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Immunomodulatory Effect of *Begonia Medicinalis* Ethanolic Extract in *Drosophila*

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Abstract: *Begonia medicinalis* is an Indonesian endemic plant that has been reported to exert multiple pharmacological effects, including antiviral and anticancer activities. These activities have been suggested to be related to the immunomodulatory properties of dried *B. medicinalis* ethanolic extract (DBME). However, this notion remains unclear. This study was carried out aiming to investigate the immunomodulatory effect of DBME in *Drosophila melanogaster* model based on the expression of genes in the Toll, Imd, and JAK-STAT pathways. Phenotypical analysis on the survival and locomotor of *D. melanogaster* was carried out to reveal whether changes in the gene expressions were observable at the phenotypical level. Four groups of *D. melanogaster* were prepared and treated with DBME (0%, 0.25%, 0.5%, and 2%). The results demonstrated that DBME could modulate the expression of *Drs* (Toll pathway), *Dpt* (Imd pathway), and *TotA* (JAK-STAT pathway) in *D. melanogaster*. These molecular changes were accompanied by almost no changes in survival and locomotor activities. In brief, DBME can induce the humoral immune responses of *D. melanogaster* without significant phenotypical trade-offs.

Keywords: Begoniaceae; immunomodulatory; adjuvant; fruit fly; *in vivo*

1. Introduction

The immune system is one of the most complex systems available in many living organisms, from viruses and bacteria to humans, supporting the metazoan's life. Tight coordination of all entities in the immune system is compulsory to firmly recognize and tolerate what belongs to the self and identify and dismiss any foreign (non-self) objects [1]. This system protects the host from pathogens, using diverse host protection mechanisms to control and eliminate pathogenic microorganisms, toxins, and abnormal cells. Firmly equipped with strong effector mechanisms, the immune system can eliminate various microbial cells, harmful and allergenic compounds, and dysfunctional cells [1].

Generally, the immune system is classified into two parts: innate (non-specific) immune system and adaptive (specific) immune system. These two systems work closely to respond to many different tasks. The innate immune system offers rapid and imminent defenses against foreign objects [1]. This system includes phagocytic cells, the complement system, and various classes of pathogen- and damage-associated receptors utilized by innate cells, such as Toll-like receptors (TLRs) [2]. During innate immune responses are generally non-specific and lack the luxury of long-term memory, it provides a simple yet powerful protection to the host against everyday insults, in most cases preventing unnecessary high-cost activation of adaptive immune responses [1].

Traditional medicines, incorporating the use of natural remedies in the treatment of diseases, have been used since ancient times. Claimed as one of the approaches with little adverse effects, compounds isolated from various plants with medicinal properties have significantly contributed to the pharmacological management of infectious- and non-infectious diseases, including the ones related to the immune system [3]. A wide range of phytochemicals, as well as plant extracts, have been known potential to be used in immunomodulatory therapies to manage infections and cancers [4-6], mostly by influencing the activity of immune cells and/or humoral-mediated immunity (for example, antibody production) [7].

Begonia medicinalis is a new species of Begoniaceae, growing and distributed locally in some areas of central Sulawesi, Indonesia, and commonly known as "Benalu Batu". Traditionally, *B. medicinalis* has been used by the Wana tribe and other communities in the central part of Sulawesi for treating certain non-communicable diseases, including cancer and diabetes [8]. The anticancer activity of the methanolic extract of *B. medicinalis* herb plant was previously shown against breast and cervical cancer cell lines (T47D and HeLa cells) [8]. In addition, the antiviral activity of *B. medicinalis* was also recently been reported [9], implying the prospective use of this plant against pathogenic microorganisms. However, detailed mechanisms of the anticancer and antiviral effect of *B. medicinalis* remain elusive. Thus, a preliminary study to provide hints on the possible immunomodulatory mechanism of actions of *B. medicinalis* is critical. Not only to provide scientific explanations of the known biological event (anticancer properties of *B. medicinalis*), but also to encourage further engagements on the scientific discoveries aiming to provide better pharmacological approaches in managing immune-related disorders.

