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In vivo anti-staphylococcal activity of roselle (*Hibiscus sabdariffa* L.) calyx extract in *Drosophila* model of infection

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ABSTRACT

Introduction: The emergence of antibiotic-resistant *Staphylococcus aureus* is a major threat for worldwide communities. To overcome such serious problem, the discovery of novel antibacterial agents through exploration of diverse potential sources is essential. The aim of this research was to investigate the *in vivo* anti-staphylococcal activity of roselle (*Hibiscus sabdariffa* L.) calyx extract against *S. aureus* in fruit flies (*Drosophila melanogaster*) model of infection.

Methods: Roselle calyces were dried and extracted with ethanol using maceration method. Wildtype fruit flies were infected with *S. aureus* and subjected to survival assay, bacterial load examination, and gene expression analysis, in the presence or absence of roselle calyx extract. Survival and bacterial load analysis were subsequently performed on immunodeficient fruit flies using similar protocols.

Results: Reduction of host survivorship accompanied by increasing level of bacterial proliferation was observed in group of wildtype fruit flies infected with *S. aureus.* These phenotypic events were further augmented in mutant flies devoid of component for antibacterial immune responses. Nevertheless, reduction of bacterial load and improvement of host survival were demonstrated in either wildtype or immunodeficient fruit flies upon treatment with roselle calyx extract after bacterial challenge, irrespective of immune status.

Conclusion: Collectively, our results demonstrated the *in vivo* antibacterial activity of roselle calyx extract against *S. aureus* in Drosophila model of infection and this was not due to induction of immune response in the host.

Implication for health policy/practice/research/medical education:
Our results demonstrated the in vivo antibacterial activity of roselle calyx extract against *Staphylococcus aureus* using a novel *Drosophila* infection model platform that shall emphasize the idea to explore the use of roselle calyx extract as a source to discover novel anti-staphylococcal agents.

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Introduction

Antibiotics are considered as the most used class of drugs in the world. Between 2000 and 2010, the consumption of antibiotics reached more than 30% of the total use of medicines worldwide (1). In Indonesia itself, the utilization of antibiotics even reached 80% of total drugs consumed between 2002 to 2005 (2). Unfortunately, in some cases, antibiotics were purchased in the absence of proper examination and decision from physician or even without the needs of prescription, implicating the irrational practice of antibiotics utilization. Consequently, in addition to other contributing factors, the emergence of antibiotic-resistant bacteria has been constantly reported (1,3,4).

The emergence of bacterial strains that are resistant to antibiotics is a major concern for public health (3-5). One of the pathogenic bacteria that presents serious threat to human population is *Staphylococcus aureus* (6), especially after the emergence of methicillin-resistant *S. aureus* (MRSA) (7). To overcome this life-threatening problem, the discovery of novel antibacterial agents through exploration of diverse potential sources is essential (4,

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8). One of promising sources for such efforts is roselle (*Hibiscus sabdariffa* L.). The ethanolic extract of roselle's calyx has been shown to yield excellent *in vitro* antibacterial effect against several Gram-positive pathogenic bacteria (9-11), including *S. aureus* and MRSA (12,13). However, despite such promising antibacterial effect, no *in vivo* experiment has been carried out to further clarify the anti-staphylococcal activity of roselle calyx ethanolic extract.

Recently, we introduced an inexpensive *in vivo* platform to assess the anti-staphylococcal activity of green algae *Ulva reticulata* (14). In this platform, we used wild type and immunodeficient fruit fly (*Drosophila melanogaster*) as host organisms in *S. aureus* infection and made use of two simple phenotypic assays: survival assay and determination of bacterial load (14). To date, *D. melanogaster* has been recognized as a pioneer model organism in the revelation of important signaling pathways related to immunity against not only bacterial infection (15,16) but also viral infection (17-19). In addition to that, *D. melanogaster* has been introduced as one of emerging human disease models (20,21) and considered as one of suitable model organisms in the drug discovery (22-25).

Drosophila melanogaster offers great advantages as an *in vivo* model system in antibacterial drug discovery research. In addition to the fact that it has been shown as an appropriate host for *S. aureus* (14,26-28), *D. melanogaster* shares high degree of genetic similarity with human (up to 75%) (21) which suggests high possibility for getting the same results upon clinical trial on human subjects. Moreover, the use of fruit flies as a model organism raises almost no ethical issues and requires little effort in the stock maintenance (20,21,29). Based on such advantages, we carried out this research to investigate the *in vivo* anti-staphylococcal activity of roselle extract against *S. aureus* in fruit flies model of infection.

