFU/mL with turbidity of 0.5 McFarland standards. The bacteria suspension was diluted (1:100) with NSS to contain approximately  $10^6$  CFU/mL, then 50  $\mu$ L bacteria suspension was added into each well and then incubated under the suitable conditions.

The MIC was considered as the lowest concentration of sample that prevented visible growth. Meanwhile, the MBC was detected as the lowest concentration, which no colonies of test bacteria formed on the cultivation medium

### Time kill assay

Time kill assay was used to observe bactericidal activity of compounds against tested bacteria. Bacterial suspension in normal saline ( $1 \times 10^6$  CFU/ml) was added into BHI broth containing compounds at  $\frac{1}{2} \times$  MIC, MIC,  $2 \times$  MIC and  $4 \times$  MIC to reach final concentration ( $5 \times 10^5$  CFU/mL) and incubated at 37°C. Next, 50  $\mu$ L of culture were collected at several time intervals (0, 1, 2, 4, 6, 8, 12 and 24 h), then diluted with 450  $\mu$ L of normal saline. Afterward, 10  $\mu$ L of bacterial suspension from each serial dilution was added on BHI agar. The surviving bacteria were then incubated. The numbers of viable colonies were then calculated after 24 h incubation (Hamoud *et al.*, 2014).

### Bacteriolysis

The modified method was used to detect the bacteriolysis activity (Limsuwan *et al.*, 2012). Briefly, bacterial suspension was prepared from the overnight culture on BHI agar. The cells were then supplemented with various concentrations of compound at  $\frac{1}{2} \times$  MIC, MIC,  $2 \times$  MIC and  $4 \times$  MIC. The final concentration was  $5 \times 10^7$  CFU/mL. The cell without treatment was used as control. Afterwards, the cells were incubated at 37°C. The optical density at 620 nm (OD620) was taken at several times interval (0, 1, 2, 4, 6, 8, 12 and 24 h). Bacteriolysis was indicated by decreasing of absorbance at 620 nm. The results were a percentage of OD620 at each time versus OD620 a 0 h. The test was done in triplicate.

### Loss of 260 nm absorbing material

The release of UV-absorbing material was carried out as described previously with slight modifications (Limsuwan *et al.*, 2012) Overnight cultures of tested bacteria were washed and suspended in normal saline. Various concentrations of the compound at  $\frac{1}{2} \times$  MIC, MIC,  $2 \times$  MIC and  $4 \times$  MIC were added into the cell to reach the final count approximately  $5 \times 10^7$  CFU/mL.

The cell without treatment was used as control. All the samples were then incubated at 37°C. The cells were removed at 0, 1, 2, 4, 6, 8, 12 and 24 h, then diluted with normal saline (1:100)

and filtered through a 0.2  $\mu\text{m}$  pore-size filter. The optical density at 260 nm was UV-Vis spectrophotometer. This assay was performed in triplicate.

### Statistical analysis

Data are presented as average value and standard deviation. Data were analyzed using ANOVA for multiple comparison, followed by Tukey's HSD post hoc test denoted the presence of a statistically significant difference.  $P < 0.01$  was considered as statistically significant.

## RESULTS AND DISCUSSION

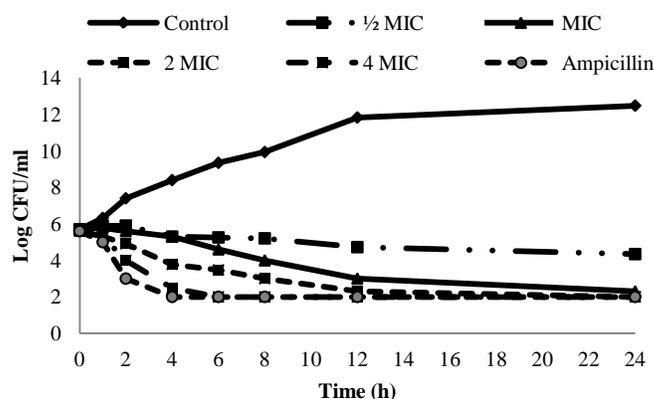
Antibacterial activity of artocarpone and antibiotic against diarrhea pathogenic bacteria were presented as MIC and MBC (Table 1). Artocarpone exhibited the strongest antibacterial activity against *E. coli* with MIC value of 3.9  $\mu\text{g/mL}$  and MBC value of 7.8  $\mu\text{g/mL}$ . This compound only had weak activity against *V. cholerae* and *S. sonnei* with MIC values of 62.5 and 250  $\mu\text{g/mL}$ , and MBCs of 62.5 and 250  $\mu\text{g/mL}$ , respectively. However, artocarpone had no effects against *S. typhi* and *S. typhimurium*. On the other hand, ampicillin also showed strong antibacterial activity against *E. coli* with MIC value of 0.9  $\mu\text{g/mL}$ , and MBC value of 1.95  $\mu\text{g/mL}$ . This reference antibiotic only produced moderate antibacterial activity against *V. cholerae*, *S. Sonnei*, *S. typhi* and *S. typhimurium* with MIC values of 62.5 -125  $\mu\text{g/mL}$ , and MBC values of 125-250  $\mu\text{g/mL}$ . The results revealed that *E. coli* was more sensitive to artocarpone, therefore this bacteria was selected for further experiments.