Experimental investigation on the immunomodulatory properties of *B. medicinalis* can be done using a simple yet elegant *Drosophila* model. Many immune pathways, including molecular pathogen detection and nuclear factor-κB (NF-κB) signaling, are evolutionarily conserved between fruit fly *Drosophila melanogaster* and humans [10]. Furthermore, physiological responses triggered in the host upon infection and how dysregulation of these responses contributes to disease are also consistently conserved from flies to mammals, including humans [10, 11]. *Drosophila melanogaster* and the human immune system have similar signaling pathways, including Toll and IMD that are connected to the NF-κB transcription factor, JAK/STAT, apoptosis, autophagy, and RNA interference (RNAi) [11, 12]. We have also recently demonstrated the utility of flies in screening potential immunomodulators [13]. Therefore, in this study, in vivo experiments were performed to examine the immunomodulatory activity of the ethanolic extract of *B. medicinalis* on innate immune responses using *D. melanogaster* as the model organism.

2. Materials and Methods

2.1. Sample identification and extraction.

Parts of *Begonia medicinalis* (leaves and stem) were obtained from Toddopoli village, Soyojaya district, North Morowali, Central Sulawesi, Indonesia. The plant species were determined at Bogor Botanical Gardens (The National Research and Innovation Agency, BRIN). According to the previously published protocol, approximately 2 kg of sample in the form of dried powder was extracted by maceration with 70% ethanol as an organic solvent for 3 × 24 hours [14]. Next, the filtrates were collected and evaporated using the rotary evaporator until an obvious dried *B. medicinalis* ethanol extract (DBME) was obtained. The DBME was stored properly for further experiments.

2.2. Fly stock.

The *Oregon R* line of *Drosophila melanogaster* was used as a model organism in this research (generously provided by the Host Defense and Responses Laboratory, Kanazawa University, Japan). All experiments utilized male flies that were 7-11 days old. These flies were kept in a culture vial filled with standard cornmeal-agar under standard conditions (25 °C, a 12-hour cycle of light and darkness).

2.3. Survival test.

All fly groups were observed for their survival based on the following procedure. Briefly, newly eclosed flies were separated by sex and transferred into vials containing standard fly food. Flies were divided into four groups, and each group contained 30 flies. Group I, designated as the no treatment control (NTC) group, was given standard fly food (0% *B. medicinalis* extract). In comparison, groups II-IV were assigned to receive 0.25 % extract, 0.5 % extract, and 2 % extract, respectively. Survival was measured as the time from when an individual fly entered its experimental vial until a maximum of 8 days. The experimental design utilized in this study is illustrated in Figure 1.

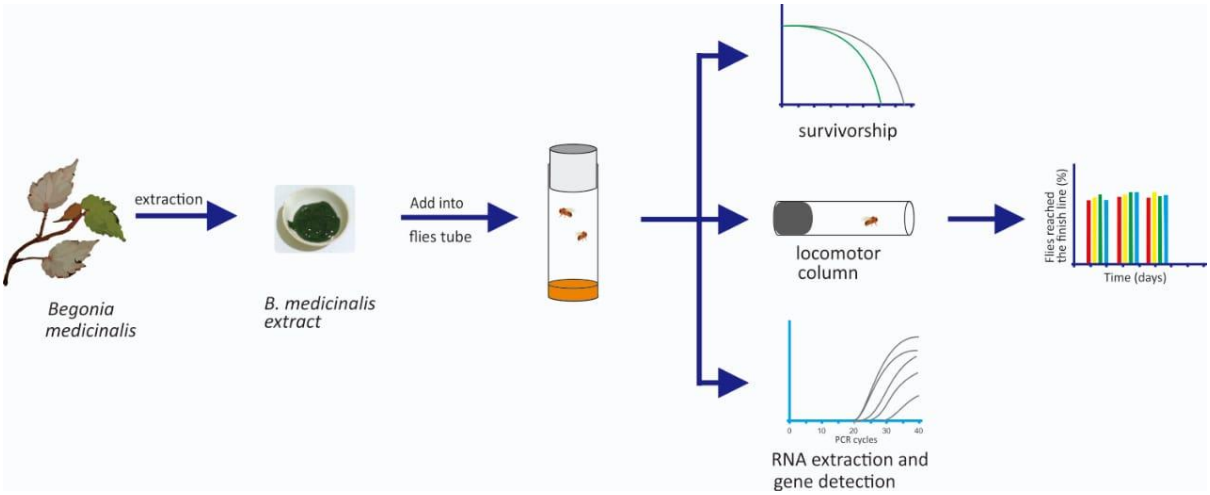

Figure 1. The experimental design used in this study.

