

Artocarpin isolated from *Artocarpus heterophyllus* heartwoods alters membrane permeability of *Streptococcus mutans*

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ABSTRACT

Artocarpin was successfully isolated from the heartwoods of *A. heterophyllus*. **Objectives:** The present study was conducted to determine the antimicrobial activity of artocarpin against cariogenic microbial including *Streptococcus mutans*, *Escherichia coli*, and *Candida albicans*. The activity of artocarpin on alteration of the cell membrane was also investigated. **Methods:** Standard microdilution method was applied to evaluate its antibacterial activity against tested microorganisms. Time-killing test was then performed to verify its antimicrobial effect. In order to know its effect on membrane permeability, bacteriolysis assay was selected, followed by determining loss of 260 nm absorbing material using UV-Vis spectrophotometer. **Results:** Artocarpin exhibited strong antibacterial against *S. mutans* with MIC and MBC values of 1.95 and 3.91 µg/mL, respectively. Time-killing curve showed that artocarpin at 2 × MIC (3.9 µg/mL) and 4 × MIC (7.8 µg/mL) inhibited bacterial growth after 4 h incubation which was comparable to ampicillin as a positive control. Artocarpin 2 × MIC (3.9 µg/mL) and 4 × MIC (7.8 µg/mL) altered membrane permeability of *S. mutans* after 24 h exposure and led to release 260 nm absorbing material as an indicator of membrane cell damage. **Conclusion:** This finding indicated that artocarpin has high potency as antibacterial compound against *S. mutans* by reducing cell membrane permeability.

INTRODUCTION

Dental caries along with periodontal disease are still a major oral infectious problem worldwide particularly in developed countries (Wang *et al.*, 2017). Many microorganisms, such as *Streptococcus mutans*, *Escherichia coli*, and *Candida albicans* have been reported to contribute to the development of oral health problem (Panyo *et al.*, 2016). Amongst those microorganisms, *S. mutans* is been known as the most cariogenic bacteria which usually cause oral diseases. This bacterium is also the major pathogen associated with the formation of dental plaque biofilms (Choi *et al.*, 2016). Many chemicals and synthetic drugs have been used for the treatment and prevention of the diseases. Chlorhexidine, a broad spectrum antibacterial and antifungal, has been clinically used to reduce the growth of streptococci; however, it is associated

with some limitations including mouth irritation and unpleasant taste (Ahrari *et al.*, 2015). In addition, the use of antibiotic has also linked with developing of drug resistance (Ghannoum and Rice, 1999). Thus, a search for alternative source of antimicrobial for oral pathogens has been a major concern.

Natural product has been an important source of alternative medicine for the treatment of infectious diseases. Some medicinal plants, such as *Robinia pseudoacacia*, *Emblia officinalis*, *Ixora megalophylla*, and *Thymus vulgaris* have shown a significant antibacterial effect against cariogenic bacteria, including *S. mutans* (Patra *et al.*, 2015; Jain *et al.*, 2015; Panyo *et al.*, 2016; Schott *et al.*, 2017). *Artocarpus heterophyllus* Lam. has been known as one of the ingredients of ancient medicine in some Asian countries (Saxena *et al.*, 2009). It is well known as a source of some secondary metabolites, including flavonoid (Arung *et al.*, 2006). Artocarpin (Fig. 1) is a major compound in the heartwoods of *A. heterophyllus* (Septama and Panichayupakaranant, 2016a). This compound has demonstrated various biological activities such as anticancer, antioxidant and anticarcinogenic (Sato *et*

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al., 1996; Yang *et al.*, 2010; Lee *et al.*, 2013). Previously, it has been found that artocarpin could enhance antibacterial activity of some antibiotics in synergistic effect against MRSA (Septama and Panichayupakaranant, 2016b). The study was undertaken to investigate the effect of artocarpin against oral pathogen and to determine its effect towards alteration of membrane cell wall.

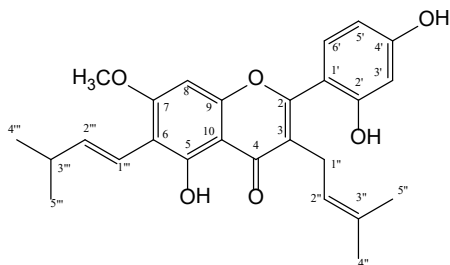


Fig. 1: Chemical structure of artocarpin.

