In vivo Antibacterial Activity of Green Algae Ulva reticulata against Staphylococcus aureus in Drosophila Model of Infection

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ABSTRACT

Background: Expansion of multi-drug resistant bacteria in social communities and health facilities has been widely reported. To overcome this ever-growing problem, new antibiotics with novel mechanisms of action are urgently required. Purpose: This research was carried out aiming to investigate the in vivo antibacterial effect of green algae Ulva reticulata against Staphylococcus aureus in fruit flies (Drosophila melanogaster) model of infection. Methods: Sample was dried and extracted with ethanol using maceration method. Wild type and mutant fruit flies were infected with S. aureus and subjected to survival and bacterial load analysis in the presence or absence of tetracycline or Ulva reticulata extract at different concentrations. All data were statistically analyzed. Results: Infection of D. melanogaster with S. aureus was characterized by two notable trends: reduction of host survival and increasing level of bacterial growth in the host during the course of infection. Such events were further augmented in mutant flies lacking normal immune responses. Nonetheless, improved survival rates and reduction of bacterial load were observed in wild type and immunodeficient mutant flies challenged with S. aureus in the presence of either tetracycline or ethanolic extracts of green algae Ulva reticulata. Conclusion: Taken together, our results suggest that Ulva reticulata yielded antistaphylococcal activity in vivo thus would be a prospective source for harvesting wide spectrum antibacterial compounds.

Key words: Antistaphylococcal, Drug discovery, Fruit flies, Infection model, Marine products.

INTRODUCTION

The growing problem of life-threatening infectious diseases caused by the emergence of antibiotic-resistant bacteria have been shown to have an adverse impact on the health of human populations.¹ To overcome this challenge, the discovery of new antibiotic drugs with a possible novel mechanism of action is urgently required.²,³ Unfortunately, despite the huge effort given by countless research groups and pharmaceutical companies worldwide, the rate of discovery of new effective antibiotics is progressively declining.⁴-⁷ which is substantially diminishing our hope of providing a solution to this ever-growing crisis. One of the reasons for the declining in the rate of antibiotic drug discovery is the high cost of in vitro testing of antibacterial activity using mammalian model systems.⁸,⁹ In addition, the impact of ethical issues raised by the use of the traditional established mammalian model of bacterial infection is a challenge when examining the effect of many antibiotic candidates in parallel, thus increasing the assessment period.¹⁰ To make things worse, there is no guarantee that the antibiotic candidates with positive results in an in vitro experiment will yield similar results in the trial stage using an in vivo animal model system.²,¹⁰ Therefore, there is a high chance that valuable time and costs that have been spent during the discovery process may not bring a profitable outcome.

At present, there is a vast number of original articles reporting the antibacterial activity of crude extracts (or compounds isolated) from a diverse array of natural products,¹¹-¹³ including Indonesian medicinal plants.¹⁴-¹⁵ Although most of these extracts and compounds yielded promising results in the in vitro stage, many are not further characterized or even tested in the pre-clinical in vivo stage,¹² thus jeopardizing the whole reason for such research to be done in the first place.

To circumvent these money- and time-wasting risks and difficulties, there is a need for an alternative platform to assess the antibacterial activity of drug candidates with low-cost and high-throughput results. Reasoning that some of the above-mentioned obstacles can be overcome by the usage of low-cost live-animal infection model system, we used fruit fly (Drosophila melanogaster) model of bacterial infection as a platform to screen the antibacterial effect of crude extracts prepared (or compounds isolated) from a diverse array of natural products,¹¹-¹³ including Indonesian medicinal plants.¹⁴-¹⁵ Although most of these extracts and compounds yielded promising results in the in vitro stage, many are not further characterized or even tested in the pre-clinical in vivo stage,¹² thus jeopardizing the whole reason for such research to be done in the first place.

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MATERIALS AND METHODS

Bacterial strains and fly stocks

The S. aureus ATCC 29213 strain was used as the infectious agent. The bacteria were cultured in Nutrient Broth (NB) medium at 37°C, separately. When the cultures reached full growth, it was harvested, washed with PBS, and used in the experiments. The following lines of Drosophila were used in this study: w^1118 as genotype (background) control, drpr^4 clone 15 which has no detectable expression of Draper (a gift from Yoshi nobu Nakashiki, Kanazawa University), and Tl[r3] (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN) with undetected level of Toll, a known receptor that triggers innate immunity of the D. melanogaster. All flies were maintained with standard cornmeal-agar medium at 25°C.

