

Solanum melongena* (Eggplant) Crude Anthocyanin Extract and Delphinidin-3-glucoside protects *Caenorhabditis elegans* against *Staphylococcus aureus* and *Klebsiella pneumoniae

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RESEARCH ARTICLE

Abstract

Background and Objective: During infection, ROS signaling is activated to protect the cells from invading microorganisms. However, high level of ROS may also damage the host tissue. The anthocyanin delphinidin is known to have a strong antioxidant activity which protects cells from oxidative damage. We explored the potential of crude anthocyanin extract from the fruit of *Solanum melongena* (Eggplant) and Delphinidin-3-glucoside in enhancing the innate immunity in *Caenorhabditis elegans* against *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Methodology: We used *Caenorhabditis elegans* to study innate immune response because it lacks adaptive immunity. First, we determined the sublethal concentration of *S. melongena* crude anthocyanin extract (SMCAE) and Delphinidin-3-glucoside (D3G) in *C. elegans*. We used the sublethal concentration of SMCAE and D3G to supplement the nematodes during its exposure to *S. aureus* and *K. pneumoniae*. We then observed its survival rate until day five post-L4. We also tested SMCAE and D3G for probable antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Results and Conclusion: We found out that both SMCAE and D3G showed no inhibitory effect on the growth of the bacteria. However, both SMCAE and D3G enhanced the survival of the nematode when exposed to *S. aureus* and *K. pneumoniae*. Overall, we speculate that the anthocyanin delphinidin in *S. melongena* crude extract protected the *C. elegans* against *S. aureus* and *K. pneumoniae* infection through its antioxidant activity.

Keywords: Anthocyanin, delphinidin, innate immunity, *Caenorhabditis elegans*, *Staphylococcus aureus*, *Klebsiella pneumoniae*

Introduction

Multidrug resistant bacteria persist in hospitals and in community settings which challenges the health care system of various countries, especially the developing ones [1]. Among the various pathogens that are multi-drug resistant, two of the most common reported cases were *Staphylococcus aureus* (*S. aureus*) and *Klebsiella pneumoniae* (*K. pneumoniae*) [2]. Bacteria like *S. aureus* causes skin infection, lung infection, and urinary tract infection [3]. Meanwhile, *K. pneumoniae* is another pathogen which causes nosocomial infections such as pneumonia, urinary tract infections, and bacteremia which

are becoming prevalent in hospitals. These bacteria are usually resistant to drugs such as penicillin and most cephalosporins [4].

There are several ways to alleviate the prevalence of multidrug resistant bacteria, one of which is to find an alternative medication in the form of natural product. *Solanum melongena* (Eggplant) is known for its high content of flavonoids and other phenolic compounds which accounts for its high antioxidant activity [5]. Flavonoids such as anthocyanins were known to increase lifespan of various organisms and protect them from oxidative damage [6].

During bacterial infection, reactive oxygen species and nitric oxide are being produced in the host cells which activates oxidative stress signalling to combat oxidative damage [7]. Humans have a complex innate immune response which also intercede its adaptive immunity [8]. Meanwhile, *Caenorhabditis elegans* (*C. elegans*), has a simple innate immune response which is associated with its stress response pathway, mitogen activated protein kinase (MAPK) signaling [8]. The simplicity of *C. elegans* makes it susceptible to bacterial infections which causes death [9].

In the present study, we aimed to evaluate the effects of crude anthocyanin extract from *S. melongena* and Delphinidin-3-glucoside on the mid-lifespan of *C. elegans* upon exposure to *S. aureus* and *K. pneumoniae*.

Methodology

Caenorhabditis elegans and Bacterial strains handling procedures

Bristol N2 (wildtype) *C. elegans* and *Escherichia coli* OP50 were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota (MN, USA). Nematodes were grown at 25 °C on nematode growth medium (NGM) agar plates following Stiernagle's protocol [6]. Worms were age-synchronized by allowing each 50 adult worms to lay eggs for 4 hours on an NGM plates and were fed with *E. coli* (OP50). The adult worms were removed from the plates and the eggs were allowed to grow until L4. Strains of *Staphylococcus aureus* (ATCC-25923) and *Klebsiella pneumoniae* (BAA-1705) were used in the experiment.

Preparation of the *S. melongena* crude anthocyanin extract

Thin-sliced *S. melongena* fruits were oven dried at 60 °C for 48 hours and grinded into fine powder afterwards. The powder was soaked in 95% ethanol for 24 hours, following the 10:1 solvent (mL) to solute (g) ratio. The ethanolic extract was filtered and placed in a rotary evaporator at 60 °C to obtain crude anthocyanin extract. It was then diluted to 1% Dimethyl sulfoxide (DMSO) with a starting concentration of 1000 µg/ml. The solution was placed in an amber bottle and stored at 4 °C before further use. The extraction protocol was based on a previous work by Todaro, *et al.* [10].