MATERIALS AND METHODS

Chemicals

Artocarpin was isolated from ethyl acetate extract of *Artocarpus heterophyllus* heartwoods as previously describe (Septama and Panichayupakaranant, 2015). Ampicillin and clotrimazole were purchased from Sigma (Sigma-Aldrich, UK). Brain heart infusion (BHI), Sabouraud dextrose broth (SDB) and agar were obtained from the Becton, Dickinson, and Company (Franklin Lakes, NJ).

Bacterial strains

Streptococcus mutans (DMST 26095), *Escherichia coli* (ATCC 25922), and *Candida albicans* (TISTR 5779) were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand.

Determination of minimum inhibitory concentrations (MICs)

The microdilution method with slight modification was used to determine minimum inhibitory concentrations (MICs) (NCCLS, 2008). In brief, two-fold dilution of each sample in Brain heart infusion (BHI) (*S. mutans* and *E. coli*) or Sabouraud dextrose broth (SDA) (*C. albicans*) was prepared in 96-wells plate. The cell 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 colony-forming unit (CFU)/mL. This suspension was diluted with normal saline to contain 1×10^6 CFU/mL, and it was then added to each well. The final cell concentration was 5×10^5 CFU/mL. The plates were incubated at 37°C for 24 h. The MIC was considered to be the lowest concentration of the sample that produced suppression of visible growth. The lowest concentration of the sample that inhibits the growth of bacteria in the medium was reflected as the minimum bactericidal concentration (MBC).

Time-kill assay

Time-killing test was conducted to confirm any bactericidal effect of the sample against tested microbial. This assay was performed eight times (0, 1, 2, 4, 6, 8, 12 and 24 h). The microbial suspensions contained 1×10^6 colony-forming unit (CFU)/mL was added to a broth containing the mixture of

samples to reach the final cell concentration of 5×10^5 CFU/mL, then incubated at 37°C. Afterward, aliquots (50 μ L) of the cultures were removed at eight-time intervals of incubation, and ten-fold serial dilutions were prepared in normal saline. Then, 20 μ L of each dilution was cultured on Brain heart infusion (BHI) agar. The numbers of viable colonies were then calculated after 24 h incubation. Dimethyl sulfoxide (DMSO) was used as negative control. The lower limit quantification was 100 CFU/mL (Hamoud *et al.*, 2014).

Bacteriolysis

Bacteriolysis assay was carried out as described by Limsuwan (Limsuwan *et al.*, 2012). Briefly, a suspension of an overnight culture of bacteria was prepared in normal saline. Afterward, artocarpin at $\frac{1}{2} \times$ MIC, MIC, $2 \times$ MIC and $4 \times$ MIC, were mixed with the bacterial suspensions to obtain the final concentration of 5×10^7 colony-forming unit (CFU)/mL, incubated at 37°C. Dimethyl sulfoxide (DMSO) was used as a control. Ampicillin was used as positive control. The absorbance of the supernatant at 620 nm (OD₆₂₀) was quantified by a UV-Vis spectrophotometer. The measurement of supernatants was carried out eight times (0, 1, 2, 4, 6, 8, 12 and 24 h). Reducing of absorbance at 620 nm was considered as bacteriolysis of tested bacteria. The result was displayed by percentage relative absorbance.

Loss of 260 nm absorbing material

The concentrations of released 260 nm absorbing material were quantified using a UV-Vis spectrophotometer (Devi *et al.*, 2010). The bacterial suspension was prepared from overnight cultures of tested bacteria in normal saline. Artocarpin at $\frac{1}{2} \times$ MIC, MIC, $2 \times$ MIC and $4 \times$ MIC, were added to the suspension, incubated at 37°C. The final concentration of bacteria was 5×10^7 colony-forming unit (CFU)/mL. Dimethyl sulfoxide (DMSO) was used as a control. Ampicillin was used as positive control. Each measurement was performed eight times (0, 1, 2, 4, 6, 8, 12 and 24 h). The bacterial suspension was then diluted with normal saline (1:100) and filtered. The optical density at 260 nm was then quantified using UV-Vis spectrophotometer. The value 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. Data was displayed as mean and SD. One-way ANOVA was used for multiple comparisons between these groups, followed by Tukey's test to identify any significant difference at $P < 0.01$.

