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Dual Antibacterial and Immunostimulatory Effects of Green Tea Extract in *Drosophila melanogaster*

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Abstract: Bacteria resistant to antibiotics are a major threat to the world community. To overcome this serious problem, discovering new antibacterial agents through the exploration of various potential sources is very important, one of which is using natural ingredients. This study was conducted aiming to determine the *in vivo* antibacterial and immunostimulant activities of green tea extract (GTE) (*Camellia sinensis*) in the *Drosophila melanogaster* larval infection model. Green tea was extracted using n-hexane and 50% ethanol by a multilevel extraction method. Assessments for GTE toxicity as well as its effect on the survival and the modulation of gene expression in *D. melanogaster*, were carried out. The results showed that the administration of GTE was relatively safe for *D. melanogaster* larvae. Furthermore, the administration of GTE to the wildtype Oregon R line of *D. melanogaster* experienced an improved survival rate after being infected with Staphylococcus aureus, suggesting that GTE may provide antibacterial protection against *S. aureus*. A similar phenotypical observation, improvement in the survival rate upon *S. aureus* infection, was evident in the immunodeficient mutant larvae that were treated with GTE. In addition, gene expression analysis demonstrated increased expression of Drs, an antimicrobial peptide produced by *D. melanogaster* in response to infection by *S. aureus*, a Gram-positive bacteria. This result indicates the prospective immunostimulatory activity of GTE in *D. melanogaster*. Taken together, our phenotypic and molecular analysis revealed the dual immunostimulatory and antibacterial activities of GTE against *S. aureus* in a Drosophila larval infection model.

Keywords: green tea; fruit fly; infection model; antibacterial; immunostimulant

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1. Introduction

Green tea is a widely used material containing chemical compounds, including various secondary metabolites such as saponins, alkaloids, and polyphenols, with numerous pharmacological potentials [1-3]. One of the polyphenolic compounds, epigallocatechin-3-gallate (EGCG), has been reported to yield immunomodulatory effects in mice, particularly efficacious on the innate arms of the immune system [4]. EGCG can influence cytokine production and expression, thereby influencing downstream signal transduction. Early research has shown that the effect of EGCG on T cell function is primarily manifested in the change in

lymphocyte proliferation after in vitro EGCG supplementation [5]. Another report described the immunostimulatory effect of green tea extract (GTE) in broiler chickens [6], further supporting the notion that GTE is an herbal medicine with immunomodulatory potentials [7] that could be useful in the battle against infection, including COVID-19 [8].

Green tea's polyphenol composition has been shown to have immunological benefits in addition to preventing the development of microorganisms, such as methicillin-resistant *Staphylococcus aureus*, *Candida albicans*, *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhi, *Shigella dysentery*, *Shigella flexneri*, *Streptococcus mutans*, *Streptococcus sobrinus*, and *Vibrio cholera* [1]. Further analysis revealed that the mechanism of antibacterial activity of the EGCG, epicatechin gallate (ECG), and epicatechin (EC) in the green tea are mediated by direct binding of the compound(s) to the peptidoglycan layer, thus interfering with the synthesis of the cell wall that leads to the damage of bacterial protective layer and structural changes in the teichoic acid formation [9], two features that are important for pathogenic bacteria [10]. Previous research demonstrates the antibacterial activity of green tea with a minimum inhibitory concentration (MIC) of 0.3 mg/mL or equivalent to a concentration of 0.03% against *S. aureus* [11]. The ethanolic extract of green tea, with a concentration of 200 mg/mL, was shown to exert an inhibitory zone against *S. aureus* with a diameter of 20 mm [12]. Another study reported that the combination of GTE-amoxicillin can potentially increase amoxicillin's antibacterial activity with a fractional inhibitory concentration index (FICI) value of 0.28 [13].