2.4. Locomotor test.

All fly groups were observed for locomotor analysis based on the negative geotaxis method, as previously described [15, 16], with slight modifications. Briefly, all flies were assigned into

groups based on their gender orientation prior to subsequent placement into empty vials, designated as the locomotor testing vials. These vials were then set in front of the climbing wall in an upright vertical position. At the beginning of each locomotor test, the testing vials were tapped downward to ensure that, at the same time, every fly began to ascend the vial from the bottom. At the end of this experiment, we monitored and recorded events that occurred in the testing vial for up to 15 seconds. The number of flies that climbed up to pass a marked finish line was counted. Each trial was repeated three times in total.

2.5. Gene expression assay.

Total RNA isolation was performed on all fly groups on days 2 and 4 post-treatment using live flies. Five live flies were subjected to an RNA isolation procedure from each group using Pure Link™ RNA Mini Kit (Invitrogen™, Thermo Fisher Scientific Inc.). The concentration of RNA in all samples was determined using a nano spectrophotometer (BioDrop, Biochrom, Ltd.) and processed using the Reverse Transcriptase Quantitative PCR (RT-qPCR) method. The expression of *Drs, Dpt,* and *TotA* genes was separately examined in all treatment groups by RT-qPCR, in a reaction volume of 10 µl each, using the SuperScript™ III Platinum® SYBR® Green One-Step RT-qPCR kit with ROX (Invitrogen™, Thermo Fisher Scientific Inc.), according to the manufacturer's protocols. RT-qPCR was carried out using the Rotor-Gene Q thermal cycler (Qiagen, Germany), and the level of ribosomal protein rp49 was used as the internal control in the RT-qPCR assay. The running profile of RT-qPCR was as follows: 37°C for 15 minutes, 95°C for 10 minutes, followed by 40 cycles of amplification (each cycle was carried out at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds). The expected amplified product was verified based on the standard melting curve profiles spanning from 60°C to 95°C. All generated data were then processed using Q-Gene® and subjected to gene expression analysis. A list of primers used in the RT-qPCR is provided in Table 1.

Table 1. Primers used in the RT-qPCR assay.

Genes	Forward primer	Reverse primer
Drs	5' – TTG TTC GCC CTC TTC GCT GCT CT – 3'	5' – GCA TCC TTC GCA CCA GCA CTT AC – 3'
Dpt	5' – GTT CAC CAT TGC CGT CGC CTT AC – 3'	5' – CCC AAG TGC TGT CCA TAT CCT CC – 3'
TotA	5'– CCA AAA TGA ATT CTT CAA CTG CT – 3'	5'– GAA TAG CCC ATG CAT AGA GGA C – 3'
rp49	5' – AGA TCG TGA AGA AGC GCA CCA AG – 3'	5' – CAC CAG GAA CTT CTT GAA TCC GG – 3'

2.6. Data analysis.

Data derived from the survival assay is visualized using the Kaplan-Meier approach and statistically analyzed using the Log-Rank approach. At the same time, data obtained from the locomotor experiments were processed and statistically analyzed using a One-Way ANOVA approach followed by post hoc analysis. Data are presented as mean ± S.D, and p-values of less than 0.05 are considered significant. All data were processed and visualized using GraphPad Prism® 9.