RESULTS

Minimum inhibitory concentrations (MICs)

Antimicrobial activity of artocarpin against tested microorganisms was expressed as MIC and MBC values. As shown in Table 1, artocarpin demonstrated strong antibacterial activity against *S. mutans* with MIC and MBC values of 1.95 and 3.91 μ g/mL, respectively. The activity of this compound was also comparable to ampicillin against *S. mutans* (Fig. 2). It indicated that Gram-positive bacteria, *S. mutans* was susceptible to artocarpin. This bacterium was then used as targeted bacteria

in order to know the mechanism of action of artocarpin on cell membrane permeability. In contrast, this compound only exhibited moderate activity against Gram-negative, *E. coli* and yeast, and *C. albicans* (MIC and MBC of 31.25 and 62.5 µg/mL).

Time-kill assay

Time-kill curves of *S. mutans* after exposure with

artocarpin are presented in Fig. 3. Artocarpin at 2 × MIC (3.9 µg/mL) and 4 × MIC (7.8 µg/mL) showed strong inhibition effect against *S. mutans* after 2 h. It was comparable to a standard drug, ampicillin as a positive control. This compound at MIC (1.95 µg/mL) enabled to inhibit the growth of *S. mutans* within 4 h exposure, while the sub-MIC (0.98 µg/mL) of artocarpin did not exhibit any inhibition activity after 24 h incubation.

Table 1: MIC and MBC values of artocarpin, ampicillin, and clotrimazole.

	Artocarpin		Ampicillin		Clotrimazole	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
<i>Streptococcus mutans</i>	1.95	3.91	0.5	0.5	nd	nd
<i>Escherichia coli</i>	31.25	62.5	0.9	1.95	nd	nd
<i>Candida albicans</i>	31.25	62.5	nd	nd	0.9	1.8

nd: not determined.

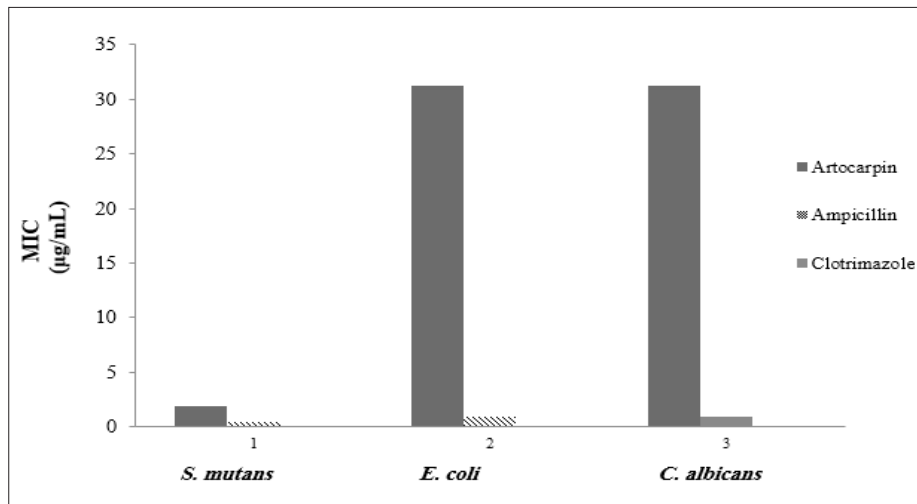


Fig. 2: Inhibitory of artocarpin, ampicillin, and clotrimazole against tested microbial.

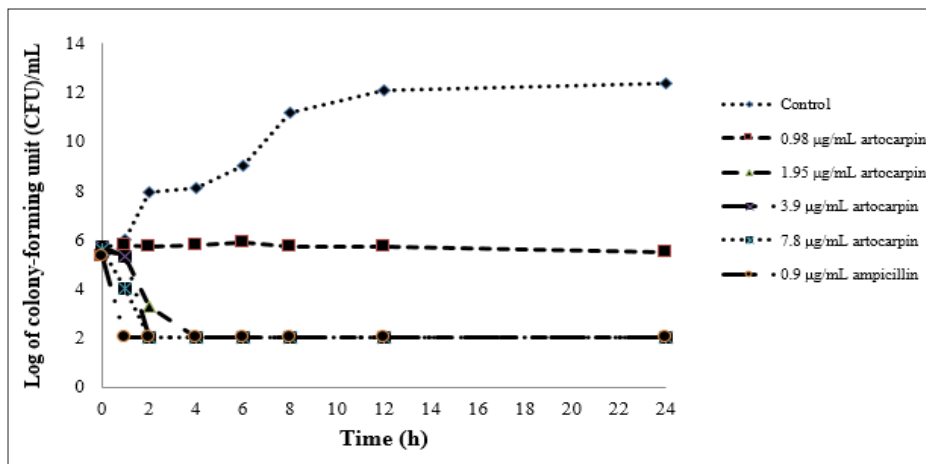


Fig. 3: Time-kill curves of artocarpin and ampicillin against *S. mutans*.