Materials and Methods

Bacterial strains and fly stocks

Fresh culture of *S. aureus* ATCC 29213 strain was used as an infectious agent in the entire experiments. To obtain the appropriate inoculum, the bacteria was cultured in Nutrient Broth (NB) medium at 37°C for 1 × 24 hours, collected and washed thoroughly with PBS followed by quantification using spectrophotometry method. The inoculum of *S. aureus* was suspended in PBS prior to use in the experiments. The following lines of Drosophila were used in this study: w^{1118} as genotype (background) control and $spz^{rm7/TM6}$ as immunodeficient fly line (gifts from Prof. Yoshinobu Nakanishi, Kanazawa University) which has impaired activity of Spätzle. All fly lines were maintained with standard cornmeal-agar medium at 25°C.

Extract preparation

Samples of roselle (*Hibiscus sabdariffa* L.) calyces were obtained from Makassar area, South Sulawesi, Indonesia. These samples were sorted and subjected to maceration procedure using 96% ethanol for 1 x 24 hours and re-macerated for 1 x 24 hours. All filtrates were collected and further processed using rotavapor until extract with appropriate thickness were obtained. The resulting extract was stored in a brown silica container.

Fly infection and assays for survival and bacterial growth

Infection experiment was carried out on either background control or immunodeficient fly line using pricking method, in which bacteria was introduced into the thorax of male adult flies, as described previously (14). In brief, flies at age of 4–7 days after eclosion (10 flies per vial, 3 vials in each experiment) were subjected to bacterial pricking (6×10^5 cfu/mL per fly) and further maintained at 29°C. Pricked flies were then subjected to either survival assay or colony forming assay as described previously (14). For the survival assay, the flies were pricked with *S. aureus* and maintained in vials in the presence or absence of treatments and number of live flies at each group was recorded daily during experiment. For the colony forming assay, flies were pricked with *S. aureus* and maintained in vials in the presence or absence of treatments. At a given time, live flies were taken from each group and mechanically processed using a micropestle in PBS solution. Homogenates obtained from each group were subsequently prepared at a serial dilution and then plated on Vogel-Johnson agar medium. Number of colonies appeared after incubation was expressed as colony-forming unit (cfu) per ml. Groups of healthy flies pricked with PBS were used as controls in both survival and colony forming assays.

Gene expression analysis

Total RNA was extracted from five live *Drosophila* harvested from each group of treatments at 48 hours post infection and homogenized manually in the Treff tube using Micropestle followed by further processing using SV Total RNA Isolation System (Promega) according to the manufacturer's protocol. Analysis of *drosomycin* (*drs*) level was carried out using one set of *drs* primer (sequence of *drs* forward primer: 5' - CGT GAG AAC CTT TTC CAA TAT GAT G - 3' and sequence of *drs* reverse primer: 5' - TCC CAG GAC CAC CAG CAT - 3') in a 20 µl reaction volume using GoTaq® 1-Step RT-qPCR System (Promega), as per manufacturer's instruction. Ribosomal protein *rp49* was used as host reference gene and examined by using one set of *rp49* primer (sequence of *rp49* forward primer: 5' - GAC GCT TCA AGG GAC AGT ATC TG - 3' and sequence of *rp49* reverse primer: 5' - AAA CGC GGT TCT GCA TGA G - 3') using the same protocol. Rotor-Gene Q thermal cycler (Qiagen, Germany) was used with the following profile: 37°C for 15 minutes, 95°C for 10 minutes, and 40 cyclic repeats of 95°C for 10 seconds and 60°C for 30 seconds, and 72°C for 30 seconds. A

standard melt curve analysis was carried out to confirm that only the expected product had been amplified. The abundance of *drs* relative to the host reference gene *rp49* was determined using qGENE software.

Data processing and statistical analysis

Results obtained from at least three independent biological replicates were processed using GraphPad Prism® 7. Survival curve was prepared as a Kaplan-Meier curve and followed by log-rank analysis. For analysis of CFU and mRNA level of *drs,* data were prepared as bar graphs and analyzed statistically using one-way ANOVA. For all statistical analysis, data were presented as mean ± S.D and *P* values of less than 0.05 were considered significant.