In general, pharmacological assessments on the potential antibacterial activities of new drug candidates/compounds remain focused on the in vitro screening methods, which obviously have not been able to provide a comprehensive representation so far [14]. Hence, many drug candidates/compounds with promising results in the in vitro screening failed to exert the expected results in the *in vivo* tests using experimental animals, further creating psychological burdens to the researchers and worldwide economic problems [15]. To prevent this, fruit fly *Drosophila melanogaster* was introduced as another option for the infection model [14], especially in the *in vivo* screening of drug candidates with antibacterial activities [16-20].

Drosophila melanogaster has a similar genetic composition, around 65% -75%, to humans [14]. In addition, the easiness of maintenance to obtain a new generation of flies, the lower cost of testing, and the absence of ethical clearance requirements in research using fruit flies have made this invertebrate an ideal model for drug discovery research [14,21]. Moreover, the availability of immunodeficient mutant flies and the highest degree of similarities between the human innate immune system and *D. melanogaster* [22,23] are two important features offered by the fruit fly. Therefore, by using this model organism, one may be able to assess the pharmacological potentials of drug candidates with immunostimulatory and/or antimicrobial activities in *in vivo* settings, as has been shown in some studies [18,24]. By taking advantage of the availability of this *in vivo* platform in our lab, we hereby examined the *in vivo* immunostimulatory and antibacterial activities of GTE (*Camelia sinensis* (L) O. Kuntze).

2. Materials and Methods

2.1. Extract and sample preparation.

100 g of Simplicia was weighed and immediately extracted using 1:5 n-hexane (supported by ultrasonication) for 15 minutes and kept for 24 hours. This extraction method

yielded two parts, the parts that were soluble in the n-hexane and the one that was n-hexane insoluble. The n-hexane-insoluble part was subsequently subjected to the second extraction process using 50% ethanol (1:5) with ultrasonication for 15 minutes and kept at room temperature for 24 hours, resulting in the formation of ethanolic liquid extract. To create a viscous green tea ethanol extract, the ethanolic liquid extract was then subjected to a further process using a rotary evaporator. For sample preparation, 2.5 g GTE were dissolved in 50% ethanol, and the GTE solution was diluted to obtain a series of concentrations, designated as 0.5%, 0.25%, 0.125%, and 0.0625%.

2.2. Bacterial strain and fly stocks.

In all infection experiments, a fresh culture of *S. aureus* ATCC 25923 served as the infectious agent. Brain heart infusion broth (BHIB) was used to cultivate the bacteria medium at 37°C for 1 × 24 hours to produce a sufficient inoculum. In this investigation, the second instar larvae of Oregon R (wildtype) and psh[1];;modSP[KO], an immunodeficient mutant line, were both utilized, as previously reported [25]. The larvae were kept in culture vials with regular fly food under normal circumstances (25 °C, a 12-hour cycle of light and darkness).

2.3. Toxicity and survival assay.

The toxicity test was carried out by exposing the larvae to GTE with concentrations of 0.5%, 0.25%, 0.125%, and 0.0625%. The purpose of this test was to determine the safest concentration of GTE that was non-toxic to flies. Two life-changing forms of fruit fly were assessed: from larvae to pupae and from pupae to adult flies. The obtained data was recorded and processed. In the survival assay, flies were subjected to a whole survival test after treatment was made. This assay was carried out to examine whether GTE can exert pharmacological effects on the larvae of the Oregon R (wildtype) line of *D. melanogaster* and the immunodeficient mutant line of psh[1];;modSP[KO] in the presence or absence of infection. Larvae were divided into six groups, with each group containing ten larvae. For the antibacterial experiment, larvae' survival in all groups was observed every three hours and continued until 4-5 days (when larvae are expected to grow into pupae and emerge as adult flies). For an experiment to assess the immunostimulatory activity of GTE, larvae were treated with GTE at different concentrations, and the survival of all groups was observed until flies in all groups died. A group of larvae that were not given any treatment, designated as the untreated control, was used as a comparison.

