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Antimicrobial Effect of Roselle (*Hibiscus sabdariffa* L.) Water Fraction against *Pseudomonas aeruginosa* using *Drosophila* Infection Model

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Abstract: *Pseudomonas aeruginosa* is one of the most common pathogenic bacteria that cause nosocomial infection. Unfortunately, the irrational use of antibiotics has created a surge in *P. aeruginosa* resistance nowadays. To overcome this situation, new antibacterial compounds are urgently needed. One of the potential sources to obtain such antibacterial compounds is roselle calyx. This research was carried out using two experimental approaches, survival assay and gene expression analysis, to examine the *in vivo* antibacterial effect of water fraction of roselle calyx (WFR) against *Pseudomonas aeruginosa* in *Drosophila* model of infection. Survival assay was used to demonstrate the impact of treatment on the lifespan of the infected host. The measurement of immune-related *Dpt* mRNA levels by reverse-transcriptase quantitative PCR (RT-qPCR) was used to assess whether immunostimulation is involved in the antibacterial protection of WFR against *P. aeruginosa*. The result demonstrated that WFR at concentrations of 0.8% and 2% were able to enhance P. aeruginosa-infected flies' survival. Furthermore, gene expression analysis showed the insignificant difference between WFR-treated flies and healthy control flies at all tested concentrations, implying the non-involvement of *Imd-Dpt*-mediated pathway immunity in the antipseudomonal protection of WFR. Taken together, our data suggested the *in vivo* antibacterial activity of WFR against *P. aeruginosa* in the fruit fly model of infection.

Keywords: roselle; *Hibiscus sabdariffa*; antimicrobial; *Pseudomonas aeruginosa*; *Drosophila melanogaster*; drug screening.

1. Introduction

Pseudomonas aeruginosa is one of the most frequent pathogenic bacteria recovered from hospitalized patients, thus regarded as an important cause of nosocomial infections [1, 2]. In general, infections by this pathogen develop rapidly, and optimal treatment heavily depends on appropriate therapy initiation in patients [2, 3]. However, recent reports have urged the emergence of antibiotic resistance problems globally [4, 5]. Indeed, misusing and overusing antibiotics has promoted multi-drug antibacterial resistance in many bacteria, including P. aeruginosa, which ultimately makes the treatment less effective [5].

At present, the emergence of bacteria with an antibiotic-resistant profile is an urgent public health threat [5, 6]. Such a situation has encouraged the search for effective antibacterial agents, including harvesting compounds with new structures and/or novel mechanisms of

action from diverse sources such as plants, marine organisms, and others [7-9]. Plant extracts have been one of the very interesting resources for scientists to achieve such purposes. Compounds derived from plant extracts have been shown to yield direct activities on bacterial growth and metabolism [10, 11], and/or through indirect activity, combined with the selection of established antibiotics, to modify the antibiotic-resistant profile of certain bacteria [12].

Roselle, *Hibiscus sabdariffa* L., is an annual tropical and sub-tropical plant with a promising use as herbal medicine remedy [13]. Previous studies have demonstrated that the calyces of roselle exhibit several pharmacological activities such as hypocholesterolemic, antioxidant, antihypertensive, antimicrobial, anti-inflammatory, antidiabetic, and anticarcinogenic [13-17]. A number of chemical compounds have been isolated from roselle flower extract and subsequently characterized by phytochemical analysis. Some of them are alkaloids, flavonoids, saponins, steroids, triterpenoids, tannins, anthocyanins and hibiscus protocatechuic acid, which all could be used as a resource for therapeutically useful products [13, 18]. Interestingly, a study by Liu *et al.* (2005) has reported that protocatechuic acid effectively inhibits the growth of pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* [19].