**Table 1:** MIC and MBC values of artocarpone and ampicillin.

	Artocarpone		Ampicillin	
	MIC ( $\mu\text{g/mL}$ )	MBC ( $\mu\text{g/mL}$ )	MIC ( $\mu\text{g/mL}$ )	MBC ( $\mu\text{g/mL}$ )
<i>E. coli</i>	3.9	7.8	0.9	1.95
<i>V. cholerae</i>	62.5	62.5	62.5	62.5
<i>S. sonnei</i>	250	250	62.5	62.5
<i>S. typhi</i>	500	>500	125	125
<i>S. typhimurium</i>	500	>500	125	125

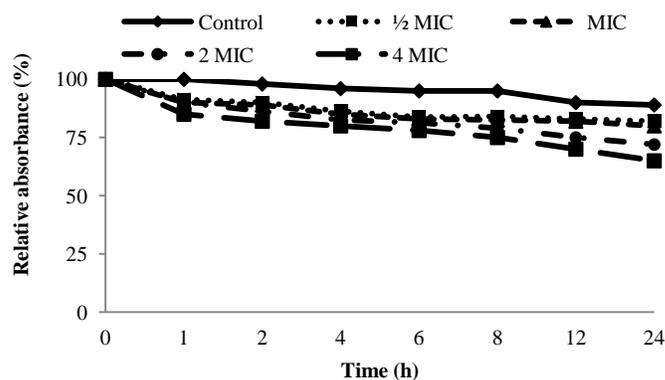
Time kill assay was performed to confirm bactericidal activity of artocarpone against *E. coli*. Four serial concentrations were used. The killing curve of tested bacteria after treatment with compounds was demonstrated in Fig. 2. It was observed that artocarpone at 4  $\times$  MIC, completely inhibited the bacterial growth within 6 h incubation, which is comparable to the activity of ampicillin showing growth inhibition of *E. coli* within 4 h. This indicated that artocarpone at supra-MIC (4  $\times$  MIC) had powerful antibacterial activity against *E. coli*. Artocarpone at 2  $\times$  MIC and MIC only inhibited the bacterial growth after 24 h incubation. It has been reported that artocarpone had satisfactory antibacterial activity against several bacteria including *E. coli* (Septama and Panichayupakaranant, 2015). This compound also

enhanced the antibacterial activity of norfloxacin by altering membrane permeability of MRSA (Septama *et al.*, 2017).



**Fig. 2:** Time-kill curves of artocarpone and ampicillin against *E. coli*.

It has been known the Gram-negative bacteria are surrounded by outer membrane which provides a selective barrier to protect the cell from harmful agents (Lambert, 2002). These bacteria were also difficult to control using commercial antibiotics due to the low permeability on their membrane. Cell membrane alteration is one of the mechanisms of action of flavonoid (Tsuchiya and Iinuma, 2000). Therefore, this study also focused on exploring the alteration of membrane permeability by artocarpone. According to bacteriolysis assay, the result showed that artocarpone at 4  $\times$  MIC and 2  $\times$  MIC generated bacteriolytic effect after 24 h (Fig.3). Compound at MIC and 1/2  $\times$  MIC had no effect on membrane permeability. The experiment was then confirmed by measuring release of absorbing materials as an indicator of cytoplasmic membrane leakage (Oonmetta-aree *et al.*, 2006). As shown in Fig. 4, after 24 h incubation, the absorbance of artocarpone at 4  $\times$  MIC was significantly higher than control. This result corresponded well with bacteriolytic activity. It indicated that artocarpone may cause membrane cell disruption and release of intracellular material form including nucleic acid as well as ion from bacterial to cell environment.



