Time-Kill Assay of 4-Hydroxypanduratin A Isolated from *Kaempferia Pandurata* Against Foodborne Pathogens

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ABSTRACT

Time–kill assay was performed for 4-hydroxypanduratin A that was isolated from *Kaempferia pandurata* rizhome against four important foodborne pathogens, namely *Bacillus cereus* ATCC 21772, *Bacillu subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29737, and *Proteus mirabilis* ATCC 21100. The methods have been investigated in term of Minimum Inhibitory Concentration (MIC) and killing time curve using methods of Clinical and Laboratory Standards Institute (CLSI) guidelines. The results showed that 4-hydroxypanduratin A rapid acting in killing bacteria as follow: *B. cereus* : 1×MIC for 4 h, *P. mirabilis*: 4×MIC for 0.5 h, meanwhile *B. subtilis* and *S. aureus* were 1×MIC for 2 h. In conclusion, 4-hydroxypanduratin A showed strong antimicrobial activity against four important foodborne pathogens.

Keywords: time-kill assay, 4-hydroxypanduratin A, foodborne pathogens

INTRODUCTION

Food is the main resource of nutrition for human so that safety and hygiene are very important for human consumption. One of the diseases is the infection caused by foodborne pathogens. It is estimated that each year has 2 million people deaths which has 1.5 million is children because of foodborne and waterborne diseases in the world (WHO, 2015). Up to now, microorganisms have become resistance to many common antibiotics due to decreasing the drug efficiencies. Research in recent years has focused on finding active molecules from natural sources which become leading compounds for the manufacture of various pharmaceutical products (Chen et al., 2012).

Natural products, especially from plants, have been considered interesting alternatives for treatment because they have a numerous variety of secondary metabolites with antimicrobial properties (Lu, Zhao, Wang, Chen, & Fu, 2007; Sgariglia, Soberon, Sampietro, Quiroga, & Vattuone, 2011). These secondary metabolites which are pharmacologically bioactive compounds include alkaloids (Saleem et al., 2010), flavanoids (Cushine & Lamb, 2011; Kuete, et al. 2008; Mandalari et al., 2007), tannins (Doss, Mubarack, & Dhanabalan, 2009), terpenoids (Sarıkahya et al., 2011; Ding et al., 2009), anthraquinones, and phenolic compounds (Yagi et al., 2012; Fang, Ye, Chen, & Zhao, 2008; Nawrot, Lesniewicz, Pienkowska, & Gozdzicka, 2007).

One of them is 4-hydroxypanduratin A, a chalcone derivative (flavonoid), was isolated from *Kaempferia pandurata* rizhome. This rizhome is known as ‘‘temu kunci’’ in Indonesia. Some studies indicated various biological activities of 4-hydroxypanduratin A, including antioxidative, antimutagenic, and antivirus activities (Trakoontivakorn, 2001; Shindo, Kato, Kinoshita, Kobayashi, & Koike, 2006; Cheenpracha, Karalai, Ponglimanont, Subhadhirasakul, & Tewtrakul, 2006). Previously, we have been reported that 4-hydroxypanduratin A showed good antibacterial activity against foodborne pathogens, namely *Bacillus cereus* ATCC 21772, *Bacillus subtilis* ATCC 6633, *Escherichia coli* O157:H7, *Listeria monocytogenes* ATCC 15313, *Proteous mirabilis* ATCC 21100, *Vibrio parahaemolyticus.* ATCC 17802, *Samonella typhi* ATCC 14028, *Klebsiella pneumoniae* ATTC13733 and *Staphylococcus aureus* ATCC 29737 (Marliyana, Rukayadi, Ismail, Mujahidin, & Syah, 2015).

Here in, this study is aimed to continue our investigation to find bactericidal properties with time-kill assay. This assay was done against *B. cereus, B. Subtilis, S. aureus,* and *P. mirabilis*. This study is part of an advanced

research to find new bioactive natural products from traditional Indonesian medicine.

EXPERIMENTAL SECTION

Materials

Rhizomes of *K. pandurata* were purchased from the herbal shop 'Babah Kuya', Pasar Baru, Bandung, Indonesia, and were deposited at the Laboratory of Natural Product, Institut Teknologi, Bandung. Materials for isolation were silica gel 60 (Merck, 1.07733.1000), thin layer chromatography (TLC) silica gel 60 F254 (Merck (1.05554.0001), and silica gel 60 G (Merck, 1.07731.1000). The solvents used in this study were obtained commercially and were used further purification. There were n-hexane, acetone, ethyl acetate, methanol, and chloroform ($CHCl_3$, E. Merck). Reagent for antibacterial activity was DMSO (dimethyl sulfoxide), NaCl (p.a), and chlorhexidine (E. Merck).