Bacteriolysis

As shown in Fig 4, exposure with artocarpin at 2 × MIC (3.9 µg/mL) and 4 × MIC (7.8 µg/mL) caused bacteriolysis effect

after 24 h incubation, which was similar to ampicillin as a positive control. Artocarpin at sub-MIC (0.98 µg/mL) slightly affected cell lysis over 24 h.

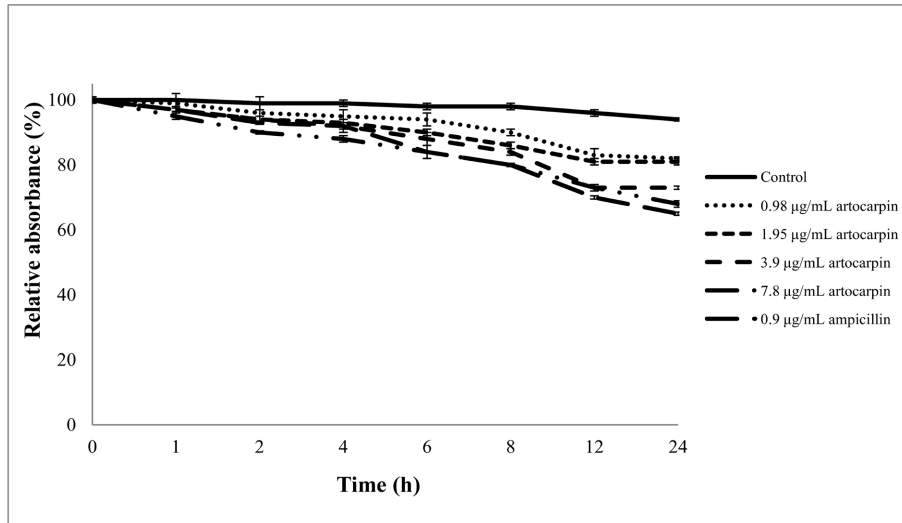


Fig. 4: The bacteriolytic activity of artocarpin and ampicillin against *S. mutans*.

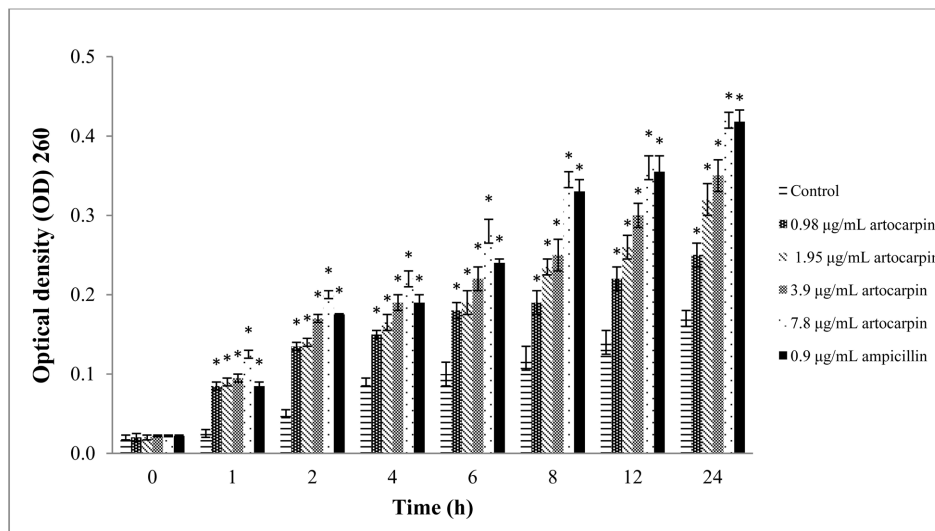


Fig. 5: Optical density (OD) 260 nm of *S. mutans* treated with artocarpin. *Significant difference ($P < 0.01$) compared to control.

Loss of 260 nm absorbing material

The release of intracellular material from *S. mutans* after treated with artocarpin was monitored as absorbance reading at 260 nm. As presented in Fig. 5, the absorbance of cells treated with artocarpin at $2 \times$ MIC (3.9 µg/mL) and $4 \times$ MIC (7.8 µg/mL) were significantly higher than normal cell after 24 h incubation ($P < 0.01$).