Drosophila melanogaster has been used as a model organism to explore numerous biological spectacles, including the role of host innate immune responses to combat bacterial [20] and viral [21, 22] infections, the protective effect of viral infection against cancer [23], the mechanistic basis of sterile inflammation in metazoan species [24] and even the translational relevance of fruit fly in drug repurposing and drug discovery [25]. In the field of drug discovery, *D. melanogaster* has attracted researchers to use this insect as a model organism in the investigation of therapeutic activities of natural compounds derived from plants, including examining their antimicrobial effect against certain pathogens [15, 26-28]. The application of *D. melanogaster* as an *in vivo* model organism in anti-infective drug discovery has been suggested to be useful due to its simplicity, inexpensiveness, and powerful applicability in different types of experiments [20, 29]. Furthermore, *Drosophila* is an effective infection model in studying the antistaphylococcal activity of roselle calyx extract [15] and green algae *Ulva reticulata* against *S. aureus* [26] and *P. aeruginosa* [27]. In the end, the use of *Drosophila* in the investigation of chemicals and/or natural compounds effects on signaling pathways responsible for antimicrobial pharmacological effects is a feasible and economical approach to undertake, especially in low- to middle-income countries [20, 29, 30].

2. Materials and Methods

2.1. Bacterial strains and fly stocks.

The infectious agent, *Pseudomonas aeruginosa* ATCC 27853 strain, was used in all bacterial infection experiments conducted in this study. The bacteria were periodically subcultured in a new Nutrient Broth (NB) medium at 37°C. The w^{1118} line of *Drosophila* was used in this study. Male flies were used in the entire experiment, and they were anesthetized with CO_2 before carrying out bacterial infection experiments. Flies were maintained in standard conditions (25°C, 12 hours light, 12 hours dark cycle, standard cornmeal-agar food).

2.2. Plant extract preparation.

Roselle (*H. sabdariffa* L.) calyx samples were obtained from the garden of the Faculty of Pharmacy Makassar, South Sulawesi, Indonesia. The water fraction of roselle calyx extract (WFR) was prepared by maceration using 96% ethanol for 1×24 hours and re-maceration for 1×24 hours. All filtrates were further evaporated in rotary evaporators until suitable thickened extracts were obtained. The resulting extract was then subjected to a fractionation procedure using ethyl and water to obtain the water fraction before further processing to reduce the water content. The obtained WFR was stored in a light-protected silica container before use.

2.3. Fly infection and survival assay.

Several methods are generally used to inoculate adult flies with bacterial cells: needle pricking, injector pumping, and feeding [31]. In this study, we applied wound infection of *P. aeruginosa* using the needle pricking method due to its simplicity, robustness and requiring only a tungsten needle for pricking. According to the established procedures, the bacteria were locally pricked into the thorax of adult male flies [27, 31]. We used a 0.4 mm (diameter) needle that had been dipped in a bacterial suspension to inflict a wound at the flies' dorsolateral thorax. Briefly, as shown in Fig. 1A, flies at the age of 4–7 days after eclosion (10 flies per vial, 6 vials in each experiment) were pricked with a bacterial suspension (1×10^5 cfu/ml bacteria per fly). The w^{1118} flies infected with bacteria were maintained at 29°C and subjected to survival assay. A Group of healthy flies was also included. In the assay, the survival ability of flies, in the presence or absence of treatments, was observed during the course of infection.

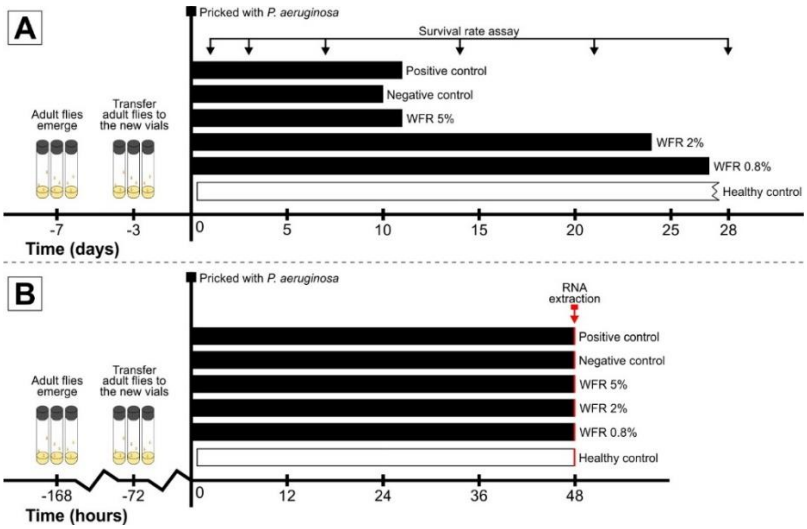

Figure 1. Timeline of the experimental design. (A) Adult flies of each group except the healthy control group were pricked with *P. aeruginosa* then transferred to new vials containing fly food with tetracycline or different concentrations of WFR. Survival rates were observed daily after bacterial injection. The end of each bar means all flies in that group were completely succumbed to infection. (B) Aligned with survival rate assay, adult flies were pricked with *P. aeruginosa* then maintained in the vials according to their treatment groups. RNA extraction was carried out 48 hours after bacterial pricking, followed by an amplification process using RT-qPCR method.