Preparation of Delphinidin-3-glucoside

Delphinidin-3-glucoside (D3G) (>97%) was obtained from AS Polyphenols (Norway). It was reconstituted with distilled water to 1 mg/mL and was stored at 4 °C until further use. It

was placed in a dark room and protected by a foil to avoid direct contact with light. It was dissolved in 1% DMSO to prepare 100, 10, and 1 µg/mL.

Minimum Inhibitory Concentration: Broth Macrodilution

The media was prepared by mixing 2.8 g of Trypticase Soy Broth (TSB) with 100 mL of distilled water, transferred into screw top tubes then autoclaved. The tubes were cooled at room temperature before use. *Staphylococcus aureus* and *Klebsiella pneumoniae* were inoculated in the TSB tubes following a 0.5 McFarland Standard. The tubes were then compared for the turbidity by looking through them on a paper with printed black lines.

The preparation of the concentrations of SMCAE extract and D3G was done by serial dilution with 10-fold lower from the precedence. With a starting concentration of 1000 µg/mL, the SMCAE was serially diluted seven times. On the other hand, D3G was serially diluted three times with a starting concentration of 1000 µg/mL. The tubes were incubated and read with UV-Vis spectrophotometer at 600 nm. Following the same preparations done, SMCAE and D3G were replaced with 1% DMSO which serves as the negative control.

Caenorhabditis elegans sublethal assay

Dilutions of SMCAE and D3G were prepared with the concentrations of 1000 µg/mL, 100 µg/mL, 10 µg/mL, and 1 µg/mL. The negative control received 0 µg/mL of SMCAE and D3G, instead 1% DMSO was given as the vehicle. Fifty µl of the treatment with *Escherichia coli* OP50 was placed on a new Nematode Growth Media (NGM) plate. Thirty L4 worms were transferred on the plate per different treatment groups across varying concentrations as previously mentioned. The plates were then observed for any live, dead and missing worms for 24, 48, and 72 hours. Worms were considered dead when they do not move or respond to fine touch.

Caenorhabditis elegans survival assay against *Staphylococcus aureus*

Freshly prepared *S. aureus* suspension was made by suspending the bacteria in varying concentrations of SMCAE and D3G solutions. All bacterial suspensions used in the experiment have an absorbance of 0.450 at 600 nm. Fifty µl of the bacterial suspension was dispensed onto NGM plates. Thirty L4 nematodes were transferred every day until day 5 post-L4. Live worms were scored when moving after a gentle touch.

Caenorhabditis elegans survival assay against *Klebsiella pneumoniae*

Freshly prepared *K. pneumoniae* suspension was made by suspending the bacteria in varying concentrations of SMCAE and D3G solutions. All bacterial suspensions used in the experiment have an absorbance of 0.450 at 600 nm. Fifty μ l of the bacterial suspension was dispensed onto NGM plates. Thirty L4 nematodes were transferred every day until day 5 post-L4. Live worms were scored when moving after a gentle touch.

Statistical Analysis

In the experiment all individuals were treated as replicates and each set-up was done in two trials. Kaplan Meier Statistics was used for the measurement of the mid-lifespan for the survival assay. Mid-lifespan is the estimated day where 50% of the population survived. Further, log-rank test was used for post-hoc analysis to determine significant differences between the treatment groups. All data were analyzed using OASIS version 2 (Korea). Statistical significance was set at * for 0.05, ** for 0.01, *** for <0.001.

Results

SMCAE and D3G have comparable MIC against gram-positive and gram-negative bacteria

Four concentrations of SMCAE were prepared and tested on the growth of *S. aureus* and *K. pneumoniae* with 1000 μ g/mL as the highest concentration. This was the maximum concentration soluble in 1% DMSO. All the concentrations treated with *S. aureus* and *K. pneumoniae* shown in Table 1 have greater turbidity compared to the 0.5 McFarland which denotes bacterial

growth. Similarly, the highest concentration of D3G available, 1000 μ g/mL, was also more turbid in contrast with 0.5 McFarland when treated with *S. aureus* and *K. pneumoniae*. These data suggest that ≤ 1000 μ g/ml of SMCAE and D3G may have no inhibitory effect on gram-positive and gram-negative bacteria.

Sublethal Assay

Nematodes were exposed to three SMCAE concentrations for the sublethal assay. Under the three concentrations (1000 μ g/ml, 100 μ g/ml, and 10 μ g/ml), the *C. elegans* consistently had 100% survival rate (Fig.1-A). The latter was determined sublethal if the survival rate is >90% (N>27) after 72 hours. Meanwhile, D3G at 1000 μ g/ml has <90% survival rate after 72 hours (Fig.1-B). It is notable that the pure compound has a lower sublethal concentration compared to the crude extract. This may be possible due to D3G innate enhanced efficacy – hence toxicity, since there is a lack of synergism.

