Research Article

Anti-MRSA activity, Mode of Action and Cytotoxicity of 2, 4-Di-tert-butylphenol Produced by *Streptomyces* sp. KB1

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ABSTRACT

Rapid emergence of vancomycin resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) has made the search of a novel bioactive compound a necessity. This study aimed to determine the anti-MRSA activity of 2, 4-Di-tert-butylphenol which produced by *Streptomyces* sp. KB1, analyze the mode of action, including cytotoxicity against mature brine shrimp. Totally 10 clinical isolates of MRSA were used for the anti-MRSA activity testing by broth microdilution method. The mode of action and cytotoxicity of 2, 4-Di-tert-butylphenol was performed by observing the tested MRSA cells with a scanning electron microscope and evaluated by the brine shrimp lethality bioassay respectively. The results indicated that 2, 4-Di-tert-butylphenol has the potent anti-MRSA activity, and the mode of action at the cell wall and also show the moderate cytotoxic activity. Its MIC, MBC and LC_{50} values were 31.25, 31.25 and 64.85 µg/ml respectively. The obtained data also demonstrated that this compound show bactericidal anti-MRSA activity at the concentration level lower than LC_{50} (P-value < 0.05). This study concluded that 2, 4-Di-tert-butylphenol, produced by *Streptomyces* sp. KB1, is the new anti-MRSA compound that can be used as a model for novel anti-MRSA drug development.

Keywords: *Streptomyces* sp. KB1, 2, 4-Di-tert-butylphenol, Anti-MRSA activity, Mode of action, cytotoxicity.

INTRODUCTION

Among all known multi-drug-resistant pathogenic bacteria, MRSA is possibly the greatest concern of all health-care-associated pathogen due to its ability to cause a wide variety of life-threatening infections. MRSA has the ability to rapidly adapt to different environmental conditions.[1] The problem of MRSA resisted to the approved antibiotics that increased rapidly of several areas has become a worldwide problem with serious consequences of the treatment of MRSA infection.[2,3] MRSA was first reported in the United Kingdom in 1961, soon after the introduction of the penicillinase-resistant β-lactam antibiotic and methicillin.[4] Initially, it was described as a nosocomial pathogen and a problem confined to institutionalized patients. More recently, spread of MRSA to the community setting has been described in injection drug users, prisoners and children. Today, MRSA has been recognized as one of the important pathogenic bacteria in community-acquired and hospital-acquired infections.[3] Even though MRSA infections are no more a scourge than infections caused by methicillin-sensitive *Staphylococcus aureus* (MSSA). Whereas, available antibiotics for treating MRSA infection is more limited. Vancomycin is a drug of choice for the treatment of infections caused by MRSA. Unfortunately, there is much research reported vancomycin failures.[5] The increased use/misuse of antibiotic in a treatment of MRSA infectious disease is mainly caused to the MRSA develops a mechanism of antibiotic resistance.[6] Such, new antibiotics will have to be developed in order to treat MRSA infections. New and more efficient antibiotics will have to be sought continually because of the capacity of microorganisms to survive their action. Many different strategies for finding new anti-MRSA compounds are actually proposed and the area of bioactive compounds is under intense investigation. Among microorganisms, actinomycetes, especially the genus *Streptomyces*, are a prominent source of natural bioactive compounds that have important applications in human medicine. *Streptomyces* are chemoorganotrophic, non-fastidious, filamentous and aerobic bacteria, spore forming and non-motile with high G+C content (approximately 69-78%) in their DNA.[5] They are Gram-positive bacteria known for their capacity to produce antibiotics and other medically important agents such as anti-cancer, anti-inflammatory, antifungal, antihelminthic and herbicide agents.[7] It was found that approximately two thirds of all currently used antibiotics were developed from bioactive compounds of them.[8] According to our previous observation, *Streptomyces* sp. KB1 could produce bioactive compound and excrete into the liquid culture medium. This bioactive compound was extracted, purified and elucidated chemical structure. It was designated as 2, 4-Di-tert-butylphenol and preliminarily tested the anti-MRSA activity by agar well diffusion method.