Results

Dose-dependent toxicity of roselle calyx extract in adult *Drosophila melanogaster*

In this experiment, we used *in vivo Drosophila* platform to assess the antibacterial effect of roselle on *S. aureus.* Prior to antibacterial activity assessment, we carried out a simple survival assay to determine the safest concentration of roselle calyx extract to be used in the further experiments. As shown in Figure 1, *D. melanogaster* w^{1118} (Figure 1A) and *spz* mutant line (Figure 1B) were succumbed to immediate and gradual death upon ingestion of roselle calyx extract at high concentrations (20% and 10% w/w, respectively). However, the survival of flies was seemingly not affected once maintained in foods containing roselle calyx extracts at lower concentration (5% w/w), indicating the toxicity effect of this extract on *D. melanogaster* was occurred in a dose-dependent manner. Therefore, to rule out the possible influence of lethal effect of extract, we used roselle calyx extract at a concentration of 5% (w/w) in further experiments.

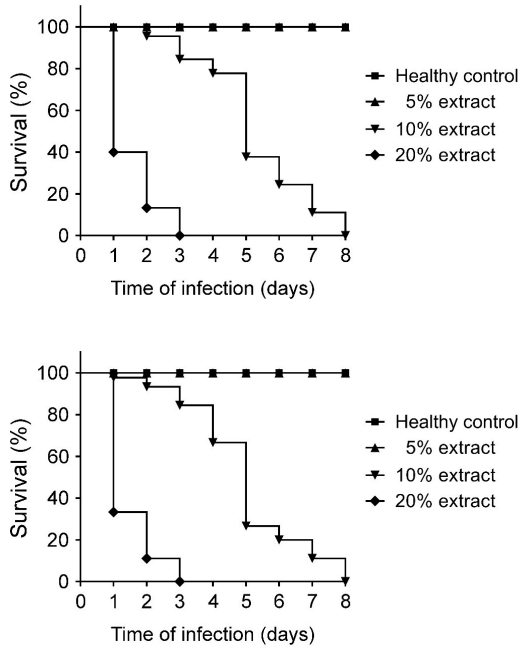

Figure 1. Ingestion of roselle calyx extract reduced the survival of *D. melanogaster* in a concentration-dependent manner. Adult flies w^{1118} (A) and $spz^{rm7/TM6}$ (B) with age of 4-7 days were fed roselle calyx extract-containing food at three different concentrations (5, 10, and 20 %) and incubated at 25°C. Number of dead flies were observed daily and fly survival was carried out using a Kaplan-Meier-Log Rank analysis.

Improvement of *Drosophila melanogaster* survival rate by roselle calyx extract under infection condition

Staphylococcus aureus is a gram-positive bacterium that has been shown to exert wide-ranging negative effect on many organisms including humans (31) and *D. melanogaster* (27). As shown in Figure 2A, infection of *D. melanogaster* w^{1118} by *S. aureus* resulted in the reduction of flies' survival rate. However, treatment of *S. aureus*-infected *Drosophila* w^{1118} with tetracycline, a potent bacteria protein synthesis inhibitor, was sufficient to prevent host early death phenotype, as documented by previous investigator (27) and recently confirmed by our group (14). In addition to that, treatment using ethanolic extract of roselle calyx at concentration of 5% was able to improve the survival of *S. aureus*-infected *D. melanogaster*, demonstrating the *in vivo* antibacterial activity of roselle calyx extract against *S. aureus* at the corresponding concentration.

Inhibition of *Staphylococcus aureus* growth by roselle calyx extract

Decreased survivorship of *D. melanogaster* upon *S. aureus* infection has been shown to be associated with increased

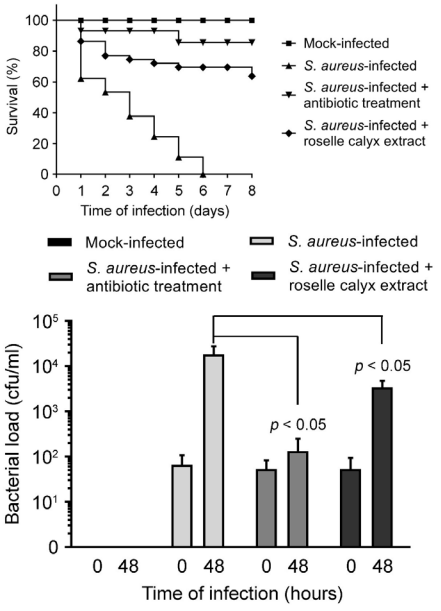

Figure 2. Improvement of host survival and reduced bacterial load in infected w^{1118} flies in the presence roselle calyx extract. Adult w^{1118} flies at 4-7 days after eclosion were infected with 6 x 10^5 cfu/ml of *S. aureus* by pricking, incubated at 25°C in the presence of 5% roselle calyx extract, and subjected to fly survival (A) and bacterial load (B) analysis. Flies treated with tetracycline at 200 µg/ml were used as a positive control group.

bacterial load (27) and inhibition of bacterial growth by antibiotics improved the survival of infected host (27, 32). Taken this into account, we anticipated the reduction of bacterial load will be seen in the infected flies treated with roselle calyx extract. To examine this, we carried out a colony forming assay. As a result, we found that treatment of *S. aureus*-infected flies with either tetracycline or roselle calyx extract was certainly beneficial to reduce the bacterial load (Figure 2B), further supporting the notion that inhibition of bacterial growth by antibiotic or certain extract with potent antibacterial activity could lead to increased survivorship of infected flies.