Sample preparation

Samples of the green alga Ulva reticulata were purchased from Pun tondo, Takalar, South Sulawesi, Indonesia and processed as described previously, with modifications. Samples were sorted and subjected to maceration procedures using 96% ethanol for 3-24 hours. The resulting extracts were then further processed to reduce the water content and kept in a brown silica container prior to use. The voucher specimen was deposited at Biofarmaka Laboratory, Faculty of Pharmacy, Hasanuddin University.

Fly infection and assays for survival and bacterial growth

The introduction of bacteria into the thorax of male adult flies, known as prick ing, was carried out according to the established procedures with modifications. Briefly, at 4-7 days after eclosion, the flies (10 flies per vial, 3 vials in each experiment) were pricked with bacterial suspension containing given numbers of bacteria (1 x 10^5 cfu/ml) per fly. Flies infected with bacteria were maintained at 29°C and subjected to either survival assay or colony forming assay. In the survival assay, fly groups were observed for survival during the course of infection, in the presence or absence of treatments. In the colony forming assay, the growth of bacteria in flies was analyzed by determining the colony-forming activity of injected bacteria as described previously, with some modifications. Homogenates of infected flies were plated at serial dilutions on Vogel-Johnson agar medium and the number of colonies that appeared after incubation was expressed as CFU per ml. Groups of healthy flies were also included in both survival and colony forming assays.

Data Processing and Statistical Analysis

Results from quantitative analysis are expressed as the mean ± S.D. of the data from at least three independent experiments, unless otherwise stated in the text. Statistical analyses were performed using Kaplan-Meier log-rank analysis (for survival curve) and one-way ANOVA or Student's t test (for CFU analysis), and p values of less than 0.05 were considered significant and are indicated in the figures. All results were processed using Graph Pad Prism 7.

RESULTS AND DISCUSSION

Drosophila melanogaster is a suitable model for S. aureus infection

Staphylococcus aureus is a Gram-positive bacterium that have broad negative effects on organisms, including humans. To examine the virulence properties and possible (novel) drug targets available in this bacteria, scientists have tried to cultivate it in different types of hosts, including lower invertebrates such as Drosophila melanogaster. Here, we used Drosophila melanogaster as an alternative in vivo platform to assess the antibacterial effect of green algae Ulva reticulata on S. aureus. As shown in Figure 1, injection of Drosophila melanogaster with S. aureus resulted in the decrease of infected flies’ survival rate in a dose-dependent manner. It is apparent that S. aureus was able to propagate in D. melanogaster in vivo, supporting the notion that D. melanogaster can be used to explore aspects related to S. aureus infection, including virulence factors and possible of treatments, as reported by other investigators.

Improvement of S. aureus-infected Drosophila melanogaster survival rate by either antibiotics or ethanolic extract of Ulva reticulata

A class of drugs that can inhibit the growth of bacteria, known as antibiotics, has been widely introduced as one of the potent arsenals in the treatment of infection in humans. In this experiment, the incorporation of tetracycline, an antibiotic that inhibits protein synthesis in bacteria, into the food of S. aureus-infected Drosophila w^1118 was able to prevent the early death of infected host (Figure 2), similar to the ones observed by Needham et al. (2004), suggesting that tetracycline which function well on humans can yield a similar effect in our Drosophila infection model system. In addition to that, 25 mg/ml ethanolic extract of Ulva reticulata also rescued S. aureus-infected D. melanogaster from early death phenotype that was seen in the untreated control group. This result implicates the in vivo antibacterial of ethanolic extract of Ulva reticulata against S. aureus at the tested concentration.