**Fig. 3:** Bacteriolytic activity of artocarpone against *E. coli*.

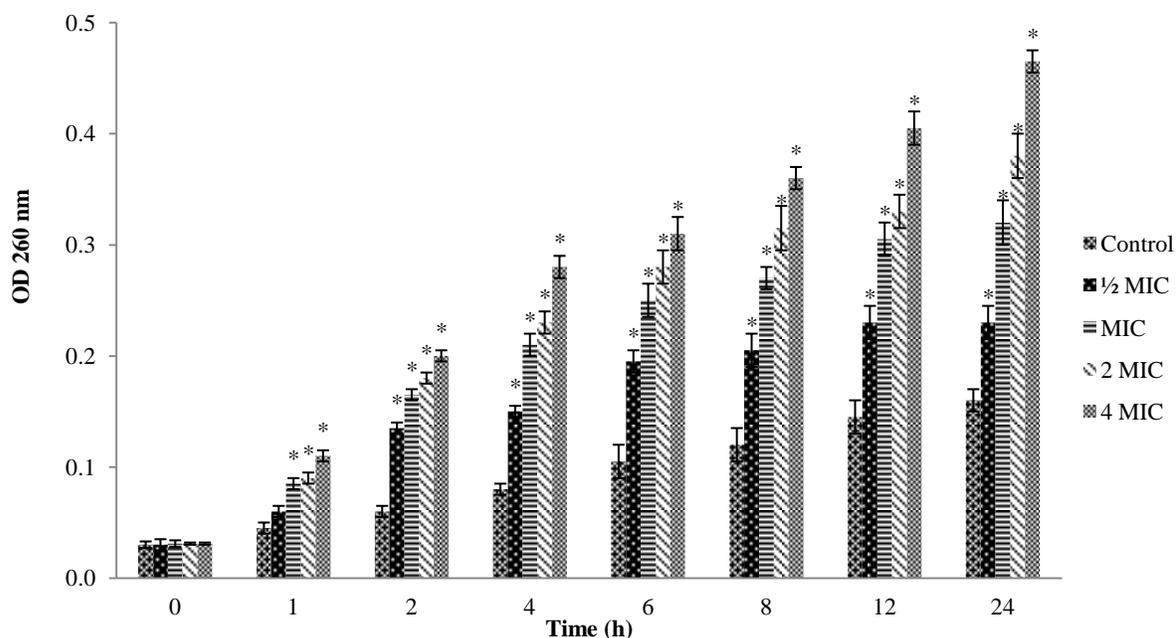


Fig. 4: Optical density (OD) 260 nm of *E. coli* treated with artocarpanone. \* Significant difference ( $P < 0.01$ ) compared to control.

Natural product has a promising prospect as a source of novel antibacterial agents which can be used to overcome resistant problem. Many isolated compounds including flavonoid had strong antibacterial activity. The mechanism of antibacterial action of isolated compound is still unclear. However, membrane cell is probably one of the sites of action (Cushnie and Lamb, 2011). It has been known that flavonoid may interact with lipophilic side of the Gram-negative bacteria. This interaction may reduce the membrane fluidity which causes cytoplasmic membrane damage (Hendrich, 2006). Some studies also suggested that the inhibition of synthesis of nucleic acid as well as energy metabolism as the target sites of flavonoid (Cushnie and Lamb, 2011). According to previous study, sophoraflavanone G isolated from *Sophora exigua* displayed an antibacterial activity against MRSA by decreasing membrane permeability (Mun *et al.*, 2014). Study by Cushnie and Lamb showed that galangin induced the cytoplasmic membrane damage in *Staphylococcus aureus* (Cushnie and Lamb, 2005). In addition, quercetin also performed strong antibacterial activity against *E. coli* by increasing inner membrane permeability and dissipation of the membrane potential (Simoes *et al.*, 2009).

## CONCLUSION

In conclusion, artocarpanone displayed an antibacterial activity against *E. coli* by disrupting membrane permeability. This finding implied that artocarpanone enabled to inhibit the enteropathogenic bacteria and provide the evidence that this compound has a potency as antibacterial agent. Nevertheless, further investigations are still needed to elucidate the mechanism

of action of artocarpanone towards diarrheal pathogenic bacteria including *E. coli*.

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