Anti-staphylococcal activity of roselle calyx extract was not due to increased expression of antimicrobial peptide Drosomycin

Upon infection with gram-positive bacteria, *Drosophila* produced several antimicrobial peptides (AMP) that serve as part of humoral immune responses (15,33). One of the well-characterized AMPs in *Drosophila* against gram-positive bacteria such as *S. aureus* is called drosomycin (33), which is expressed by *drs* gene. To confirm whether drosomycin plays a role in the anti-staphylococcal activity of roselle extract, we examined the expression level of *drs* gene using RT-qPCR method. We found that the expression of *drs* was significantly induced upon *S. aureus* infection and such state was also observed in the tetracycline-treated or the extract-treated *S. aureus*-infected groups (Figure 3). Furthermore, the expression of *drs* was not statistically significant among all treatments, suggesting that treatments using either tetracycline or roselle calyx extract did not aggravate increased induction of *drs*. This result indicates that antibacterial activity of tetracycline or roselle calyx extract at the given concentration was not related to the increased production of drosomycin.

Anti-staphylococcal effects of roselle calyx extract in the immunodeficient model system

Improvement on the survival rate of *S. aureus*-infected host and reduction of the *in vivo* bacterial load upon treatment with roselle calyx extract were most likely not due to the increased production of drosomycin. Therefore, we considered that the protection might be conferred as the result of interaction between antibacterial compounds contained in the extract and *S. aureus* found in infected flies. To further clarify this, we conducted infection experiments on a mutant fly line lacking for Spätzle, an important component in the Toll-mediated humoral immune response against *S. aureus*. As shown in Figure 4, Spätzle-immunodeficient mutant flies succumbed earlier (Figure 4A) with a little higher bacterial load (Figure 4B) than the genotype control flies (compared to Figure 2A and 2B) upon infection with *S. aureus*, clarifying the important role of Toll-mediated protection against *S. aureus* infection. Nevertheless, treatment of *S. aureus*-infected mutant fly line lacking for Spätzle with ethanolic extract of roselle calyx at concentration of 5% (w/w) was sufficient to inhibit bacterial proliferation (Figure 4B) and led to the improvement of mutant flies' survivorship

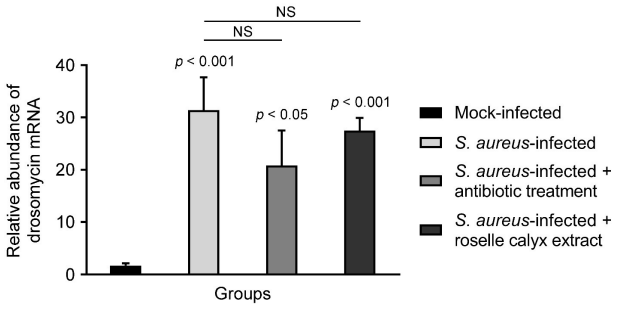

Figure 3. Anti-staphylococcal protection of roselle calyx extract was achieved independent of humoral immunity stimulation. Adult w^{1118} flies at 4-7 days after eclosion were infected with 6 x 10^5 cfu/ml of *S. aureus* by pricking, incubated at 25°C in the presence of 5% roselle calyx extract, and subjected to RNA isolation followed by drosomycin mRNA level quantification by RT-qPCR. Expression of reference gene *rp49* was used as the internal control.

