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Original Article

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

Firzan Nainu[1*], Rangga Meidianto Asri[1], Aryadi Arsyad[2], Marianti Anggreni Manggau[1], Muhammad Nur Amir[1]

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.

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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.[1-5] To overcome this challenge, the discovery of new antibacterial drugs with a possible novel mechanism of action is urgently required.[6,7] 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,[7-9] 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 vivo* testing of antibacterial activity using mammalian model systems.[10,11] 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.[7,10] 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 prepared (or compounds isolated) from a diverse array of natural products,[12,13] 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 samples. In these past years, *Drosophila melanogaster* has been used extensively to uncover important biological pathways,[16,17] especially the ones related

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to immunity against bacteria[18,19] and viral infection.[20-22] To the latest, *D. melanogaster* has been recognized as a promising disease model to discover new drug candidates and/or their respected targets.[23-25]

Drosophila melanogaster have been widely suggested as a suitable host for several pathogenic bacteria known to cause devastating infections in humans such as *Staphylococcus aureus,*[11,26] *Pseudomonas aeruginosa,*[27] *Listeria monocytogenes,*[28] *Burkholderia spp.,*[29] and *Bacillus anthracis.*[30] In addition to that, with its high degree of genetic similarity with human, cheap maintaining costs, and poses almost no ethical issues,[10,16,17] *D. melanogaster* offers great advantages as an *in vivo* model system in antibacterial drug discovery research. Capitalizing on such advantages, in the current study, we tested the application of *Drosophila* model of bacterial infection as an *in vivo* platform to assess the antibacterial effect of *Ulva reticulata* extract, a particular sample that was shown to have a strong *in vitro* antibacterial activity against *S. aureus* in the preliminary *in vitro* experiment.[31,32]

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, $drpr^{\Delta 5}$ clone 15 which has no detectable expression of Draper (a gift from Yoshinobu Nakanishi, 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 Puntondo, Takalar, South Sulawesi, Indonesia and processed as described previously,[32] 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 pricking, was carried out according to the established procedures[33] 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 (1×10^5 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,[34] 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.*[26,27] 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, infection of *Drosophila melanogaster* w^{1118} by *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.[26,27,35]

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.[26] 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

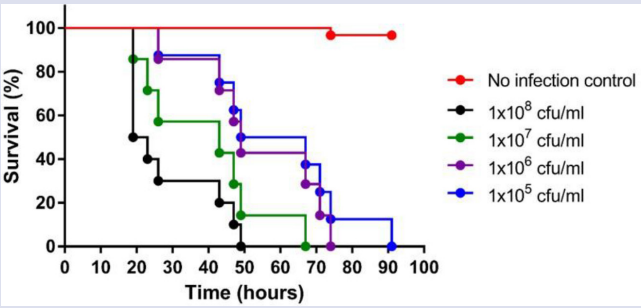

Figure 1: Dose-response of *S. aureus* in the infection experiment. Adult flies *w1118* at 4-7 days after eclosion were infected with a range of doses of *S. aureus* ATCC 29213 by pricking and subjected to fly survival analysis.

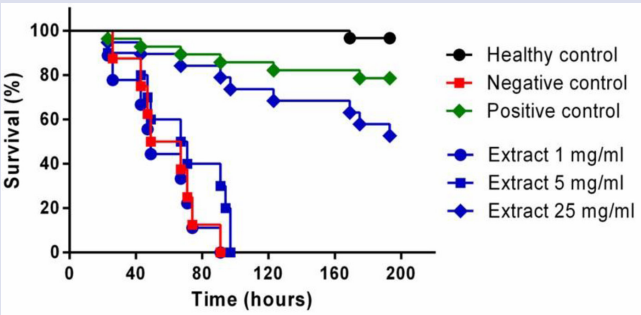

Figure 2: Survival of infected-*w1118* D. melanogaster in the presence or absence of treatments. Adult flies *w1118* at 4-7 days after eclosion were infected with 1 x 10^5 cfu/ml of *S. aureus* ATCC 29213 by pricking and incubated at 25°C in the presence of 1, 5, or 25 mg/ml of *Ulva reticulata* extracts then followed by fly survival analysis. Flies treated with tetracycline at 200 µg/ml were used as a positive control group.

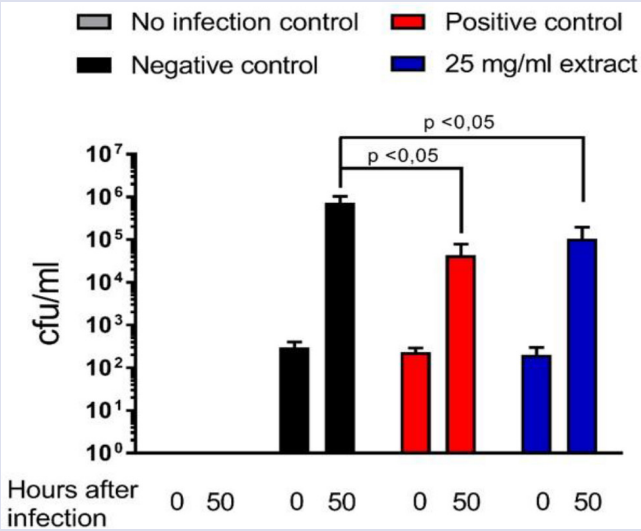

Figure 3: Growth of bacteria in the infected- *w1118* D. melanogaster in the presence or absence of treatments. Adult flies *w1118* at 4-7 days after eclosion were infected with 1 x 10^5 cfu/ml of *S. aureus* ATCC 29213 by pricking and incubated at 25°C in the presence of 25 mg/ml of *Ulva reticulata* extracts then followed by bacterial load analysis. Flies treated with tetracycline at 200 µg/ml were used as a positive control group.

