DELPHINIDIN PROTECTS *CAENORHABDITIS ELEGANS* AGAINST BACTERIAL INFECTIONS

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ABSTRACT: *Delphinidin-3-glucoside (D3G) is a pigment widely represented in various fruits and vegetables. Being an anthocyanin compound, D3G offers an antioxidant property which could influence oxidative damage. In this paper, the researchers are interested in pathogen-associated oxidative damage. Hence, this paper investigates the protective property of D3G on Caenorhabditis elegans (C. elegans) against different pathogens such as Staphylococcus aureus (S. aureus), Enterococcus faecalis (E. faecalis), Klebsiella pneumonia (K. pneumonia), and Proteus mirabilis (P. mirabilis). The C. elegans were nourished with varying concentrations of D3G and were infected with different pathogens. The lifespan of the nematode was observed in these other conditions. Results show a significant increase ($p \leq 0.05$) in the lifespan of C. elegans when exposed to S. aureus, E. faecalis, K. pneumonia, and P. mirabilis. However, the efficiency of D3G against the different pathogens differ. Taken together, the researchers speculate that D3G may have an additional protective mechanism against various pathogens to lengthen the lifespan of the C. elegans.*

Keywords: anthocyanin, delphinidin, lifespan, Caenorhabditis elegans, innate immune response

INTRODUCTION

The innate immune response plays a pivotal role in maintaining homeostasis [1]. This system functions to recognize pathogens through pathogen-associated molecular patterns (PAMPS) or damage-associated molecular patterns (DAMPS) [2-3]. These are the two critical concepts that influence the host's first defense line against an invading entity.

Recent studies have shown that anthocyanins exhibit positive impacts on the immune response, particularly in interleukin 1, beta (IL-1β), interleukin 8 (IL-8), tumor necrosis factor (TNF-α), heat shock protein 70 (HSP70), and interferon-gamma (IFN-γ) [4-5]. Moreover, another study observed that anthocyanin increased macrophages' phagocytic activity [6]. In this study, the researchers are interested in a particular anthocyanin, delphinidin, which may potentially affect the innate immune response.

C. elegans is a non-parasitic nematode that lacks adaptive immune response [7]. Basically, the first line of defense of the *C. elegans* against different pathogens lies in its pattern recognition receptors (PRRs). Interestingly, studies show that *C. elegans* have a different innate immune response against foreign pathogens [8-9]. Meanwhile, other studies suggest that *C. elegans* may have a unique DAMPs, which can recognize exotoxins from a particular pathogen [10]. The ability of *C. elegans* to withstand pathogenic infections may indicate influence from the host's immune response [11]. Besides, other studies have supported this idea that highlights the significant role of the innate immune response to promote longevity in *C. elegans* [12-13].

In this study, the researchers investigate the effects of delphinidin on *C. elegans* longevity after infection with various pathogens, such as *S. aureus*, *E. faecalis*, *K. pneumoniae*, and *P. mirabilis*. Hence, the researchers used the D3G concentrations that do not pose lethal effect to the nematode nor inhibit the bacterial growth.

MATERIALS AND METHODS

Caenorhabditis elegans and bacterial strains handling procedures

The researchers obtained the wildtype *C. elegans* and its food, *Escherichia coli* (*E. coli*) OP50, from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota (MN, USA). The worms were maintained on a nematode growth medium (NGM) agar at 25 °C [14]. The researchers incubated *E. coli* OP50 at 37 °C. The researchers age-synchronized the nematode by collecting the eggs within 4 hours to ensure ±4 hours of age differences. The researchers also maintained *S. aureus* (ATCC-25923), *E. faecalis* (ATCC 29212), *K. pneumoniae* (BAA-1705), *P. mirabilis* (ATCC 29906) at 37 °C.

Preparation of D3G

The delphinidin-3-glucoside (D3G) which has >97% purity was purchased from AS Polyphenols (Norway). This compound was dissolved in distilled water to obtain 1 mg/mL. It was stored at 4 °C until further usage.

Caenorhabditis elegans sublethal assay

Fifty µl of different concentrations 0, 1, 10, 100, and 1000 µg/mL of D3G were given to the nematodes to determine the sublethal concentration. The negative control 0 µg/mL of D3G only contains distilled water. Twenty worms were placed on each plate, and the number of surviving worms were counted after 24 and 48 hrs. The researchers determine if the worms are dead worms if no movement is observed after slight poking.