2.4. Infection experiment.

A modified oral infection technique is used in the *S. aureus in vivo* infection model [25,26]. Briefly, *S. aureus* was mixed with smashed bananas in a microcentrifuge tube with a total volume of 500 µL. Drosophila larvae were transferred into the bacteria-banana tube (10 per tube) and incubated for three hours at 29°C. After three hours, the larvae were removed from the tube and rinsed with PBS before further experiments: survival analysis or gene expression analysis. In comparison, a group of larvae that were not given any treatment (untreated control) and a group of larvae that were infected with *S. aureus* but not given GTE treatment (infection control) were used.

2.5. Gene expression assay.

The total RNA isolation was performed on all groups of flies after treatment on ten larvae. From each group, ten live larvae were subjected to RNA isolation procedure 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 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). Verification of the expected amplified product was done based on the standard melting curve profiles spanning from 60°C to 95°C. All generated data were 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'– CGT GAG AAC CTT TTC CAA TAT GAT G – 3'	5'– TCC CAG GAC CAC CAG CAT – 3'
rp49	5' – GAC GCT TCA AGG GAC AGT ATC TG – 3'	5' – AAA CGC GGT TCT GCA TGA G – 3'

2.6. Data processing and statistical analysis.

Survival data were visualized as Kaplan-Meier graphs and statistically analyzed using Log Rank approach. Gene expression data were processed, visualized as bar graphs, and statistically analyzed using the One-Way ANOVA approach followed by Tukey post hoc analysis. Data are presented as mean ± SD for all statistical analyses, and a p-value of less than 0.05 was considered significant. All data is processed and visualized using GraphPad Prism® 9.

3. Results and Discussion

3.1. GTE treatment is relatively safe in D. melanogaster larvae.

A toxicity test was carried out to ensure that the GTE is safe for *D. melanogaster*. The observed parameters were growth from larva to pupa and then pupa to fly. In this experiment, six treatments were used, namely control (without treatment), solvent control, GTE 0.5%, 0.25%, 0.125%, and 0.0625%. The results showed that GTE with varying concentrations did not affect growth and was safe in *D. melanogaster* wildtype Oregon R and psh[1];;modSP[KO] mutants (Fig. 1).