Bacterial strain

The antibacterial activity of isolated compound of *K. pandurata* rizhome was evaluated against four bacterial species. They were *Staphylococcus aureus* ATCC 29737, *Proteous mirabilis* ATCC 21100, *Bacillus subtilis* ATCC 6633, and *Bacillus cereus* ATCC 21772. All of them were obtained from the American Type Culture Collection (Rockville, MD, USA). All the bacteria were grown in Muller Hinton Broth (Difco Becton Dickincon, Sparks, MD, USA) at 37 ^oC and maintained on Muller Hinton Agar (Difco Becton Dickincon, Sparks, MD, USA). The experiments were performed with two replications and the results were expressed as average values

Instrumentation

Isolation and purification of compounds were used vacuum liquid and radial chromatography. Identification of the pure compounds were determined based on spectroscopic analysis covering NMR (^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), HMBC, HSQC) Agilent DD2. The time-kill assay used autoclave, laminar, orbital shaker at 200 rpm, and incubator Shimadzu at 37^oC.

Compound isolation and identification

The white powder of 4-Hydroxypanduratin A was isolated and identified from dried rizhome of *K. pandurata.* Briefly, *K. pandurata* dried rizhome (2.0 Kg) was powdered and extracted in maceration apparatus using acetone as solvent for 3 × 24 h at room temperature. The extract was then concentrated under reduced pressure, yielding 256 g of the crude extract. The crude extract (20 g) was fractionated using vacuum liquid chromatography with mix solvent *n*-hexane : ethyl acetate (10:0; 9:1; 8:2; 7:3; 1:1; 4:6; 0:10) to give 16 fraction (F1-F16). The purity test of isolated compound was analysed by TLC using different types of eluent. To know how the compound was pured, it can be seen from the single spot with various eluent.

The purification of fraction F13 was done using the radial chromatography (first run with *n*-hexane-$CHCl_3$ as eluent at 2:8; second run with *n*-hexane-EtOAc as eluent at 8:2) to obtain 4-hydroxypanduratin A (12.2 mg). This compound was identified by analysis NMR data and comparised with data reported previously.

Characteristic data of compound

The characteristic data of this compound was white amorphous powder, melting point (mp.) 158-160 ^oC. The **^{1}H NMR** Spectrum ($CDCl_3$, 500 MHz, CD_3OD), δ (ppm): 5.78 (5.88*) (2H, *s*, H-3, H-5); 4.73 (4.82*) (1H, *dd*, *J*=4.7; 11.6 Hz, H-1'); 2.62 (2.69*) (1H, *m*, H-2'); 1.76 (1.76*) (3H, *s*, H-3'-Me); 5.41 (5.41*) (1H, *br s*, H-4'); 1.98; 2.37 (1.95; 2.35*) (1H, *m*, H-5'); 3.41 (3.45*) (1H, *ddd*, J= 11.9; 10.6; 6.1 Hz, H-6'); 2.09; 2.24 (2.10; 2.26*) (1H, *m*, H-1''); 4.89 (4.92*) (1H, *t*, H-2''); 1.51 (1.51*) (3H, *s*, H-4''); 1.51 (1.51*) (3H, *s*, H-5''); 7.06-7.21 (7.04-7.19*) (5H, *m*, H-1'''- H6'''), and the **^{13}C NMR** Spectrum (125 MHz, CD_3OD), δ (ppm): 105.5 (106.2*) (C-1); 163.2 (164.8*) (C-2); 95.0 (95.9*) (C-3); 163.2 (164.8*) (C-4); 95.0 (95.9*) (C-5); 163.2 (164.8*) (C-6); 53.5 (54.5*) (C-1'); 42.3 (43.4*) (C-2'); 137.4 (137.9*) (C-3'); 22.7 (23.0*) (C-3'-Me); 120.8 (121.7*) (C-4'); 35.8 (36.8*) (C-5'); 37.0 (37.8*) (C-6'); 28.9 (29.5*) (C-1''); 124.3 (125.4*) (C-2''); 131.5 (131.7*) (C-3''); 17.8 (17.9*) (C-4''); 25.6 (25.9*) (C-5''); 147.3 (148.3*) (C-1'''); 127.0 (128.0*) (C-2''', C-6'''); 128.2 (128.9*) (C-3''', C-5'''); 125.4 (126.2*) (C-4'''); 206.6 (207.0*) (C=O). (Note: * the literature data: Tuchinda et al., 2002, CD_3COCD_3, 300 MHz (^{1}H); 75MHz (^{13}C)).