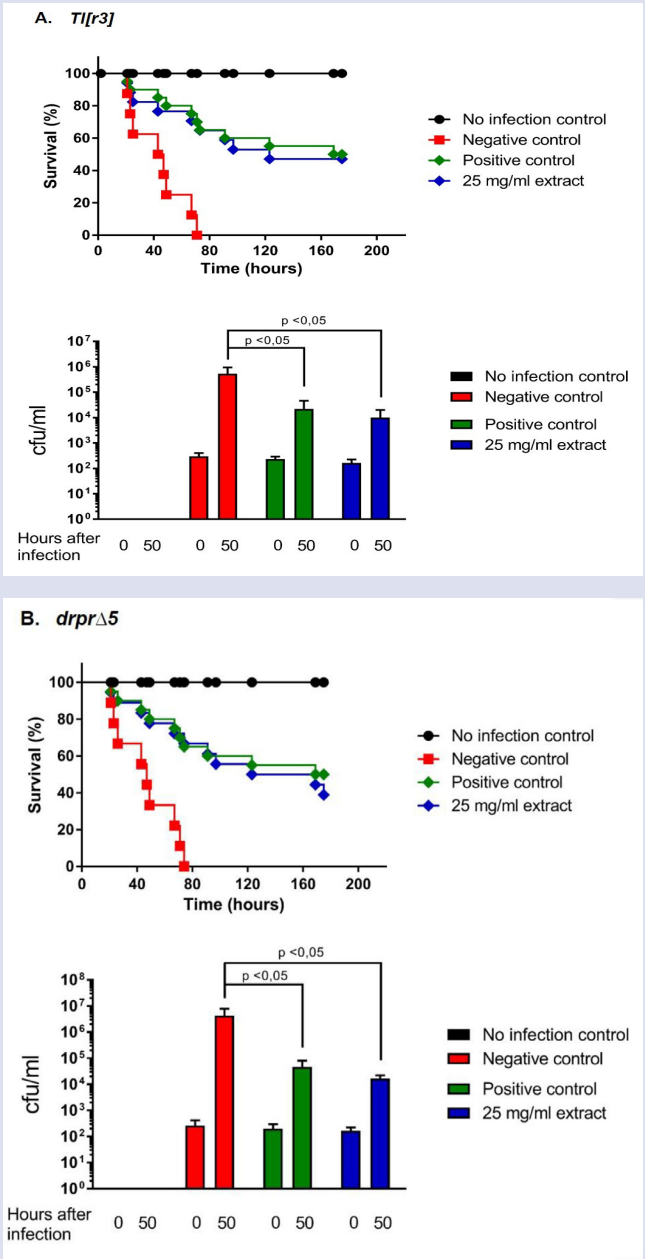

Figure 4: Analysis of host survival and enumeration of bacterial load in infected immunodeficient flies in the presence or absence of treatments. Adult immunodeficient Toll-lacking (A) or Draper-lacking (B) flies at 4-7 days after eclosion were infected with 1 x 10^5 cfu/ml of *S. aureus* (ATCC 29213) by pricking, incubated at 25°C in the presence of either 200 µg/ml tetracycline or 25 mg/ml of *Ulva reticulata* extract, and subjected to fly survival and bacterial load analysis. Flies treated with tetracycline at 200 µg/ml were used as a positive control group.

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-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.[33,35] 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 tetracycline 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.

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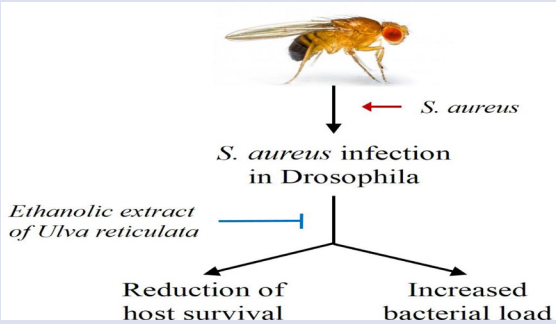
GRAPHICAL ABSTRACT



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.

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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.

Rangga Meidianto Asri is a junior lecturer in the Faculty of Pharmacy at Hasanuddin University. He completed his education in Pharmaceutical science at Hasanuddin University in 2011 and have been involved in Department of Pharmaceutics since 2014. His current research interests include investigation of extracts and fractions containing potential bioactive compounds from natural plants and their mechanism of actions as anti-infection.

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