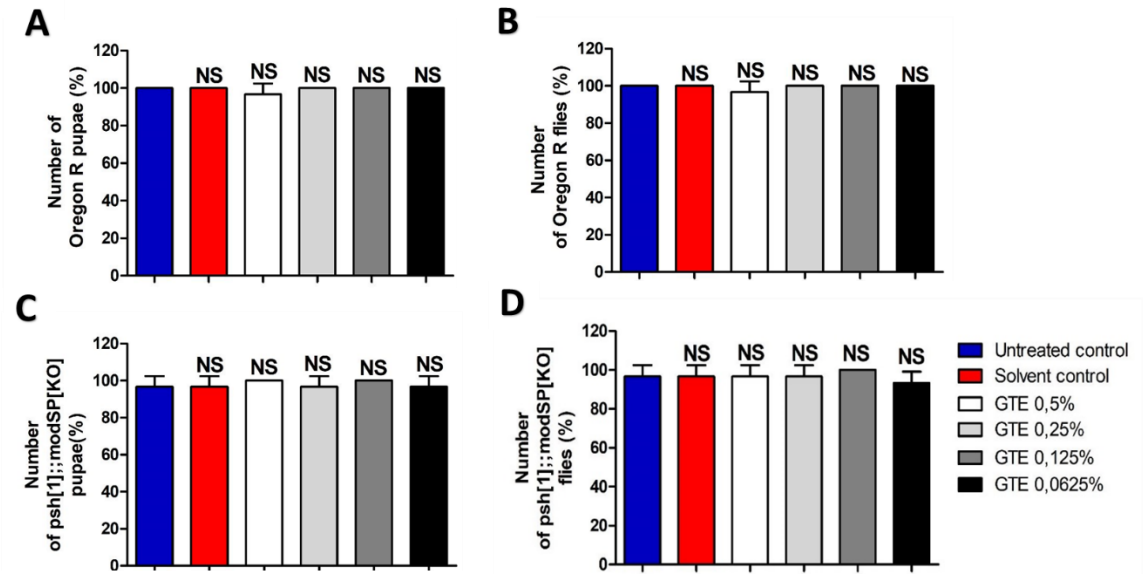

Figure 1. Developmental profile of Drosophila larvae to become pupae and adult in the presence of green tea extract. (A & C) number of Oregon R **(A)** and *psh[1];;modSP[KO]* (**C**) larvae that can develop into pupae. (B & D) Number of Oregon R **(B)** and *psh[1];;modSP[KO]* (D) pupae that can eclose into adult flies. GTE, green tea extract; NS, not significant.

3.2. GTE treatment increases the lifespan of D. melanogaster.

Lifespan is one of the easiest but most critical phenotypic parameters that can be observed in the presence of challenges (infection) or pharmacological treatments [18,27-29]. Survival assay is a simple approach to evaluate treatment effect on the lifespan, including in Drosophila [27-29]. As shown in Fig. 2, treatment of either Oregon R or psh[1];;modSP[KO] mutant flies with GTE-containing food experienced longer lifespan, suggesting that GTE can extend the lifespan of both fly lines. Apparently, the improvement of fly survival occurred in a manner independent of the GTE concentration used in the study. Strikingly, flies treated with the lowest concentration of GTE (0.0625%) had the longest lifespan compared to other groups. Since the results of the survival test provide an insight that GTE may improve the lifespan of *D. melanogaster*, it is tempting to speculate that such an effect may be achieved with the help of antioxidant compounds present in the GTE, as what has been observed in our previous study using different sample [29]. Nevertheless, this assumption shall be one of the objects of future studies.

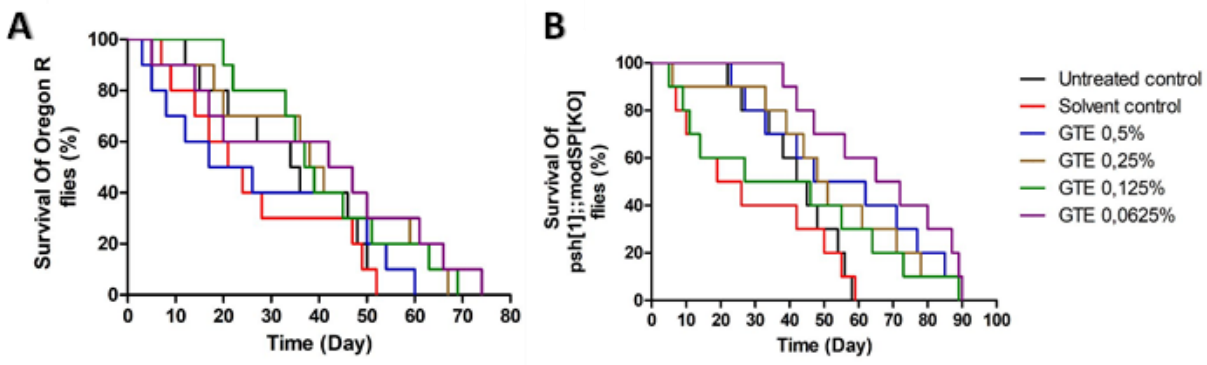

Figure 2. Survival of flies in the presence of GTE treatment. **(A)** Oregon R and (B) *psh[1];;modSP[KO]* **(B)** experienced improvement of survival in the presence of GTE. The increased survival was evident in all GTE-treated fly groups. GTE, green tea extract.

3.3. Improved survival of D. melanogaster larvae upon administration of GTE post-S. aureus infection.

In this study, the oral infection method was used to provoke infection in the larvae. Using this method, we can assess the antibacterial potential of GTE. As shown in Fig. 3, the survival of Oregon R larvae was dramatically reduced in the presence of *S. aureus*, indicating that *S. aureus* infection is lethal to Drosophila larvae in our current condition. Nevertheless, treatment of *S. aureus*-infected Oregon R larvae with GTE at varying concentrations was able to enhance the viability of the larvae, suggesting that GTE may exert antibacterial or immunostimulant activity in GTE.