It was found that 2, 4-Di-tert-butylphenol could show anti-MRSA activity. Therefore, the purpose of this study was to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of 2, 4-Di-tert-butylphenol against clinical isolates of MRSA, and also observe the mode of action, along with evaluating its cytotoxicity against the mature brine shrimp.

MATERIALS AND METHODS

Bioactive compound

The pure compound, 2, 4-di-tert-butylphenol, obtained from producing strain of *Streptomyces* sp. KB1 (TISTR2304) which reported elsewhere.[9]

Microorganisms, media and culture conditions

The 10 clinical isolates of MRSA were kindly obtained from the Maharaj Nakhon Si Thammarat Hospital, Thailand. MSSA TISTR 517 was purchased from Thailand Institute of Scientific and Technological Research, Thailand. Both MRSA and MSSA were cultured in Luria Bertani (LB; Himedia, India) agar medium at 37 °C in static incubator for 24 h. Single colony of each isolate was inoculated into 10 ml of LB broth medium in 25 × 150 mm of the screw cap test tube, incubated at 37 °C, 200 rpm in shaking incubator for 24 h and stored in 15 % glycerol at -80 °C until use.

Determination of the Minimum inhibitory concentration (MIC)

Vancomycin hydrochloride and oxacillin sodium salt monohydrate were obtained from Sigma-Aldrich, USA. They were prepared as a stock solution at a concentration of 200 µg/ml of sterile double-distilled water. While, 2, 4-Di-tert-butylphenol was prepared as a stock solution at a concentration of 1 mg/ml by dimethyl sulfoxide (DMSO). The MIC was determined by using a microplate assay according to published protocol.[10] The MRSA strains were grown to mid-log phase in LB broth medium at 37 °C. Each MRSA strain was correspondingly adjusted to obtain turbidity comparable to 0.5 McFarland standard, then a 10-fold serial dilution was made to dilute out the cells, approximately residue of 1.5×10^6 cells/ml. Two-fold dilutions of 2, 4-Di-tert-butylphenol, vancomycin and oxacillin were freshly prepared in 96 wells plate (Nunc, USA), the wells containing only 100 µl of DMSO or double-distilled water was designed as a control. One hundred microliter of 1.5×10^6 cells/ml of MRSA suspension was added to each well to a final volume of 200 µl. The plates were incubated in a moist chamber at °C for 24 h. After the incubation, the optical density (OD) was measured at 690 nm with a microplate spectrophotometer (Multiskan GO, Thermo Scientific, USA). The MIC value was interpreted from the highest dilution showing no growth of MRSA. The MIC determinations were repeated independently three times. MSSA TISTR 517 was used as a control.

Determination of the Minimum bactericidal concentration (MBC)

The MBC value was determined by using subculture technique.[11] One hundred microliter of each MRSA strain and MSSA of well that showed MIC value and 3 wells with greater than the MIC value were spread on fresh MH agar medium without antibiotic and incubated at 37 °C for 24 h. After incubation, if the growth of MRSA was observed lower than 5 colonies, it indicated that the concentration of 2, 4-Di-tert-butylphenol, vancomycin or oxacillin of these well was MBC value.