Determination of minimal inhibitory concentration (MIC)

The MIC was determined according to previous standardization as recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines. The 4-Hydroxypanduratin A was tested for antibacterial activity against four species of bacteria mentioned above in a 96-well microtiter plate using two fold standard broth microdilution method with an inoculum of approximately 10^6 CFU/mL. Shortly, a 100 µL of each compounds stock solution (1 % or 10000 µg/mL) was stirred and diluted two-folds with the test organism in 100 µL of Mueller Hinton broth (MHB, Difco, Sparks, MD, USA). The highest concentration of the compound was in column 12 of the microtiter plate, while column 3 contained the lowest concentration. The first column served as negative growth control (only MHB, no inoculum and antibacterial agent) while the second column is the positive growth control for all samples (only MHB and inoculum). The microtiter plate was then incubated aerobically at 37°C for 24 h. The MIC was defined as the lowest concentration of antibacterial agent that resulted in the complete inhibition of visible growth (Rukayadi, Lee, Han, Yong, & Hwang, 2009). In this assay, 500 µg/mL of chlorhexidine (CHX) was used as positive control.

Time-kill assay

The time-kill kinetics antibacterial study of the 4-hydroxypanduratin A of *K. pandurata* rizhome was carried out to assess the killing rate of the compound within a given contact time. In this study, the assay was done according to CLSI reference method, with slightly modification. The inoculum suspension of these bacteria was approximately 10^6 CFU/mL. The compound was diluted with the MHB medium containing inoculum to get final concentrations of 0 × MIC, 0.5 × MIC, 1 × MIC, 2 × MIC, and 4 × MIC for each bacterial species. Cultures (1 mL final volume) were incubated at 37°C with 200 rpm agitation. At pre-determined time points (0, 0.5, 1, 2, and 4 h), 100 µL aliquots were displaced and transferred to microcentrifuge tubes. The aliquot was serially diluted 1:100 in 0.9 % NaCl and plated onto NA. The bacterial subculture plates were incubated for 18 to 24 hours at 35–37 ^{o}C. After incubation, the agar plates were observed visually for the presence of growth. The colony forming units (CFUs) were enumerated, and the number of survivors at each exposure time was determined. The number of colonies formed on the plates after incubation at 37°C for 24 h was counted and the number of CFU/mL was calculated. Assays were carried out in duplicate. Time-kill curves were constructed by plotting the log CFU/mL versus time (Rukayadi et al., 2010).

RESULTS AND DISCUSSION

Nowadays, antibiotic resistance is one of the greatest threats to public health, so it is absolutely necessary to search new antibacterial agents. Antibiotic resistance can be intrinsic or acquired and can be transmitted within the same or different species of bacteria. The mechanisms can be divided into different categories: (i) modification of the active site of the target resulting in a reduction in the efficiency of binding of the antibiotic; (ii) direct destruction or modification of the antibiotic by enzymes produced by the bacterium; (iii) efflux or removal of antibiotic from the cell cause a reduced amount of antibiotic; or (iv) production of an alternative target that is resistant to inhibition by the antibiotic (metabolic by-pass) (Hemaiswarya, Kruthiventi, & Doble, 2008; Langeveld, Veldhuizen, & Burt, 2014).

Investigating the natural products from plants could be a fascinating alternative therapy (Langeveld et al., 2014; Miklasin´ska et al., 2016; Schäfer, & Wink, 2009). Medicinal plants have been unique sources of medicines and constituted the most common human use of biodiversity (Hiremath & Taranath, 2010). The traditional use of medicinal plants are used worldwide for thousands of years with more than 80% of the world's population depending on traditional medicines for various diseases, included they will be useful for the treatment of bacterial infections (Patel & Coogan, 2008). Previous studies have been implemented in different parts of the extract plant products for screening antibacterial activity (Olajuyigbe & Afolayan, 2012). Plants produce highly bioactive compounds that permit them to interact with other organisms in their environement. Many of these substances contribute to the resistance to diseases. Many researchers have evaluated and reported the bioactivity of plant extracts and the isolated constituents against the serious infectious organisms (Saleem et al., 2010; Konaté et al., 2012).