2.4. Gene expression analysis.

RNA was extracted from five live *Drosophila* harvested from each treatment group at 48 hours post-infection and transferred into the Treff tubes (Fig. 1B). Total RNA was extracted using RNA Isolation System (Promega) according to the manufacturer's protocol. The Diptericin (Dpt) expression level was quantitatively determined using the reverse transcriptase

quantitative PCR (RT-qPCR) method. Analysis of *Dpt* level was carried out using one set of *Dpt* primer (sequence of *Dpt* forward primer 5' – GTT CAC CAT TGC CGT CGC CTT AC– 3' (20-mer) and sequence of *Dpt* reverse primer: 5' – CCC AAG TGC TGT CCA TAT CCT CC– 3' (19-mer) in a 20 µl reaction volume using GoTaq® 1-Step RT-qPCR System (Promega) as per manufacturer's instruction. To confirm that only the expected product had been amplified, a standard melt curve analysis was carried out in every run. Using a similar RT-qPCR protocol, RNA level of host ribosomal protein *rp49* (used as an internal control) were examined by using one set of *rp49* primer (sequence of *rp49* forward primer: 5' – AGA TCG TGA AGA AGC GCA CCA AG – 3' (23-mer) and sequence of *rp49* reverse primer: 5' –CAC CAG GAA CTT CTT GAA TCC GG– 3' (19-mer). RotorGene Q thermal cycler (Qiagen, Germany) was operated 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.

2.5. Data processing and statistical analysis.

Results obtained from at least two independent biological replicates were processed using GraphPad Prism® 8. The survival assay data was summarized as a Kaplan-Meier graph and analyzed statistically using the Log-Rank approach. The calculated level of *Dpt* mRNA obtained in this study was prepared as a bar graph and analyzed statistically using one-way ANOVA followed by Tukey HSD posthoc analysis. For all statistical analyses, data were presented as mean ± S.D, and p values of less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Survival of Drosophila model of P. aeruginosa infection.

Several available routes for administering drugs to the adult flies can vary depending on the type and form of the drugs to be given [32]. Drugs in the form of gas or aerosols such as ethanol and cocaine can be administered by vaporization. Alternatively, drugs can be mixed into the fly food then fed to the flies. It also can be directly injected into the specific body part of the flies that have been dissected (decapitation) or be injected into the abdomen so that the drug can quickly diffuse through the whole body of *D. melanogaster* [32]. In this experiment, the water fraction of roselle (WFR) was prepared as a mixture with the fly food and then administered through feeding. The main reasons for using this method are the ease of measurement of the acute effects of a drug and the highest throughput that can be achieved by this administration.

We previously used *Drosophila* as a simple model organism in the *in vivo* platform for assessing the antibacterial effect of roselle extract against Pseudomonas *aeruginosa* [Unpublished Data]. We observed that *Drosophila* succumbed to *P. aeruginosa* infection, similar to a published report by another investigator [31], possibly due to the increasing pathogenic burden that occurred in the infected host body. A survival assay is often used to observe the effect of infection on host lifespan [30]. This assay is useful to measure the ability of *D. melanogaster* to survive after infection with *P. aeruginosa* in the presence or absence of treatments.

