Mode of Action and Cytotoxicity of Bioactive Compounds Produced by *Streptomyces* sp. KB1

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Abstract: Rapid emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) has led to search of a novel bioactive compounds from natural resources. This study was aimed to determine the mode of action of bioactive compounds produced by *Streptomyces* sp. KB1 TISTR 2304 against MRSA, including cytotoxicity against mature brine shrimp. The mode of action and cytotoxicity of bioactive compounds were 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 bioactive compounds had the mode of action at the cell wall and also showed the moderate cytotoxic activity. This study concluded that the bioactive compounds produced from strain KB1 can be used as a model for novel anti-MRSA drug development.

Key words: Anti-MRSA activity, bioactive compounds, mode of action, cytotoxicity, *Streptomyces* sp. KB1 TISTR 2304.

1. Introduction

The problem of methicillin-resistant *Staphylococcus aureus* (MRSA), the greatest concern of all health-care-associated pathogen, 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. It is possibly due to its ability to cause a wide variety of life-threatening infections. MRSA has the ability to rapidly adapt to different environmental conditions [1-4]. Formerly, 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]. That 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 [5, 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 (secondary metabolites) 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. 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 [5, 7]. Approximately, two thirds of all currently used antibiotics were developed from their bioactive compounds [8]. *Streptomyces* sp. KB1 could produce bioactive compounds and excrete into

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the liquid culture medium. These bioactive compounds in culture broth were extracted with ethyl acetate solvent. The preliminary data showed that partially purified bioactive compounds had the anti-MRSA activity when tested by agar well diffusion method. Therefore, the purpose of this study was to determine the mode of action of bioactive compounds against clinical isolates of MRSA, along with evaluating its cytotoxicity against the mature brine shrimp.

2. Method and Materials

2.1 Microorganisms, Media and Culture Conditions

The *Streptomyces* sp. KB1 TISTR 2304 was collected from air at Aonang, Krabi province by using air bio-sampler (Microflow 90) at a flow rate 100 L/min for 30 min. It was used as a source of bioactive compounds. The partially purified bioactive compounds were extracted from culture broth of strain KB1 with ethyl acetate solvent in a ratio 1:1, dried using rotary evaporator apparatus. It was kept at -20 °C until use.

The MRSA clinical isolate of 2468 (MRSA 2468) was kindly obtained from the Maharaj Nakhon Si Thammarat Hospital, Thailand. Methicillin sensitive *Staphylococcus aureus* (MSSA) TISTR 517 was purchased from Thailand Institute of Scientific and Technological Research (TISTR), 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.

2.2 Mode of Action of Bioactive Compounds

Mode of action of bioactive compounds was performed by applying from published protocol [8]. Single colony of MRSA 2468 was inoculated into 10 mL of Mueller-Hinton (M-H) 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 M-H 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 bioactive compounds 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 M-H 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 CO₂ 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).

2.3 Release of UV Absorbing-Materials 260 nm Assay

The release of UV absorbing-materials was measured using spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) which was modified from previously published protocol [9]. Supernatant from previous process was immediately filtered through a 0.45 µm pore-size filter (Millipore, Germany) to remove residual bacteria cells. The absorbance reading at 260 nm (A_{260}) from clear supernatant was recorded.

2.4 Cytotoxicity Assay

Cytotoxicity of bioactive compounds was

investigated against the mature brine shrimp, *Artemia salina*, in one day *in vivo* according to published protocol [10]. The mature brine shrimp(s) were obtained by hatching brine shrimp eggs which were 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, bioactive compounds were dissolved to prepare as a stock solution at a concentration of 10 mg/mL of dimethyl sulfoxide (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 bioactive compounds 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 was counted. The findings were graphically presented by plotting the concentrations of bioactive compounds versus percentages of the mortality rate of the mature brine shrimp from which LC₅₀ (50% lethal concentration) was determined by extrapolation.