Minimum Inhibitory Concentration Assay

For the broth microdilution assay, the researchers followed a previous study [15]. They grew different bacteria such as *S. aureus*, *P. mirabilis*, *K. pneumoniae*, and *E. faecalis* in the trypticase soy broth (TSB) tubes following the 0.5 McFarland standard. The turbidity of the bacterial suspensions before and after the treatment of D3G was compared with the 0.5 McFarland standard. Varying concentration of D3G was prepared and mixed with the bacterial solution.

***Caenorhabditis elegans* lifespan assay**

The bacterial suspensions with an absorbance of ~0.450 at 600 nm were replenished every day. Fifty µl of the bacterial suspensions were distributed onto each NGM plate. Different bacterial treatments were prepared each day using the following bacteria (*E. coli, S. aureus, E. faecalis, K. pneumoniae, P. mirabilis*). The researchers started to observe thirty L4 nematodes for each treatment at day 0 and counted the live, dead, and missing worms every day until all worms were deceased [15].

Statistical Analysis

The researchers presented the results as mean ± SEM. The nematodes' lifespan was measured through Kaplan Meier and log-rank test for post-hoc analysis in determining significant differences between the treatment groups. All the statistical analysis was accomplished using OASIS version 2 (Korea). Statistical significance was set at $p \leq 0.05$.

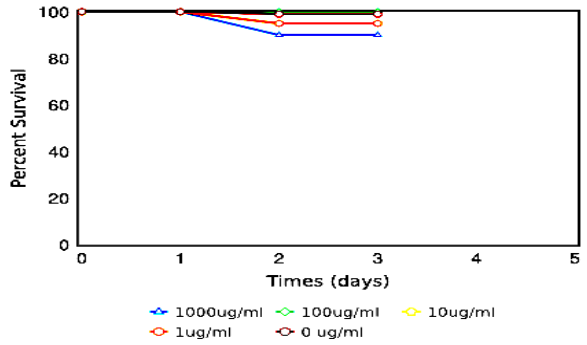

Figure 1. D3G shows no chronic toxicity in *C. elegans* up to 100 µg/ml. A total of 30 L4 nematodes were given various concentrations (0, 1, 10, 100, 1000 µg/mL) of D3G suspended in *E. coli* OP50 every day for 48 hours. Surviving worms were counted at 0, 24, and 48 hours. Worms that did not respond to light touch were considered dead. Sublethal concentration was set at the concentration where ≥90% of worms survived.

RESULTS

Sublethal Assay

The highest possible concentration of D3G available in the laboratory was used for the sublethal assay. The nematodes were given 1000, 100, 10, 1, and 0 µg/ml D3G concentrations, and their survival after 24 hours and 48 hours was observed. As shown in Figure 1, the nematodes given up to 100 µg/ml have ≥ 90% survival after the 24 and 48 hours in each concentration, which implies that these concentrations do not pose acute and chronic toxicity to the *C. elegans*.

D3G up to 100 µg/mL does not inhibit bacterial growth

Varying concentrations of D3G were tested on different gram-positive bacteria, such as *S. aureus* and *E. faecalis*, and gram-negative bacteria, namely *K. pneumoniae* and *P. mirabilis*. As shown in Table 1, ≤100 µg/ml D3G did not exhibit inhibitory property against *S. aureus, K. pneumoniae, E. faecalis,* and *P. mirabilis* bacterial growth, as indicated by comparable turbidity to 0.5 McFarland.

Various pathogens lead to shorter lifespan in *C. elegans*

In Figure 2, the researchers demonstrated that *C. elegans* fed with *S. aureus, K. pneumoniae*, and *P. mirabilis* have a significantly shorter lifespan than those provided with *E. coli* ($p \leq 0.05$).

Table 1. The inhibitory concentration of D3G

	Gram-positive		Gram-negative	
	S. aureus	*E. faecalis*	*K. pneumoniae*	*P. mirabilis*
0 µg/ml	+	+	+	+
1 µg/ml	+	+	+	+
10 µg/ml	+	+	+	+
100 µg/ml	+	+	+	+

+/- absence or presence of turbidity

The result shows that *P. mirabilis* has a 55.1% decline, whereas *K. pneumoniae* exhibited 42.6% shorter lifespan in *C. elegans* ($p \leq 0.05$). On the other hand, while *C. elegans* fed with *S. aureus* has a significant 36.5% reduction in mean lifespan; the 50% decrease in mean lifespan of those provided with *E. faecalis* was not significant ($p \leq 0.05$). These results suggest that most of these bacteria are pathogenic to *C. elegans*.