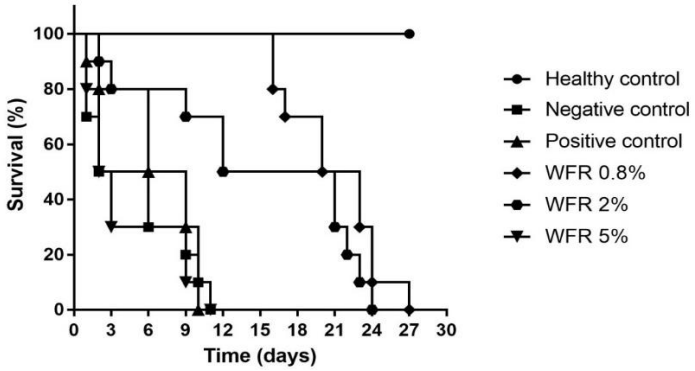

Figure 2. Survival rate of *Pseudomonas aeruginosa*-infected flies after administration of water fraction of roselle (WFR). Adult, 4-7 days after eclosion, w^{1118} flies were infected with 1.0 x 10^5cfu/ml of *P. aeruginosa* by needle pricking followed by treatment with fly food containing 0.8%, 2%, and 5% WFR or tetracycline (TET) at 200 µg/ml (as the positive control). All groups were subjected to fly survival assay.

In this study, P. aeruginosa-infected flies' survival was assessed in the presence or absence of different concentrations of water fraction of roselle (*Hibiscus sabdariffa* L.). Flies in the positive control group were treated with tetracycline. The results of *survival assay* using adult male of w^{1118} *D. melanogaster* demonstrated that around 30 % of *P. aeruginosa*-infected flies (negative control group) were succumbed to the infection on the first day of observation, whereas uninfected healthy flies remain 100% alive, even until the end of study (Fig. 2). This result clearly suggests that bacterial infection posed a deadly effect on *Drosophila* lifespan. In addition to that, flies in the positive control group (with tetracycline administration) demonstrated a survival percentage of as much as 90% on the first day of observation, indicating that tetracycline provided antibacterial protection against *P. aeruginosa* (Fig. 2), similar to our previous observation [27]. Tetracycline hydrochloride is a broad-spectrum bacteriostatic antibiotic that prevents bacterial protein synthesis via competitive binding to the 30S and possibly 50S of bacterial ribosomal subunits [33].

Further analysis on Fig. 2 revealed that treatment of *P. aeruginosa*-infected flies with WFR did not occur in a dose-dependent manner. While the highest protection was provided by treatment using the lowest concentration of WFR used in the study (0.8%), higher concentrations of WFR (2% and 5%) did not provide better outcomes, suggesting that some components in the WFR may exhibit a toxic effect on *Drosophila*. It is quite peculiar but remains steadfast as a possible explanation. Compounds responsible for the protective properties of WFR against *P. aeruginosa* infection remain unknown but possibly highly related to the water-soluble bioactive compounds in WFR, for example, anthocyanins and phenolics that have been widely known for their antimicrobial activities [34].

3.2 Antipseudomonal protection of roselle is independent of diptericin stimulation.

Increased survivorship of *P. aeruginosa*-infected flies upon treatment with WFR indicates that WFR may directly inhibit the growth of *P. aeruginosa* in the host body and/or by collaterally impair bacterial growth via stimulation of the host immune response toward *P. aeruginosa*. Future investigation on bacterial growth inhibition would be an important issue to consider. However, based on our experience using the *Drosophila* model system, experiments to assess whether the given treatment stimulates the immune response of infected hosts will provide better value in searching for antimicrobial candidates.

In response to infection by Gram-negative bacteria, *D. melanogaster* activates its specific antibacterial defense so-called Immune deficiency (IMD) pathway [35]. IMD pathway

regulates Antimicrobial Peptide production (AMP) as Peptidoglycan Recognition Proteins (PGRPs) sense the signal produced by a bacterial infection. For instance, the regulation of *Diptericin, Dpt* gene expression after flies being infected with Gram-negative bacteria [35], including *P. aeruginosa* [27]. Assessment of *Dpt* gene expression via IMD pathway in *D, melanogaster* could help to determine the mechanism of action by which WFR exerts its antibacterial effect, whether it is only produced through the possibility of direct interaction with *P. aeruginosa* or also through modulation of host immune system.

As can be seen in Fig. 3, expression of *Dpt* in *P. aeruginosa*-infected *D. melanogaster* in the absence of any treatments (negative control group) were dramatically enhanced compared to the uninfected ones (healthy control group), similar to our previous observation [27]. On the contrary, the low level of *Dpt* expression in the positive control group suggests that tetracycline administration could protect *Drosophila* from infection, possibly via the reduction of *P. aeruginosa* as what was seen in our previous study [27], thus causing the host to suddenly reduced its immune response.