Mode of action of 2, 4-Di-tert-butylphenol

Mode of action of 2, 4-Di-tert-butylphenol was performed by applying from published protocol.[12] Single colony of MRSA clinical isolate 142 (MRSA 142) was inoculated into 10 ml of MH broth medium in 25 × 150 mm of the screw cap test tube, incubated at 37 °C, 200 rpm in shaking incubator for 24 h. The cell suspension was inoculated into 50 ml of MH broth medium in 250 ml of duran bottle to obtain a final concentration of 1.5×10^8 CFU/ml or turbidity comparable to 0.5 McFarland standards, added the 2, 4-Di-tert-butylphenol at a concentration of MIC and designed as the experimental group. MRSA cell suspension without bioactive compound was designed as a control group. Both experimental and control groups were incubated at 37 °C, 200 rpm in shaking incubator for 24 h. Every 8 h, the cell suspension was harvested and centrifuged to separate the cells sediment and supernatant at 5,000 rpm for 5 min. The cell sediment was washed three times with fresh MH broth medium and then fixed with 2.5 % glutaraldehyde in phosphate buffer (pH 7.2) at 4 °C for 1 h, washed three times with phosphate buffer for 10 min and fixed again with 1 % osmium tetroxide for 2 h. This was followed by three washings in phosphate buffer for 10 min and subsequently dehydrated in a series of ethanol concentrations (30, 50, 70, 90 and 95 %), for 15 min each. The samples were subjected to 100 % ethanol and CO_2 to achieve the critical point and then coated with gold ion in a pressure metallic chamber. At the end of the process, the samples were submitted for analysis by Scanning electron microscope (SEM) (Quanta 400, FEI, Czech Republic).

Release of UV absorbing-materials 260 nm assay

The release of UV absorbing-materials were measured using Spectrophotometer (Nanodrop 2000, Thermo scientific, USA) which was modified from previously published protocol.[13] Supernatant from previous process was immediately filtered through a 0.2 µm pore-size filters (Millipore, Germany) to remove residual bacteria cells. The absorbance reading at 260 nm (A_{260}) from clear supernatant was recorded.

Cytotoxicity assay

Cytotoxicity of 2, 4-Di-tert-butylphenol was investigated against the mature brine shrimp, *Artemia salina*, in a one day *in vivo* according to published protocol.[14] The mature brine shrimp were obtained by hatching brine shrimp eggs which kindly obtained from the faculty of Aquaculture Technology, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat, Thailand, in artificial sea water (3.8 % sodium chloride solution) at 25 °C for 48 h. Meanwhile, 2, 4-Di-tert-butylphenol was dissolved to prepare as a stock solution at a concentration of 10 mg/ml of DMSO. The stock solution was introduced into the vials by pipetting at

different volumes to prepare for the serially different concentrations of 250.00, 125.00, 62.50, 31.25, 15.62 and 7.81 µg/ml.

The vial that contained the DMSO without 2, 4-Di-tert-butylphenol was designed as a control. Both experimental vials and control vial were added the artificial sea water up to 5 ml and then transferred 10 mature brine shrimp to all experimental vials and control vial. After incubation at 25 °C for 24 h, the number of mature brine shrimp(s) was counted. The findings were graphically presented by plotting the concentrations of 2, 4-Di-tert-butylphenol versus percentages of the mortality rate of the mature brine shrimp from which LC_{50} (50 % lethal concentration) was determined by extrapolation.

Statistical Analysis

All the above assays were performed in triplicate and repeated independently three times, for consistency of results and statistical purpose. The obtained data were analyzed by using the SPSS software version 17. One-way ANOVA (Analysis of Variance) was employed and the level of significance was P value < 0.05. Post-hoc turkey analysis was investigated the differences between the data.

RESULTS AND DISCUSSION

Rapidly increasing frequency of MRSA infections have become issues of very important public health concern. Although, intravenous vancomycin remains the standard therapy, but concerns about MSSA strains with reduced sensitivity to vancomycin and about the resistant increase to vancomycin of MRSA were remained.

Therefore, the screenings of anti-MRSA compounds were continuously conducted for the development of new antibiotic. From our previous research, we obtained 2, 4-Di-tert-butylphenol from producing strain of *Streptomyces* sp. KB1. This active compound had molecular weight and molecular formula was 206.2 g/mol and $C_{14}H_{22}O$ respectively. It could also show anti-MRSA activity when observed by agar well diffusion method. Moreover, its activity was remained when treated with hotness at 121 °C for 15 min. The obtained data implied that the active compound is non protein molecule.