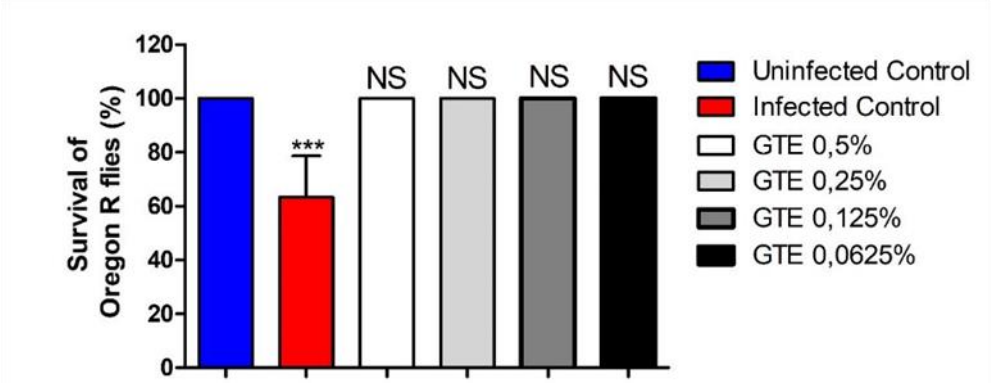

Figure 3. The survival of Oregon R larvae during infection with *S. aureus* in the presence or absence of GTE. *Staphylococcus aureus* reduced the survival of Oregon R larvae, and treatment of GTE to *S. aureus*-infected Oregon R larvae can diminish the lethal effect of *S. aureus*. GTE, green tea extract; NS, not significant; ***p < 0,001.

3.4. Significant changes in the expression of Drosomycin after GTE administration in D. melanogaster.

To examine whether the improvement of survival in the *S. aureus*-infected Oregon R larvae upon GTE treatment occurred as a result of antibacterial or immunostimulatory activities of GTE, we carried our transcriptional analysis on the expression of Drosomycin (Drs), a *Drosophila* innate immune gene that can be activated upon *S. aureus* infection, using the RT-qPCR method. The results, as shown in Fig. 4A, demonstrated that the expression of the Drs gene was enhanced in the larvae infected with *S. aureus*, compared to the non-infected control.

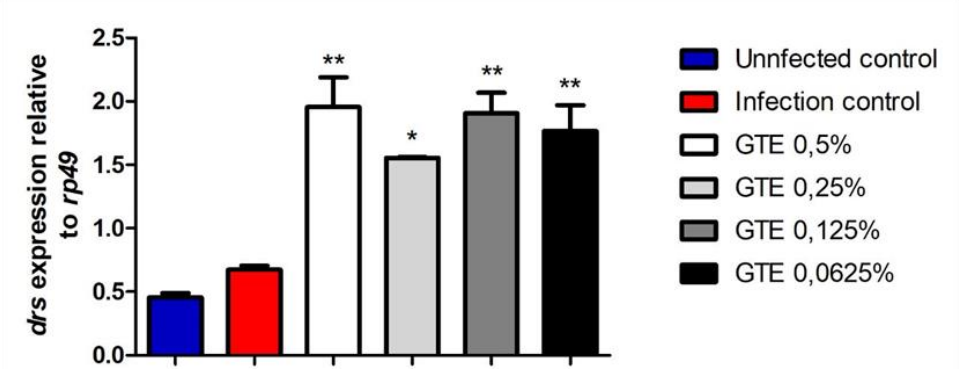

Figure 4. Transcriptional level of *Drs* in response to *S. aureus* infection and GTE treatment. **(A)** Induction of *Drs* expression in the presence of *S. aureus* infection. **(B)** Further induction of *Drs* expression upon administering GTE post *S. aureus* infection. RNA was extracted from ten live Drosophila larvae in each group, followed by RNA quantification and amplification by RT-qPCR. Target gene levels were compared with rp49 RNA levels as an internal control. GTE, green tea extract; NS, not significant; *p<0.05; ** p < 0.01.