SMCAE and D3G protect *C. elegans* against *S. aureus*

The sublethal concentration 1000 μ g/ml of SMCAE was diluted to 100 and 10 μ g/ml to determine any dose response. Both 1000 and 100 μ g/ml increased the survival rate of *C. elegans* against *S. aureus* after 5 days (Fig.2-A), thus, also extended its mid-lifespan by 0.68 (19%) and 0.66 (19%) days respectively (Fig.2-B). Meanwhile, both 100 and 10 μ g/ml D3G had higher survival rate relative to the negative control, 1% DMSO (Fig.2-C), with mid-lifespan increase by 0.76 (22%) days and 0.61 (17%) days, respectively (Fig.2-D). Furthermore, 100 and 10 μ g/ml of both SMCAE and D3G were comparable with each other in terms of survival rate and mid-lifespan of the treated nematode against *S. aureus*.

D3G enhances the defense of *C. elegans* better than SMCAE against *K. pneumoniae*

Adult N2 *C. elegans* were fed with *K. pneumoniae* mixed with varying concentrations (1000, 100 and 10 μ g/ml) of SMCAE. Only 1000 μ g/ml SMCAE was able to increase the survival rate of *C. elegans* against *S. aureus* after 5 days (Fig.3-A), with 1.06 (31%) days mid-lifespan (Fig.3-B). Interestingly, worms fed with D3G concentrations 100, 10, and 1 μ g/ml survive better compared to the negative control, 1% DMSO by 1.23 (35%) days, 1.4 (41%) days, and 4.7 (43%) days (Fig.3-D). Furthermore, 100 and 10 μ g/ml of D3G protected the nematodes better than 100 and 10 μ g/ml of SMCAE by 0.96 (26%) days and 0.73 (18%) days, respectively. This suggests that pure anthocyanin compound may have different effects on gram-positive and gram-negative bacteria.

Table 1. Minimum Inhibitory Concentration of SMCAE and D3G

	S. aureus	K. pneumoniae
SMCAE		
1000 μ g/ml	+	+
100 μ g/ml	+	+
10 μ g/ml	+	+
1 μ g/ml	+	+
D3G		
1000 μ g/ml	+	+
100 μ g/ml	+	+
10 μ g/ml	+	+
1 μ g/ml	+	+
Control	+	+

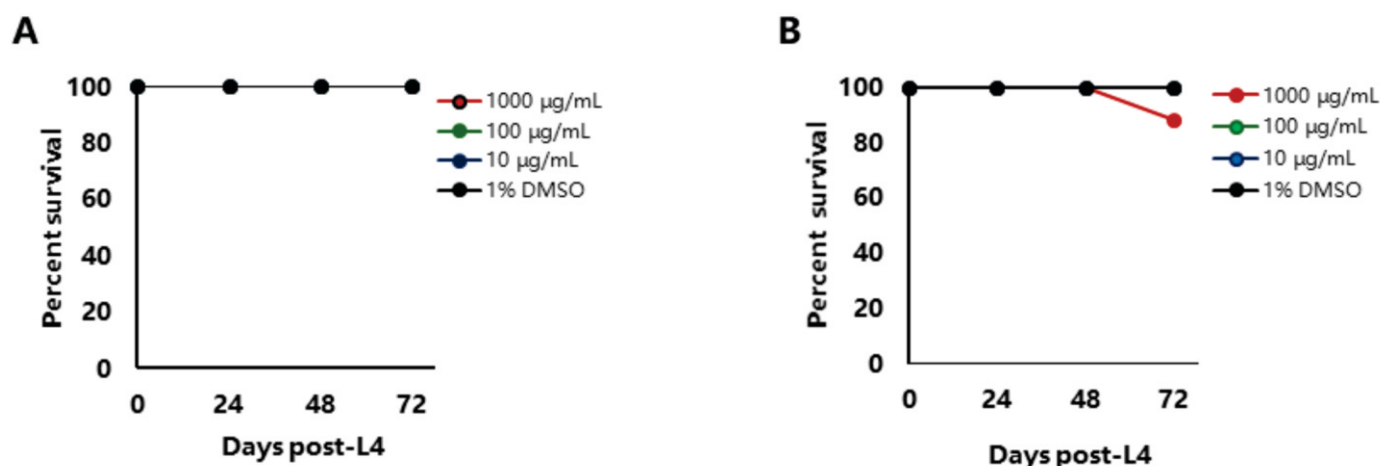


Figure 1. SMCAE and D3G posed no chronic toxicity on *C. elegans* upto 1000 mg/mL and 100 g/mL, respectively. L4 nematodes were transferred to new NGM plates seeded with *E. coli* OP50 mixed with varying concentrations of A: SMCAE and B: D3G every day for 72 hours. The control groups which were not treated with SMCAE and D3G were given 1% DMSO. Live worms were counted on 24, 48, and 72 hours. Nematodes were considered dead when no response to light touch ($n=30$, 2 trials).