Determination of the MIC and MBC values

This method, individual MRSA was grown in 96 wells plate containing different concentrations of bioactive compound or antibiotic. The wells become clear when bioactive compound or antibiotic are able to inhibit the growth of MRSA, otherwise show turbid due to presence of viable MRSA. The minimum concentration of bioactive compound or antibiotic which is able to inhibit the growth of MRSA is referred as MIC value. The wells that showed MIC value and 3 wells with greater than the MIC value are continuously sub-cultured for determination of MBC value. In this study, MIC and MBC values of 2, 4-Di-tert-butylphenol, vancomycin and oxacillin were shown in Table 1. Statistical analysis of one-way ANOVA and post-hoc turkey test revealed that MIC and MBC values of 2, 4-Di-tert-butylphenol was higher than of vancomycin, but lower than of oxacillin (P value < 0.05). Vancomycin and oxacillin were often used when other antibiotics have failed. As a result, antibiotic-resistant strains of staphylococci have appeared and caused a major clinical problem in hospitals.[10] The results in this paper could support nicely. The MICs obtained for both oxacillin and vancomycin against MSSA varied from 0.78 - 1.56 µg/ml. Those MICs are within the currently accepted range of susceptibility (≤ 2 µg/ml) by the Clinical and Laboratory Standard Institute (CLSI)[15] because MSSA or methicillin-sensitive *S. aureus* TISTR 517 is laboratory strain which used within laboratory only. Whereas, vancomycin MIC against clinical isolates of MRSA varied from 1.56 - 3.13 µg/ml. From the obtained results, it was found that vancomycin MIC of some isolate was higher than of susceptibility cutoff (≤ 2 µg/ml). This is evidence suggesting a tendency toward higher vancomycin MIC in these clinical isolate, also mentioned to as "MIC creep".[16] For 2, 4-Di-tert-butylphenol which is not appear the report of anti-MRSA activity showed the MIC value against the clinical isolates of MRSA higher than vancomycin about 20-folds. Whereas, from the obtained MBC value found that 2, 4-Di-tert-butylphenol exhibited bactericidal activity which considered from MBC/MIC value lower than or equal 4 (≤ 4).

Table 1: MIC and MBC values of 2, 4-Di-tert-butylphenol, vancomycin and oxacillin

Microorganisms	MRSA	MSSA
MIC* (µg/ml) of		
2,4-Di-tert-butylphenol	31.25 (15.63-62.50)	31.25 (15.63-62.50)
Vancomycin	1.56 (1.56-3.12)	1.56 (0.78-1.56)
Oxacillin	>100.00	1.56 (0.78-1.56)
MBC* (µg/ml) of		
2,4-Di-tert-butylphenol	31.25 (15.63-62.50)	31.25 (15.63-62.50)
Vancomycin	1.56 (1.56-3.12)	1.56 (0.78-1.56)
Oxacillin	>100.00	1.56 (0.78-3.12)

* The MIC and MBC values were expressed as the mode (range) which obtained from triplicate and three-time independence.

Table 2: Concentration of 260 nm absorbing-materials in supernatant obtained from cultivating MRSA with or without 2, 4-Di-tert-butylphenol and vancomycin at different incubation periods

Supernatant from cultivating MRSA	Genetic materials concentration (ng/ml) in supernatant at		
	8 h	16 h	24 h
Without both 2, 4-Di-tert-butylphenol and vancomycin	ND*	ND*	ND*
With 31.25 µg/ml of 2, 4-Di-tert-butylphenol	1.2	17.4	36.3
With 1.56 µg/ml of vancomycin	4.3	25.5	38.1

* Not determined, it was assigned as blank.

Table 3: Percentage of the mortality rate of mature brine shrimp after exposing with 2, 4-Di-tert-butylphenol at different concentrations for 24 h and its LC_{50} value.