D3G increases the mean lifespan of *C. elegans* with or without pathogens

In Figure 3, the researchers observed the Kaplan-Meier graph and the mean lifespan of the *C. elegans* under regular physiologic activity with *E. coli* (Figure 3A), exposed to gram-positive bacteria *S. aureus* and *E. faecalis* (Figure 3B-C), and gram-negative bacteria *K. pneumoniae* and *P. mirabilis* (Figure D-E). The mean lifespan of *C. elegans* fed with *E. coli* has a 28.4% increase compared to the untreated ($p \leq 0.05$). Similarly, 46.4% and 32.6% increase in mean lifespan in *S. aureus* and *E. faecalis* ($p \leq 0.05$). Moreover, 35.2 % and 40.5 % increase in mean lifespan in *K. pneumoniae* and *P. mirabilis* ($p \leq 0.05$).

DISCUSSIONS

The researchers have demonstrated that as much as 100 µg/mL of D3G did not inhibit the growth of *S. aureus, E. faecalis, K. pneumoniae,* and *P. mirabilis*. Besides, these concentrations do not pose acute and chronic toxicity in *C. elegans*. In addition, *C. elegans* are susceptible to bacterial infection. With these conditions, the researchers speculate that D3G protects the *C. elegans* from these pathogens independent of its antibacterial activity.

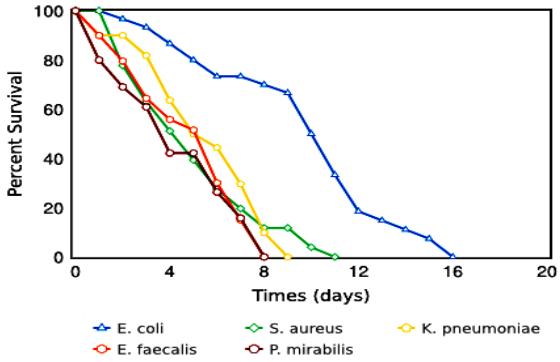

Figure 2. Lifespan of *C. elegans* fed with various bacteria. A total of 30 L4 nematodes were given 50 µL of different bacteria, such as *E. coli* OP50, *S. aureus, E. faecalis, K. pneumoniae,* and *P. mirabilis* every day until all worms are deceased. The difference in the mean lifespan of the nematode under different conditions was considered significant at $p \leq 0.05$.