DISCUSSION

A high failure rate of antibacterial therapy for the treatment of infection caused by cariogenic pathogen has been reported (Sweeney *et al.*, 2004). An appealing approach to overcome this problem is searching potential candidates from medicinal plants which have minimal side effect and high activity. Many compounds isolated from nature have been shown their

satisfactory antibacterial activity. Our present study was conducted to evaluate the antibacterial activity of flavonoid isolated from *A. heterophyllum* namely artocarpin against cariogenic pathogen including *S. mutans*. On the basis of broth microdilution method, artocarpin had strong antibacterial activity against *S. mutans*. Bactericidal activity of this compound was then confirmed using time-kill assay in which artocarpin at supra-MIC enabled to kill *S. mutans* within 4 h incubation. This finding supports the outcomes of previous studies about the antibacterial activity of artocarpin. According to a previous study, artocarpin showed broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria. Moreover, this compound enhanced antibacterial activity of some antibiotics, such as tetracycline, ampicillin, and norfloxacin against MRSA with a synergistic effect when used in combination (Septama and Panichayupakaranant, 2016b).

Some of the bacterial species, including *S. mutans* are resistant to certain antibiotics due to their attributes, such as low permeability in their cell wall and cytoplasmic (Delcour, 2009). In certain condition, particularly in bacterial resistant, overexpression of efflux pump will reduce accumulation of antibiotics inside the cells (Davin-Regli *et al.*, 2008). Little information is available about the mechanism of antibacterial action of artocarpin. Assessment of its mechanism of action will probably afford starting point for finding and development of new drugs. Membrane permeability has been known as one of the antibacterial targets of the action. Disruption of membrane cell will allow penetration of antibacterial agents across through bacterial cell and occupy their target of action inside the cell. In this study, we then focused on alteration of membrane cell as a mode of action of the antibacterial compound. Bacteriolysis assay was subjected to determine the effect of artocarpin on membrane cell damage. The test was then continued to confirm the effect of artocarpin on cytoplasmic leakage by measuring the release of absorbing materials at 260 nm. It has been known that membrane integrity is the basis for the control cytoplasmic hemostasis and important for physiological activities in bacteria (Sanchez *et al.*, 2010). This result corresponds well with previous experiment, in which alteration of membrane cell will lead to the release of intracellular material including nucleic acid as an indicator of cell damage.

The finding of this study revealed that artocarpin had strong antibacterial activity against *S. mutans*. This compound also showed a significant effect in altering membrane permeability and finally led to cell lysis. It has been known that flavonoid compound possessed various pharmacological properties including antibacterial. However, the mechanisms of antibacterial actions of flavonoid are still unclear. Their antibacterial action probably involves multiple cellular targets (Kumar and Panday, 2013). The ability of flavonoid to interact with membrane cell as well as their lipophilicity may be also important factors of their biological activity (Tarahovsky *et al.*, 2014). It has been reported that lipophilic flavonoids are able to disturb membrane permeability. Based on previous work, flavonoid from *Sophora exigua*, named sophoraflavanone G possessed anti-MRSA by altering its cell membrane (Mun *et al.*, 2014). Naringenin along with some flavonoid including genistein and rutin contributed to altering membrane cell walls (Arora *et al.*, 2000). In addition, flavonoid galangin enabled to induce cytoplasmic membrane damage in *S. aureus* (Cushnie and Lamb, 2011). Disruption of membrane cell walls in hydrophobic and hydrophilic sites can be attributed to this effect which purposes that this flavonoid compound might decrease the permeability of outer layers of membranes. On the other hand, regarding the structure-activity relationship, it can be suggested that substitutions of an isoprenyl group on C-3 and 3-methyl-1-butenyl group on C-6 as well as a free hydroxyl group on C-2' are required for antibacterial activity artocarpin. According to a previous study, cudraflavone C as analogous of artocarpin which has 3 hydroxyl group on C-2', 5 and 7, also showed satisfactory antibacterial activity (Dej-adisai *et al.*, 2014). In addition, Osawa and colleagues reported that substitution of the hydroxyl group on the 7, 2' and 4' enhanced antibacterial activity of flavonoid against *S. mutans* and *S. sobrinus* (Osawa *et al.*, 1992).

CONCLUSION

It can be concluded that artocarpin reveal strong antibacterial effect against *S. mutans* by altering its membrane fluidity. These findings provide the important insight about the potency of flavonoid compound named artocarpin to overcome the cariogenic bacterial problem. Nevertheless, further studies are still required to justify the mechanism of antibacterial action of artocarpin.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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