The result is as expected since infection of Gram-positive bacteria in Drosophila can induce the expression of several antimicrobial peptides (AMPs), including Drosomycin, that serve as humoral immune responses to limit bacterial infection [22]. However, an increased expression of Drs was also observed in the *S. aureus*-infected larval group that was treated with GTE at all concentrations (Fig. 4B), further supporting the notion that GTE may act as an immunostimulant. Indeed, GTE has been previously reported as able to activate and induce components of the immune system, including phagocytic responses, to fight microbial infection [30-32].

3.5. Enhanced survival of S. aureus-infected immunodeficient mutant larvae in the presence of GTE.

We have shown that the improvement of Oregon R larval survival in the presence of GTE, even at the lethal challenge of *S. aureus*, is, at least, mediated by the immunostimulatory activity of GTE. However, such an effect can also occur due to the antibacterial effect of GTE, as previously reported using in vitro assay [9]. To examine whether the improved survival of *S. aureus*-infected, GTE-treated Oregon R larvae was also mediated by the antibacterial effect of GTE, we carried out a survival assay using the immunodeficient *psh[1];;modSP[KO]* line in the presence of *S. aureus* challenge and with or without the presence of GTE. A double immunodeficient mutant line is known as the *psh[1];;modSP[KO]* (that are lacking for Persephone (Psh) and a modular serine protease (modSP) that are responsible in the activation of innate immune defense against Gram-positive bacteria) were used [22]. As shown in Fig. 5, GTE treatment was able to improve the survival of *psh[1];;modSP[KO]* upon *S. aureus* infection, implying that the improvement of larval survival in the event of *S. aureus* challenge was not only due to the immunostimulatory effect of GTE but also due to the *in vivo* antibacterial activity of GTE.

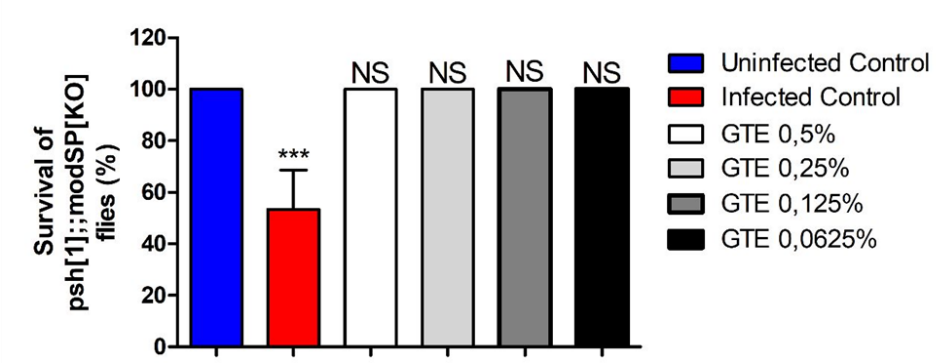

Figure 5. The survival of *psh[1];;modSP[KO]* larvae during infection with *S. aureus* in the presence or absence of GTE. *Staphylococcus aureus* reduced the survival of *psh[1];;modSP[KO]* larvae and treatment of GTE to *S. aureus*-infected *psh[1];;modSP[KO]* larvae can diminish the lethal effect of *S. aureus*. GTE, green tea extract; NS, not significant; ***p < 0,001.

4. Conclusions

In this study, for the first time, we demonstrated the dual *in vivo* antibacterial and immunomodulatory effects of GTE using *D. melanogaster* platform system. We believe that this system will serve as a powerful *in vivo* approach to clarify the antibacterial and immunostimulatory activity of not only GTE but also other medicinal plant extracts. In addition, this simple yet powerful *in vivo* approach will be useful to increase the chance of obtaining compounds with dual antibacterial and immunomodulatory effects.

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Conflicts of Interest

We declare that we have no conflict of interest.

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