Groups	Conc. of 2,4-Di-tert-butylphenol (µg/ml)	Average [a] of No. of death	% of mortality rate	LC_{50} value (µg/ml)
Control	0.00	0.00	0.00	
Experimental	7.81	0.00	0.00	64.85
	15.62	2.17	21.70	
	31.25	3.33	33.30	
	62.50	4.83	48.30	
	125.00	9.33	93.30	
	250.00	10.00	100.00	

[a] Data were obtained from triplicate and three-time independence.

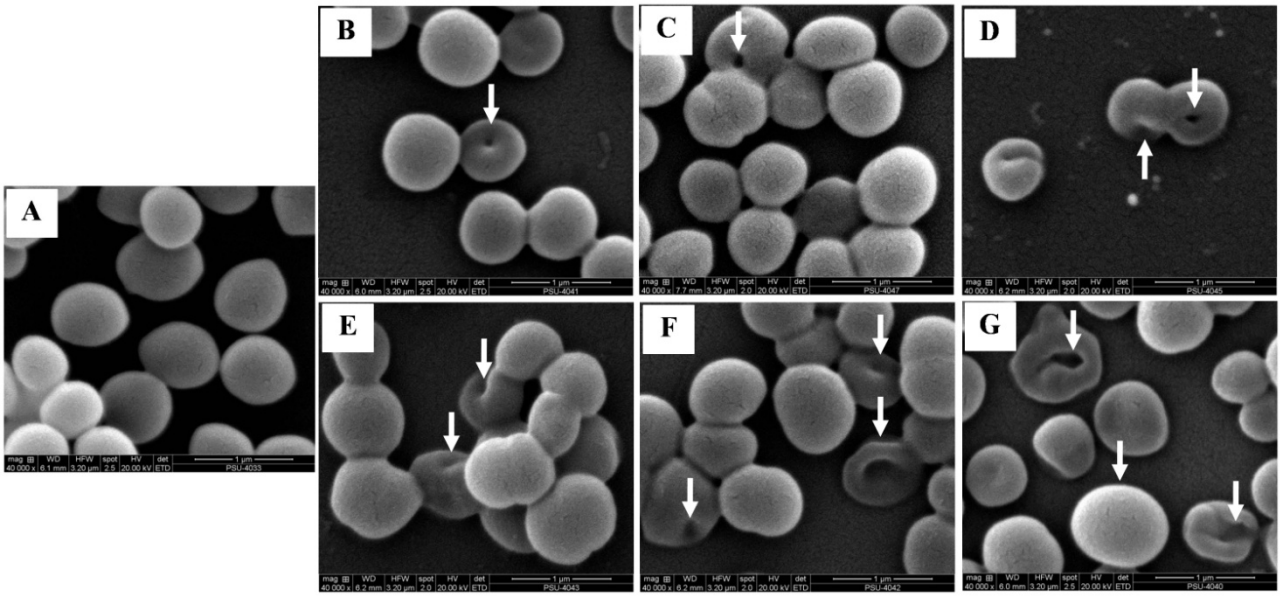

Figure 1: The SEM photograph of normal cells of MRSA 142 (A), cells of MRSA 142 after exposing with 31.25 µg/ml of 2, 4-Di-tert-butylphenol for 8, 16 and 24 h (B, C and D respectively), and cells of MRSA 142 after exposing with 1.56 µg/ml of vancomycin for 8, 16 and 24 h (E, F and G respectively). The arrow head indicated the bursting cell at cell wall of cell which treated with 2, 4-Di-tert-butylphenol or vancomycin.

Mode of action of 2, 4-Di-tert-butylphenol

MRSA 142 was cultured in MH broth medium at 37 °C until mid-log phase, supplemented 2, 4-Di-tert-butylphenol and vancomycin at the concentration of MIC value and continuously cultured at same condition. Every 8 h, MRSA cells were harvested and observed cell feature by SEM. From the SEM photograph (Figure 1), the deformation of MRSA cells was observed (pointing arrow) and this deformation resembled both MRSA cells supplement with 2, 4-Di-tert-butylphenol and vancomycin. The mode of action of vancomycin is clear that it correlates with inhibiting cell wall synthesis of bacteria by binding the pentapeptides of the peptidoglycan monomers and blocking the formation of peptide cross-links. As the autolysis continue to break the peptide cross-links and also new cross-links fail to form, the bacterium bursts from osmotic lysis.[17] For 2, 4-Di-tert-

butylphenol, it was also clear that its major target was cell wall similar to vancomycin, but the mechanism of action might be differ. For the damage confirmation of the cell wall of MRSA, its supernatant was harvested to determine the genetic material in the later time.