3. Results and Discussion

3.1. Insignificant changes in fly survival upon B. medicinalis treatment.

In this research, we investigated the immunomodulatory effect of the ethanolic extract of *B. medicinalis* using a fruit fly model. *Drosophila* is a model organism that has contributed

to many innovative and important discoveries, including identifying critical components of signaling pathways that are conserved among metazoan species, including humans [17, 18]. In this study, we explored the possible immunomodulatory effect of DBME in *D. melanogaster* at both phenotypical and molecular levels (Figure 1). At the phenotypical level, we examine the effect of DBME on the survival and locomotor of flies. Meanwhile, at the molecular level, we examined the possible changes in the expression of Drosomycin (*Drs*), Diptericin (*Dpt*), and Turandot A (*TotA*) genes. *Drosophila* lacks an adaptive immune system and instead relies solely on its innate immunity, which has both humoral and cellular components, to fend against invasive infections or foreign entities. The main components of innate immune responses take advantage of the production of antimicrobial peptides (AMPs) through core signaling pathways (Toll and Imd), antiviral response through the JAK/STAT [10], and RNA interference (RNAi) pathways [19], and pathogens as well as dead cells clearance through phagocytosis [10, 20].

In general, the life span of *Drosophila* shall not be impaired by the pharmacological effects of certain chemical entities [21, 22]. Therefore, survival assay shall serve as a simple and direct experimental endpoint to investigate the harmful effect of potential drug candidates. In this study, we first conducted a survival assay to determine whether DBME could negatively affect the lifespan of *D. melanogaster*. The assay was conducted on males of *D. melanogaster* using four different concentrations of DBME: 0.25%, 0.5%, and 2%. Flies aged 7-11 days were given orally extract *B. medicinalis* and observed for 8 days. As shown in Figure 2, treatment of males of *D. melanogaster* to DBME for 8 days resulted in insignificant changes in fly survival rate. The result suggested that various concentrations of DBME did not cause early death phenotype in flies.

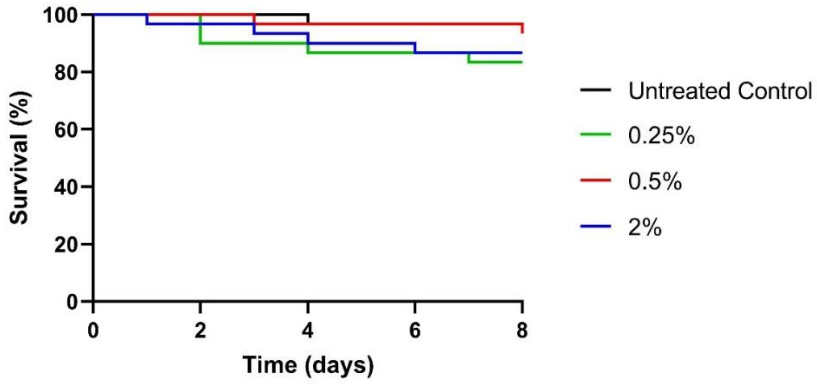

Figure 2. No changes in the survival of male *D. melanogaster* after treatment with DBME at different concentrations. Adult male flies aged of 7-11 days were separated into four groups, and each group were then treated with DBME at different concentrations. The untreated control group used is without any treatments. DBME, dried *B. medicinalis* ethanolic extract.

3.2. No changes in fly locomotor upon B. medicinalis treatment.

Based on the survival result, presented in Figure 2, it is apparent that fruit flies *D. melanogaster* did not experience any negative effect on their lifespan after DBME treatment, at least for 8 days. In addition to the survival assay, we also examined the effect of DBME on the locomotor of flies. Locomotor activity has been suggested to be correlated with longevity [23], and impaired locomotor is sometimes observable upon the introduction of certain exogenous substances/drugs [24]. Therefore, carrying out a locomotor assay may provide additional insight into the negative impact of DBME on the phenotypical trait of metazoan species.

As shown in Figure 3, treatment of male flies with DBME at all concentrations resulted in insignificant changes in locomotor activity, at least during the first four days of treatment. However, flies treated with DBME at 0.5 and 2% started to experience reduced locomotor activity six days after treatment (Figure 3) even though there were no changes in the survival rate on the same day of observation (Figure 2). This result suggested that impaired locomotor might be observable without significant impairment in the survival rate.