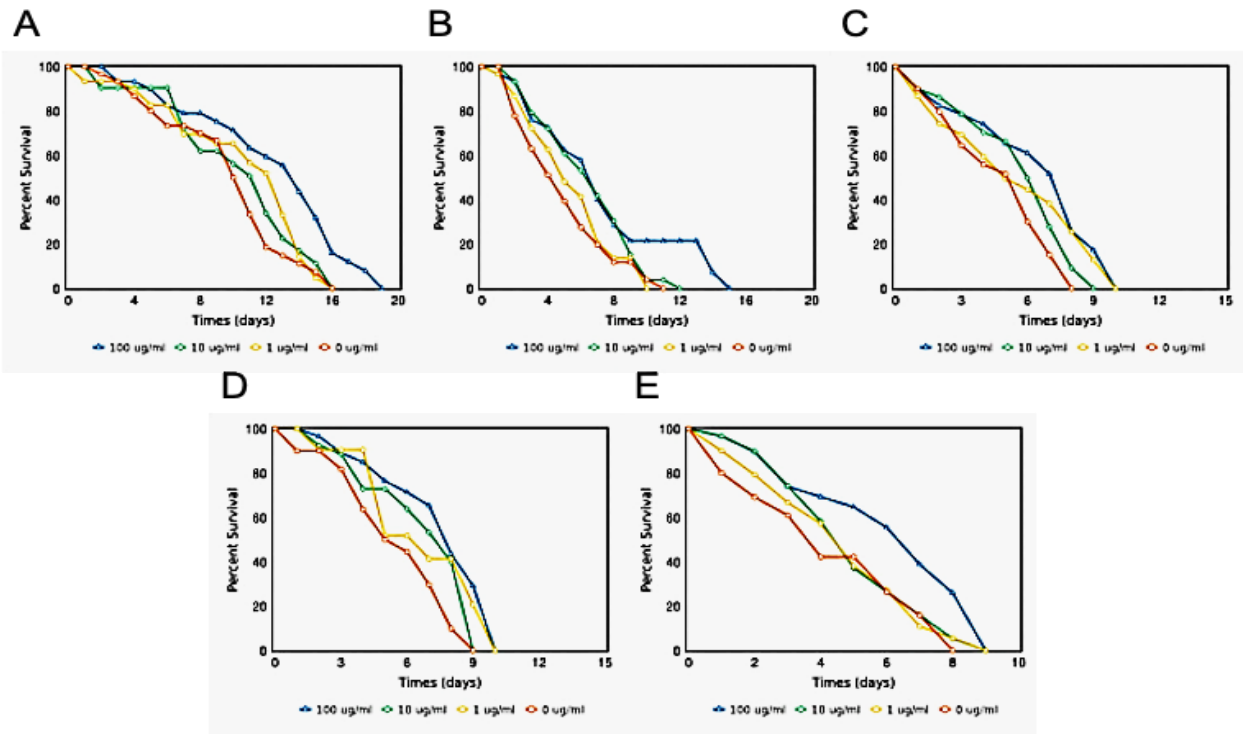

Figure 3. Survival plots of *C. elegans* fed with D3G-coated bacteria. L4 nematodes were transferred to new NGM plates daily flooded with different concentrations of D3G-coated bacteria (A. *E. coli*, B. *S. aureus*, C. *E. faecalis*, D. *K. pneumoniae*, E. *P. mirabilis*) each day until all worms are deceased. The difference in the mean lifespan of the nematode under different conditions was considered significant at $p \leq 0.05$

Anthocyanins from cranberry and Thymus were shown to have an antibacterial property [16-17]. Contrastingly, the researchers could not observe the inhibitory activity of D3G against the selected gram-positive and gram-negative bacteria. In support of this, other studies like *Myrcianthes hallii* containing anthocyanin show modest antibacterial activity [18]. Several factors influence the antibacterial property of a particular extract, including the amount, class, and possible synergistic effect of the compounds present in the extract. Another reason for the lack of antibacterial property may be the limitation in the protocol employed, which only compares the bacterial suspension's turbidity.

Previous studies show that other pathogenic bacteria exhibit detrimental effects on the health of *C. elegans* [8-9]. Remarkably, studies show that *K. pneumoniae*, *E. faecalis*, *S. aureus*, and *P. mirabilis* demonstrate a significant decline in the lifespan in *C. elegans* [19-21]. The results in this study are following the results reported from the previous studies. These studies claim various pathways involved during the oxidative damage caused by the bacteria, such as the mitogen-activated protein kinase (MAPK) pathway, toll-independent immune response, and insulin growth factor 1-like pathway [21-23]. Different studies are claiming that plants rich in anthocyanin lengthen the lifespan of *C. elegans* [24]. This lifespan extension was also observed in another model organism, such as *Drosophila* [25]. Mainly, other studies also demonstrate that delphinidin has positive effects on longevity in *C. elegans* [24]. These findings only suggest that delphinidin extends the lifespan of *C. elegans* under normal physiologic conditions.

Since pathogen-associated damage in *C. elegans* were reported to be involved with mitogen-activated protein kinase (MAPK) pathway, toll-independent immune response, and insulin growth factor 1- like (IGF1-like) pathway, the researchers determine whether these pathways are affected by anthocyanins, particularly by D3G. Studies show that anthocyanins modulate MAPK, TNF- α, and IGF1-like pathways [5, 26-27]. With regards to D3G, only a few studies were found, and most of these studies associate D3G in these pathways through the perspective of anticancer and other diseases. For instance, previous research associate delphinidin with MAPK through attenuation of cardiac hypertrophy, ovarian cancer, tenofibroblast [28-30]. This study suggests that D3G may attenuate oxidative damage caused by various pathogens through mitogen-activated protein kinase (MAPK) pathway, toll-independent immune response, and IGF1- like pathway.

CONCLUSION

Delphinidin-3-glucoside enhances the longevity of *C. elegans* despite exposure to pathogenic bacteria, despite D3G exhibiting modest antibacterial property. These findings suggest that D3G protects *C. elegans* from the bacteria without inhibiting the pathogen. The researchers speculate that the protective effect may be due to the ability of D3G to strengthen the first line of defense of the nematode against these pathogens. This speculation warrants further studies to understand the effect of D3G on the different innate immune response markers.

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