Release of UV absorbing-materials 260 nm assay

The bacterial cell wall is an extremely important component of the cell. The damage of them is usually associated with cell death. Therefore, the most bioactive compounds that has target site at here often show bactericidal or fungicidal activity.[18] This suggestion corresponds with our research which it was found that 2, 4-Di-tert-butylphenol cause the damage of cell wall of MRSA and also show bactericidal activity. The damage of the cell wall of MRSA was confirmed by genetic materials which were measured from the culture broth of them at 260 nm. The results presented in Table 2 indicated that genetic materials (DNA and RNA) were released. The release of intracellular components is a good indicator for integrating of the bacterial cell wall. If it is damaged, small ions such as potassium and phosphate tend to leach out first, followed by large molecules such as DNA, RNA, and other materials. Since these nucleotides have strong UV absorption at 260 nm, they are defined as "260 nm absorbing-materials".[19] In this study, it is clear that 2, 4-Di-tert-butylphenol has the mode of action same as vancomycin. Whereas, 2, 4-Di-tert-butylphenol has molecule very small than vancomycin. Therefore, it is highly possible that the mechanism of action of 2, 4-Di-tert-butylphenol differ with vancomycin which we will further study in the future.

Cytotoxicity assay

The cytotoxicity of 2, 4-Di-tert-butylphenol was evaluated by brine shrimp lethality bioassay. The obtained data found that the effect of 2, 4-Di-tert-butylphenol on the mortality rate of the mature brine shrimp showed cytotoxic activity dependent concentration (Table 3). After 2, 4-Di-tert-butylphenol concentration versus the percentage of the mortality rate of the mature brine shrimp was plotted as a linear correlation graph and constructed as the linear equation, it could interpret the result of 50 % lethal concentration (LC_{50}) value as 64.85 µg/ml. The obtained result demonstrated that 2, 4-Di-tert-butylphenol has moderate cytotoxic activity. When comparing with MIC value, it was found that LC_{50} value remained significantly higher than the MIC value (P-value < 0.05). For determination of the cytotoxic activity of bioactive compound, several methods are employed. A widely used method in this field is brine shrimp lethality bioassay because it is a rapid method utilizing only 24 h, inexpensive, and needs no special equipment. Moreover, the interest of this method was the use of zoological organism to investigate the cytotoxic activity.[20] In this research, brine shrimp lethality bioassay was used to preliminarily assess the cytotoxicity of 2, 4-Di-tert-butylphenol because animal cytotoxicity test shows the variations depending on the chemical nature of the compound and the route of administration to the experimental animal.[21]

Therefore, *in vivo* cytotoxicity study in rat along with the pharmacodynamics and pharmacokinetics will be performed in the next time.

CONCLUSION

Based on the results of this study, it could be concluded that 2, 4-Di-tert-butylphenol which produced by *Streptomyces* sp. KB1 has the potent anti-MRSA activity and the mode of action at the cell wall and also shows the moderate cytotoxic activity. Its MIC, MBC and LC_{50} values were 31.25, 31.25 and 64.85 µg/ml respectively. From these results also found that this compound shows the bactericidal anti-MRSA activity at the concentration level lower than LC_{50} (P-value < 0.05). All obtained results implied that 2, 4-Di-tert-butylphenol is the new anti-MRSA compound that can be used as a model for novel anti-MRSA drug development. Therefore, future our works will investigate the mechanism of action, confirm the *in vivo* cytotoxicity in rat, along with study the pharmacodynamics and pharmacokinetics of this compound.

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