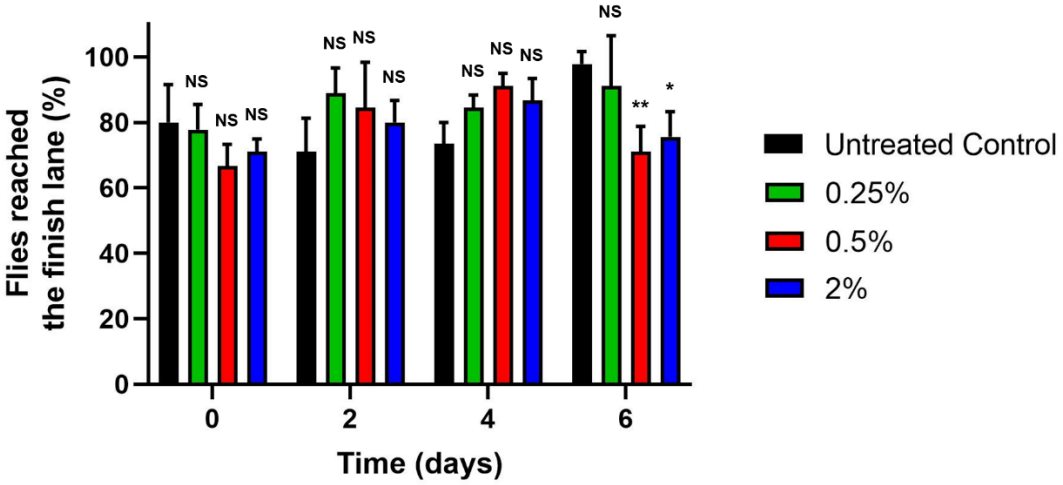

Figure 3. Insignificant changes in the locomotor of male *D. melanogaster* after treatment with DBME at different concentrations.Adult male flies aged of 7-11 days were separated into four groups, and each group were then treated with DBME at different concentrations. The untreated control group used is without any treatments. DBME, dried *B. medicinalis* ethanolic extract; NS, Not significant; * $p < 0.05$; ** $p < 0.01$.

3.3. Modulation of immune genes in Drosophila upon B. medicinalis treatment.

Overall, phenotypical assays demonstrated that DBME treatment resulted in insignificant survival rate changes and locomotor activity, at least after four days of treatment. Whether this is related to the immunomodulatory activity of DBME remains unclear. Since the impaired locomotor activity was observable at 6 days of treatment, we decided to analyze gene expression using samples prepared on days 2 and 4 post-treatment. To do this, an RT-qPCR experiment was carried out, aiming to examine the effect of DBME on the expression of NF-κB and JAK-STAT-related genes that have shown importance in *D. melanogaster* immune responses against pathogens.

In this study, RNA samples were prepared from all groups of treatment (day 2 and 4 post-DBME treatment) and subsequently subjected to RT-qPCR analysis to examine the expression of *Drs* (Toll pathway), *Dpt* (Imd pathway), and *TotA* gene (JAK-STAT pathway). As shown in Figures 4A and 4B, the treatment of male Oregon R flies with DBME at 2 and 4 days, respectively, upregulated all examined genes at certain concentrations. The expression of *Drs* was steadily enhanced in response to the DBME treatment at a concentration of 2%, but not at the other two lower concentrations tested in this study, at 2 days (Figure 4A) and 4 days (Figure 4B) post-treatment. The expression of *Dpt* and *TotA* was also upregulated in both 2 (Figure 4A) and 4 days (Figure 4B) post DBME treatment. The expression of *Dpt* has occurred in a dose-dependent manner in both 2 and 4 days post DBME treatment. However, while the expression trends of *Dpt* were similar to the ones shown by *Drs*, the expression patterns of *TotA* were quite different for both genes. At day 2 post-DBME treatment, the expression of *TotA* was highly upregulated in response to the treatment at a lower concentration (0.25%) but then slightly reduced once the DBME concentrations were increased.