Table 1. Antibacterial activity of the 4-hydroxypanduratin A

Tested bacterial isolates	4-hydroxypanduratin A	Chlorhexidine (positive control)
	MIC (μg/mL)	MIC (μg/mL)
Staphylococcus aureus ATCC 29737	1.2	0.5
Proteous mirabilis ATCC 21100	2.3	0.5
Bacillus subtilis ATCC 6633	4.7	3.9
Bacillus cereus ATCC 21772	37.5	0.9

The concentrations of 4-hydroxypanduratin A and chlorhexidine were 600 μg/mL and 500 μg/mL, respectively

Isolation work on rhizomes extracts of *K .pandurata* has led to the identification of 4-hydroxypanduratin A (white powder). Chemical structure of this compound was showed in **Figure 1**. The MICs of 4-hydroxypanduratin A against four foodborne pathogens are obtained from our previous report (Marliyana et al., 2015). The results of minimum inhibitory concentration (MIC) of the compound against the susceptible bacteria are presented in **Table 1**. Our results showed that 4-hydroxypanduratin A was significant inhibitory properties against these tested bacteria. This compound also exhibited high activity against *S. aureus* ATCC 29737, *P. mirabilis* ATCC 21100, and *B. subtilis* ATCC 6633 (MIC range at 1.2 to 4.7 µg/mL), while the MIC was good activity for *B. cereus* ATCC 21772 (MIC at 37.5 µg/mL).

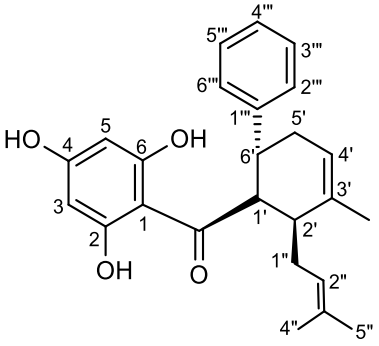

Figure 1. Chemical structure of 4-hydroxypanduratin A.

Some authors have advised criteria based on MIC values for the determination of the antimicrobial potential of compounds isolated from natural sources. The MIC values below 100 µg/mL have good antimicrobial activity (Ayres et al., 2008; Regasini et al., 2010); an MIC from 100 to 500 µg/mL represents moderate antimicrobial activity; an MIC from 500 to 1000 µg/mL represents weak activity; an MIC above 1000 µg/mL suggests that the substance is inactive. (Fonseca et al., 2013; Gibbons, 2008; Kuete, 2010). Based on these criteria, this compound was included an active compound. So we continued to investigate the Minimal Inhibitory Concentration (MIC) with time kill assay.

The time-kill assays have been widely used for in vitro investigations of new antimicrobial agents as these provide descriptive (qualitative) information on the pharmacodynamics of antimicrobial agents (Olajuyigbe & Afolayan, 2012). This assay unlike an MBC/MIC assay, allows the determination of the speed of cidal activity of the compound (Aiyegoro, Afolayan, & Okoh, 2009). In this study, the time–kill assay was done on the vegetative cells of *S. aureus* ATCC 29737, *P. mirabilis* ATCC 21100, *B. subtilis* ATCC 6633, and *B. cereus* ATCC 21772. Hence, the time-kill curve conducted to determine how long all bacteria are necessary for 4-hydroxypanduratin A to completely eliminate these pathogens. The curves were determined to assess the correlation between MIC and bactericidal activity of 4-hydroxypanduratin A at concentrations ranging from 0 fold MIC to 4 fold MIC.

The compound was rapidly bactericidal at 4 × MIC for *P. mirabilis* after 0.5 h incubation. The bactericidal endpoint for *B. cereus* was reached after 4 h at concentration of 1 × MIC of incubation. Meanwhile *B. subtilis* and *S. aureus* were completely eliminate after 2 h incubation at concentration of 1 × MIC.