Inhibition of bacterial growth by antibiotics or extract of Ulva reticulata

Bacterial load has been suggested to play an important role in the increasing death rate of the infected host. Since we observed the increasing survivorship of infected flies in the presence of either antibiotics or Ulva reticulata extract, it is tempting to speculate that such phenotype was related to the inhibition of bacterial growth in vivo. To assess this, we carried out colony forming assays to examine the rate of bacterial growth in the flies. As shown in Figure 3, treatment of infected flies with either tetracycline or 25 mg/ml Ulva reticulata extract was significantly useful to reduce the bacterial load in flies infected with S. aureus, indicating that increased survivorship of bacteria-infected flies in the presence of either tetracycline antibiotics or Ulva reticulata extract might be the result of bacterial growth inhibition.

Beneficial effects of Ulva reticulata extract in the immunodeficient model system

Increased survival rate of infected host and reduction of the bacterial load might result from direct interaction of compounds contained in the
extract with the bacteria found in the infected flies. However, previous experiments carried out in this research did not rule out the possible stimulation of host immune response that finally resulted in the inhibition of bacterial growth thus yielding the rescue effects observed in the antibiotic-treated or extract-treated bacteria-infected-\textit{w}^{1118}. To examine which of the possibilities was true, we performed infection experiments on two mutant flies lacking either humoral or cellular immune responses. We used flies with Toll-lacking (humoral immunodeficient) and Draper-lacking (cellular immunodeficient) phenotypes that have been demon-
strated to be prone to Gram-positive bacteria. As shown in Figure 4A, humoral immunodeficient mutant flies (Toll mutant flies) succumbed faster with higher bacterial load than the control flies upon infection with S. aureus. This indicates that Toll immunodeficient flies were more sensitive to bacterial infection, supporting the reports of previous investigators.15,31 Furthermore, treatment of the infected-immunodeficient flies with food containing ethanolic extract of Ulva reticulata at concentration of 25 mg/ml increased the survivorship of infected Toll mutant flies and reduced the bacterial load recovered from the corresponding mutant flies (Figure 4A). Similar results were also observed in Draper mutant flies lacking for cellular innate immunity (Figure 4B). This mutant fly lacking for cellular innate immunity known to provide protection against S. aureus could survived longer in the presence of tetra-cycline or crude extract of Ulva reticulata. Taken together, these results suggested that Ulva reticulata extract yielded its antibacterial activity against S. aureus via direct interaction of compounds available in the extract with bacteria and was not due to stimulation of Toll signaling pathway (humoral innate immune response) or activation of cellular immune responses via Draper recognition of S. aureus.

CONCLUSION

In this research, we showed, for the first time, the antibacterial effect of Ulva reticulata through the application of a genetically tractable D. melanogaster as an in vivo bacterial infection model system. Such simple and inexpensive in vivo platform can provide a high-throughput result in the screening of medicinal plant crude extracts and/or other antibiotic-producing samples prior to further processing steps such as isolation of responsible antibiotic compounds, in vivo testing using mammalian models of bacterial infection, and elucidation of antibiotic mechanisms of actions.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ABBREVIATION USED

ANOVA: Analysis of variance; ATCC: American Type Culture Collection; BMIS: Benua Maritim Indonesia Spesifik; CFU: Colony-forming unit; NB: Nutrient Broth.

REFERENCES

**GRAPHICAL ABSTRACT**

**SUMMARY**

- *Drosophila melanogaster* can be infected by human pathogen such as *Staphylococcus aureus* thus prospective to be used as infection model system in drug discovery research.
- Using *D. melanogaster* infection model, antibacterial activity of *Ulva reticulata* against *S. aureus* was assessed *in vivo*.
- Improvement of host survival accompanied by reduction in bacterial load were observed in *S. aureus*-infected *D. melanogaster* upon treatment with ethanolic extract of *Ulva reticulata*.
- Ethanolic extract of *Ulva reticulata* yield *in vivo* antibacterial effect against *Staphylococcus aureus* in *D. melanogaster* model of infection.

**ABOUT AUTHORS**

**Firzan Nainu** is a lecturer in the Pharmacology and Toxicology Laboratory, Faculty of Pharmacy, Hasanuddin University, Indonesia. He completed his Doctoral program in Pharmaceutical Sciences at Kanazawa University, Japan, in 2016. He is involved in research dealing with host defense and responses against diverse array of pathogens by using Drosophila and other available model systems. In addition, he is interested in the establishment of various *in vivo* model systems for drug discovery.

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