2.5 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 Statistical Package for the Social Sciences (SPSS) software version 17. One-way ANOVA (Analysis of Variance) was employed and the level of significance was $p < 0.05$. Post-hoc turkey analysis was used to investigate the differences between the data.

3. Results and Discussions

Rapidly increasing of MRSA infections has 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. Fortunately, the bioactive compounds from producing strain of *Streptomyces* sp. KB1 had anti-MRSA activity when observed by agar well diffusion method. Its activity was remained when treated with hotness at 60 °C for 30 min.

3.1 Mechanism of Action of Bioactive Compounds

MRSA 2468 was cultured in M-H broth medium at 37 °C until mid-log phase, supplemented with bioactive compounds and continuously cultured at same condition as described in Section 2. Every 8 h,

Fig. 1 (A) The SEM photograph of normal cells of MRSA 2468, (B, C and D) cells of MRSA 2468 after exposing with 31.25 µg/mL of bioactive compounds (MIC-value) for 8, 16 and 24 h respectively. The arrow head indicated the bursting cell at cell wall of cell treated with bioactive compounds.

Table 1 Concentration of 260 nm absorbing-materials in supernatant obtained from cultivating MRSA with or without bioactive compounds at different incubation periods.

Supernatant from cultivating MRSA	Genetic materials concentration (ng/mL) in supernatant at		
	8 h	16 h	24 h
Without bioactive compounds	ND*	ND*	ND*
With 31.25 µg/mL of bioactive compounds	1.2	17.4	36.3

* Not determined, it was assigned as blank.

MRSA, methicillin resistant *Staphylococcus aureus*.

MRSA cells were harvested and observed cell feature by SEM. From the SEM photograph (Fig. 1), the deformation of MRSA cells was observed. The mode of action of bioactive compounds is clear whose major target was cell wall. For the damage confirmation of the cell wall of MRSA, the supernatant was harvested to determine the genetic material in the later time.

3.2 Concentration of 260 nm Absorbing-Materials

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 have target site at here often show bactericidal or fungicidal activity [11]. It corresponds with our research which was found that bioactive compounds cause the damage of cell wall of MRSA and also showed 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 1, 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” [12]. In this study, it is clear that bioactive compounds have the mode of action same as vancomycin. However, it is possible that the mechanism of action of bioactive compounds differs with vancomycin which we will further study in the future.

3.3 Cytotoxicity against the Mature Brine Shrimp

The cytotoxicity of bioactive compounds was evaluated by brine shrimp lethality bioassay. The obtained data found that the effect of bioactive compounds on the mortality rate of the mature brine shrimp showed cytotoxic activity dependent concentration. After bioactive compounds 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 LC₅₀ value as 64.85 µg/mL. The obtained result demonstrated that bioactive compounds have moderate cytotoxic activity. When comparing with Minimum Inhibition Concentration (MIC) value, it was found that LC₅₀ value remained significantly higher than the MIC value (31.25 µg/mL) ($p < 0.05$). For determination of the cytotoxic activity of bioactive compounds, 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 [13]. In this research, brine shrimp lethality bioassay was used to preliminarily assess the cytotoxicity of bioactive compounds because animal cytotoxicity test shows the variations depending on the chemical nature of the compound and the route of administration to the experimental animal [14]. Therefore, *in vivo* cytotoxicity study in rat along with the pharmacodynamics and pharmacokinetics will be performed in the next time.

4. Conclusion

Based on the results of this study, it could be concluded that bioactive compounds which were produced by *Streptomyces* sp. KB1 TISTR 2304 had the potent anti-MRSA activity and the mode of action at the cell wall and also showed the moderate cytotoxic activity. All obtained results implied that bioactive compounds can be used as a model for novel anti-MRSA drug development. Therefore, future works will investigate the mechanism of action, and confirm the *in vivo* cytotoxicity in rat, along with studying the pharmacodynamics and pharmacokinetics of this compound.

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