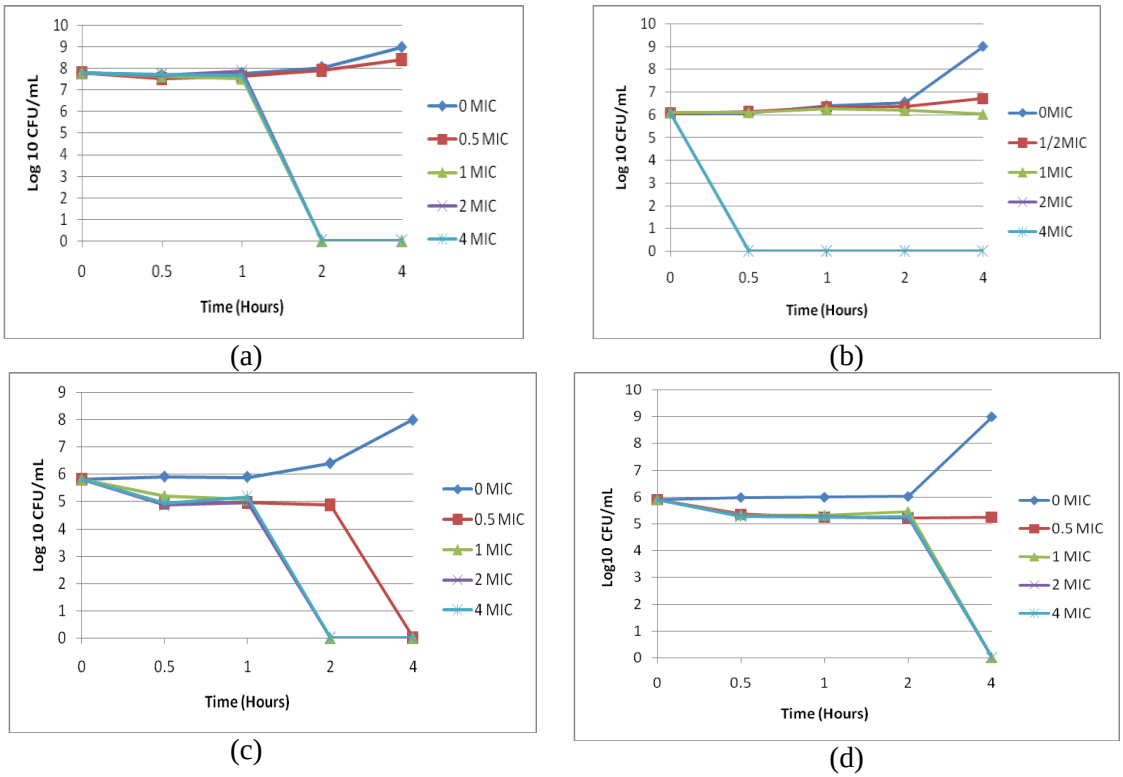

Figure 2. Time-kill curve of 4-hydroxypanduratin A against (a) *S. aureus*, (b) *P. mirabilis*, (c) *B. subtilis*, and (d) *B. cereus*.

The results of the time-kill assay are presented in **Figure 2**. In this study, our data showed that the response of the bacteria to the tested compound (4-hydroxypanduratin A) varied among the strains, concentration and time dependent. The differences in susceptibility may be due to the differences in cell wall composition and/or genetic content of their bacteria. The antibacterial activity is most likely due to the adsorption of compounds causing membrane disruption, subsequent leakage of cellular contents and cell death (Karaman et al., 2003; Negi, 2012).

Base on literature study, the 4-hydroxipaduratin A was a chalcone derivative of flavonoid. Some investigator have reported that polyphenols, such as tannins and flavonoids, are important antibacterial activity (Cushine & Lamb, 2011). The antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with bacterial cell wall while that of tannins may be related to their ability to inactivate microbial adhesions, enzymes and cell envelop proteins (Cowan, 1999). The time-kill assay was demonstrated that 4-hydroxypanduratin A showed good bactericidal activity for those bacteria and can be used to determine the dose of 4-hydroxypanduratin A for further clinical tests.

Finding new antibacterial agents is a matter of urgency as antibiotic resistance in infectious diseases has become a global issue (Gould, 2008). Although traditionally these agents have been obtained from microbes, in recent years much attention has been devoted to plants as an alternative source of the agents (Cushine & Lamb, 2011; Saleem et al., 2010). Among plant-derived compounds from traditional medicine, 4-hydroxypanduratin A isolated from the rhizome of *K. pandurata* has been proven as a promising new antibacterial agent. Thus, this paper reports for the first time the time-kill assay of 4-hydroxypanduratin A.

CONCLUSION

Phytochemical study integrated with biological assay has led to the isolation and identification of an active compound, namely 4-hydroxypanduratin A as the antibacterial activities, from the rhizome *K. pandurata*. The time-kill studies have provided valuable information on the rate, concentration and potential action of antibacterial agents in vitro. Further time-kill test showed that this compound was strong bactericidal agents against four tested foodborne pathogens: *S. aureus* ATCC 29737, *P. mirabilis* ATCC 21100, *B. subtilis* ATCC 6633, and *B. cereus* ATCC 21772. The results suggested that 4-

hydroxypanduratin A can be used as a potential candidate antibacterial agents and may serve as leads for developing new antimicrobial agents.

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