Further analysis on Fig. 3 revealed that the expression of *Dpt* in flies treated with WFR at different concentrations was much lower than those measured in the negative control group, implying that WFR seemed to have no activity in the modulation of IMD-*Dpt* immune response axis. In addition, the expression profile of *Dpt* in the positive control group and the infected groups treated with WFR at a concentration of 0.8%, 2%, and 5% were not significantly different from the healthy control group, indicating that the immune system of *D. melanogaster* was possibly returned to the homeostatic condition of which resulting in an increased lifespan in the affected *Drosophila*. Taken together, data obtained in this study indicate that WFR increased flies' survival upon *P. aeruginosa* infection, and this effect has occurred independently of the modulation of the host immune system. To better identify this possibility at a protein level, western blots analysis can be used as an alternative technique to generate supporting data in addition to the RT-qPCR data.

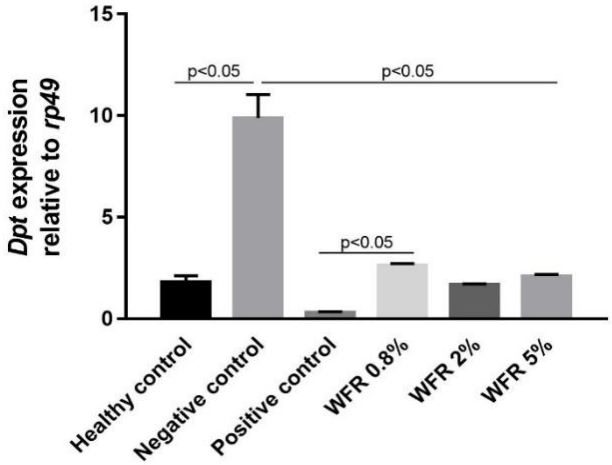

Figure 3. *Dpt* mRNA level of *D. melanogaster* after being infected with *Pseudomonas aeruginosa* (PA). Adult, 4-7 days after eclosion, w^{1118} flies were infected with 1.0 x 10^5cfu/ml of PA by needle pricking then treated with fly food containing 0.8%, 2%, and 5% WFR as well as tetracycline (TET) at 200 µg/ml as a positive control. RNA was extracted from five live *Drosophila* in each group at 48 hours post-infection, followed by *Dpt* RNA level quantification by RT-qPCR compared to *rp49* RNA level as an internal control gene. *Dpt* is expressed in response to Gram-negative bacterial infection.

While the detailed mechanism(s) of anti-pseudomonal activity of the WFR remains largely unknown, our preliminary results suggested that the effect was mainly produced via

direct interaction of the WFR component with *P. aeruginosa* without the enhancement of the IMD-mediated host immune responses. However, it is important to note that our current results could not rule out other components in the IMD pathway or even the possible involvement of other potential antibacterial pathways available in *Drosophila*. For such purpose, the use of immunodeficient *Drosophila* in the infection experiment would be the next suitable approach to consider [26, 27, 30]. In addition to that, while the importance of our data remains to be demonstrated in higher model organisms such as rodents, we believe that the high similarity of genes shared by *Drosophila* and humans [32] will provide a well-established, rapid, easily performed, and scalable approach to employ in high-throughput screening of anti-infective drug candidates from plants and/or other sources.

4. Conclusions

In this research, we demonstrated the *in vivo* antibacterial effect of water fraction of roselle (*Hibiscus sabdariffa* L.) calyx (WFR) against *Pseudomonas aeruginosa* using a fruit fly (*D. melanogaster*) model of infection. Our study was the first to report the potential *in vivo* anti-pseudomonal effect of WFR is an insect platform system. It would be essential to elucidate the characteristics of anti-pseudomonal compound(s) for further examination. Our *Drosophila* infection model can serve as an *in vivo* high-throughput screening platform to investigate certain samples' antibacterial potential such as medicinal plant crude extracts and compounds isolated from the corresponding extracts.

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Conflicts of Interest

The authors declare no conflict of interest.

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