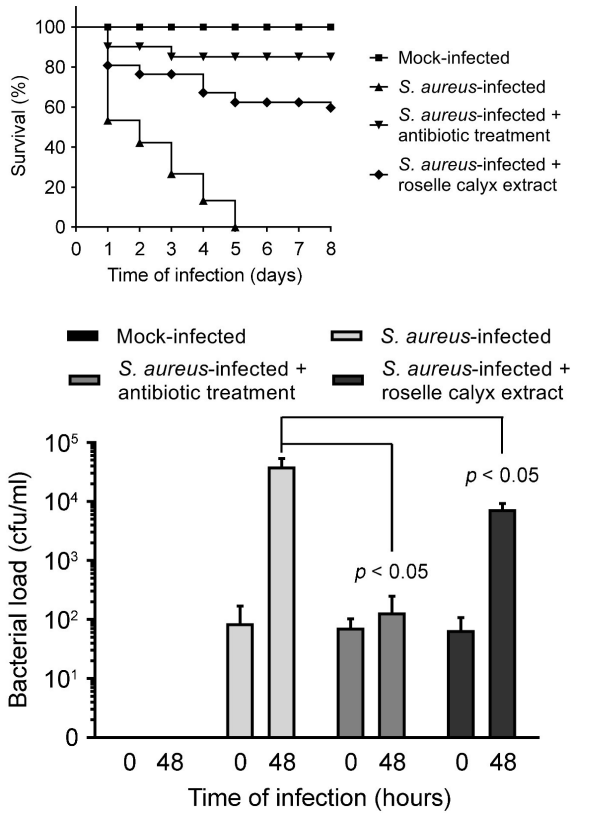

Figure 4. Enhancement of host survivorship and impaired bacterial proliferation in infected immunodeficient $spz^{rm7/TM6}$ flies in the presence roselle calyx extract. Adult $spz^{rm7/TM6}$ flies at 4-7 days after eclosion were infected with 6 x 10^5 cfu/ml of *S. aureus* by pricking, incubated at 25°C in the presence of 5% roselle calyx extract, and subjected to fly survival (A) and bacterial load (B) analysis. Flies treated with tetracycline at 200 µg/ml were used as a positive control group.

(Figure 4A). Taken together, our results demonstrated the anti-staphylococcal activity of roselle calyx extract in *Drosophila* and further clarified that its antibacterial action was not due to the increased activity in Spätzle-mediated production of drosomycin.

Discussion

Infusion prepared from roselle calyces has been generally used by populations from India and countries of Africa as a traditional remedy to treat various illnesses, including infectious diseases (30) and its extract has been shown to yield excellent *in vitro* antibacterial activities against several bacteria, including *S. aureus* (12,13). However, until now, limited information was available regarding the *in vivo* anti-staphylococcal activity of this particular preparation. Here, we used a simple and inexpensive *in vivo Drosophila* platform to assess the anti-staphylococcal activity of roselle calyx extract.

As a well-known member of the gram-positive bacteria, *Staphylococcus aureus* has been shown to cause extensive negative impacts on the health of many organisms including humans (31) and *D. melanogaster* (27), thus would be a prospective target for antibacterial drug discovery. Recently, we found that *Ulva reticulata* extract that has been demonstrated to yield a broad antibacterial activity in the *in vitro* experiment was able to inhibit *S. aureus* growth in adult *Drosophila* and this led to a decline in the infected host mortality (14). Taken this into account, we used a similar approach in this research by carrying out straight-forward survival assays and colony forming assays on the *S. aureus*-infected flies in the presence of roselle calyx extract.

Survival assay and colony forming assay are simple yet powerful methods to examine the pharmacological effect of treatment given to live objects that are under certain pressure (in this case, *S. aureus* infection). From the results, it can be seen that improved survivorship of infected flies was accompanied by the decreased propagation of *S. aureus* in genotype control and immunodeficient mutant fly groups that were treated with food containing roselle calyx extract, suggesting the antibacterial effect of this extract was extended to the individuals *in vivo* independent on their immune response status and at the same time demonstrating the applicability of survival and colony forming assays to be used together with our established *in vivo* platform to screen for medicinal plants that are potential as sources to discover antibacterial agents against *S. aureus*.

Conclusion

In this research, for the first time, we demonstrated the *in vivo* antibacterial effect of roselle calyx extract against *S. aureus* using a straight-forward and inexpensive Drosophila platform system. In conjunction with currently available *in vitro* high-throughput screening method, this system would serve as a powerful *in vivo* approach to clarify the antibacterial activity of not only roselle calyx extract but also numerous medicinal plant extracts prior to be used in further efforts to discover important antibacterial compounds from the corresponding sources. Moreover, this approach will be useful to improve our chance to obtain more appropriate compounds in the initial screening as well as to rule out toxic compounds that are harmful to the eukaryotic cells.

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Authors' contributions

MA, SS and FN designed the study and conceptualized the experiments, MA, AVG and FN performed the experiments and analyzed the data, MA and FN drafted the manuscript. SS, EW and FN contributed to research materials and supervision of the study. All authors approved the final version of manuscript.

Conflict of Interests

We declare that we have no conflict of interest.

Ethical Considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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