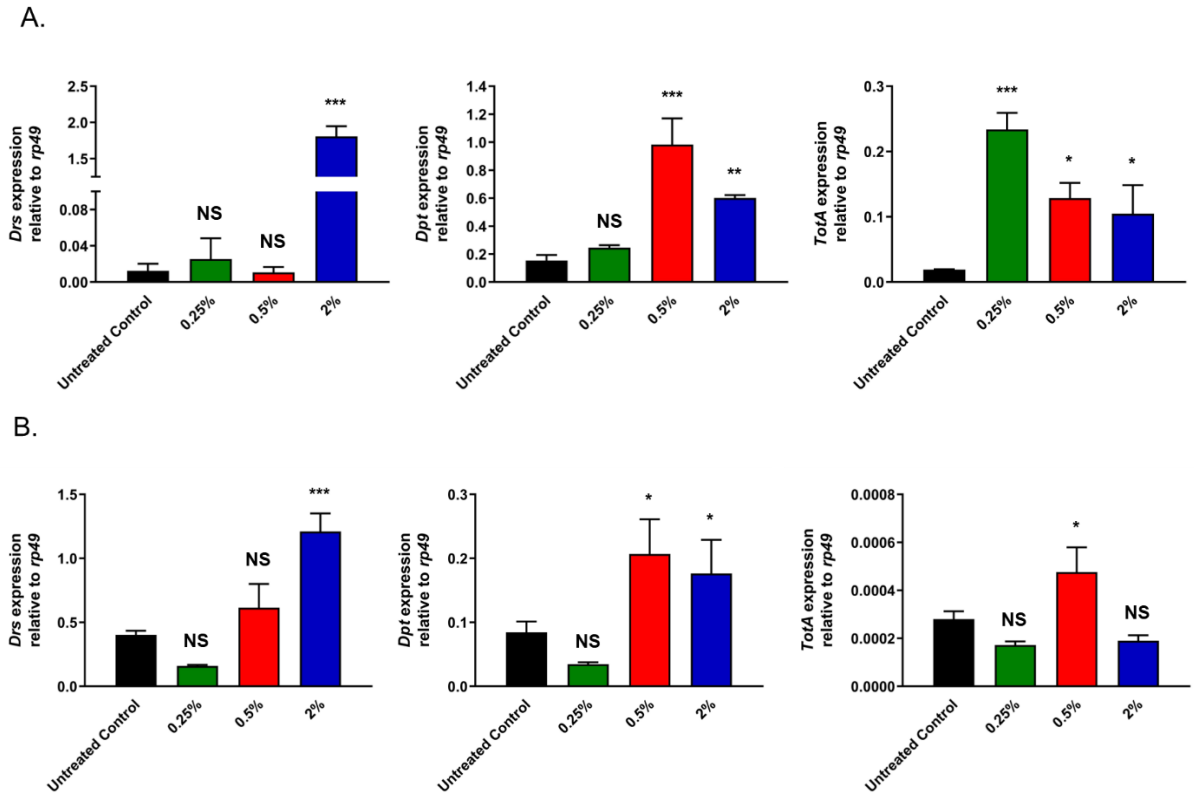

Figure 4. Modulation of *Drs*, *Dpt*, and *TotA* expressions in Oregon R flies upon treatment with DBME. Adult male flies aged 7-11 days were separated into four groups, and each group was then treated with DBME at different concentrations. The untreated control group used is without any treatments. One group maintained in the absence of treatments was used as the untreated control. DBME, dried *B. medicinalis* ethanolic extract; NS, Not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The toll pathway is induced upon the recognition of Gram-positive bacteria and fungi via secreted pattern-recognition receptors. In contrast, the Imd pathway is mainly activated in response to Gram-negative bacteria [25, 26], and the JAK-STAT pathway is stimulated in the presence of viruses [27-29]. Lastly, *TotA*, the inducible genes downstream of JAK-STAT, belongs to a group of distantly related fly genes that are all transcriptionally activated in response to a bacterial challenge or other environmental stressors and regulated by JAK/STAT pathway [30], in coordination with NF-κB-like Relish pathway [31]. These three pathways represent the major immune pathways in *D. melanogaster*, which are directly and indirectly linked to the activity of the NF-κB transcription factor [10].

4. Conclusions

In the current experimental study, we determined the immunomodulatory activity of *B. medicinalis in vivo* on NF-κB- and JAK/STAT-related pathways using *D. melanogaster*. Our findings further confirmed the potential effect of *B. medicinalis* extracts for boosting the immune system of metazoan species in a relatively insignificant phenotypical trade-off.

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Conflicts of Interest

The authors declare no conflict of interest in preparing this article.

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