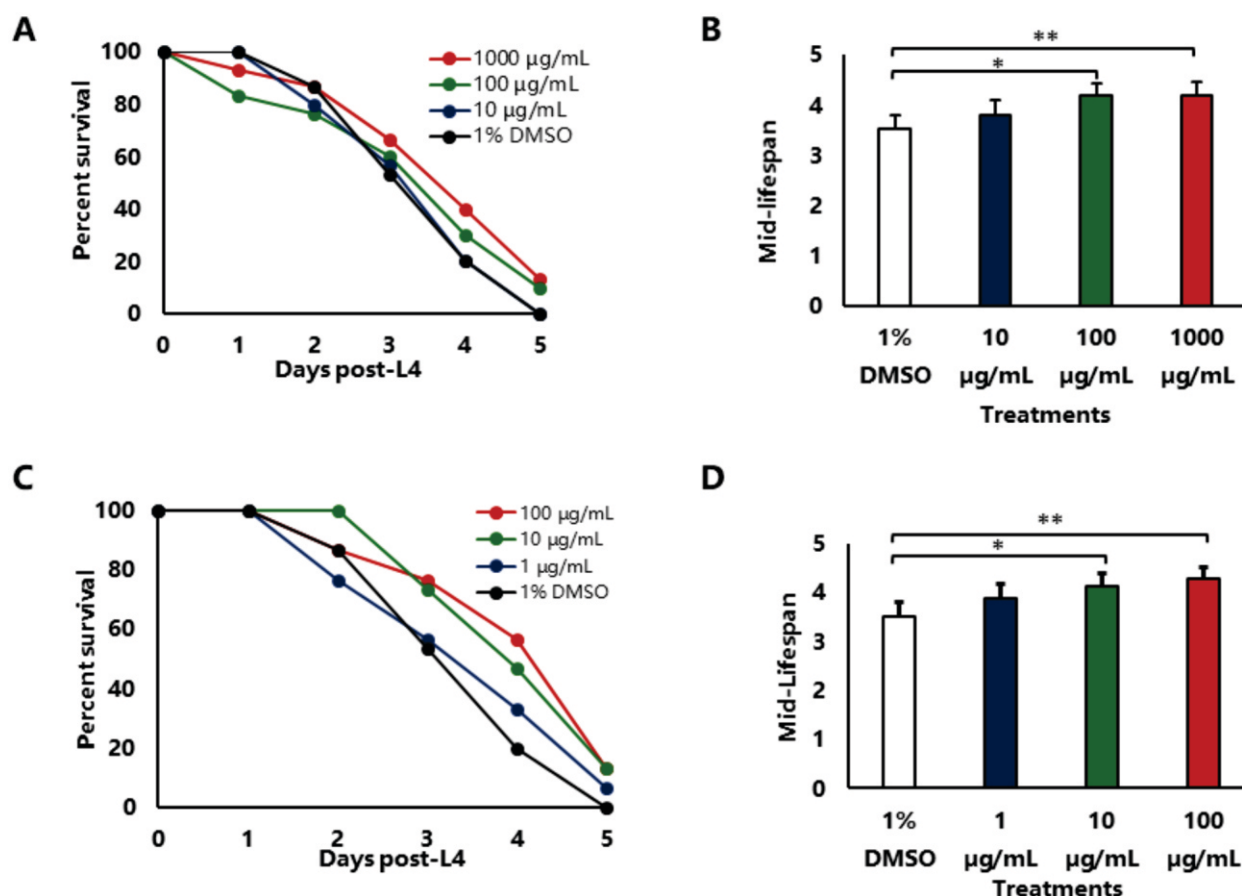


Figure 2. SMCAE and D3G protects *C. elegans* against *S. aureus*. L4 nematodes were transferred to new NGM plates seeded with *S. aureus* mixed with varying concentrations of SMCAE and D3G every day for 5 days post-L4. The median lifespan was determined by days where 50% worms survived. A. Survival plot of *C. elegans* treated with varying concentrations of SMCAE. B. Median lifespan of *C. elegans* treated with varying concentrations of SMCAE. C. Survival plot of *C. elegans* treated with varying concentrations of D3G. D. Median lifespan of *C. elegans* treated with varying concentrations of D3G ($n=30$, $p<0.05$, 2 trials). * 0.05, ** 0.01, *** 0.001 level of significance.

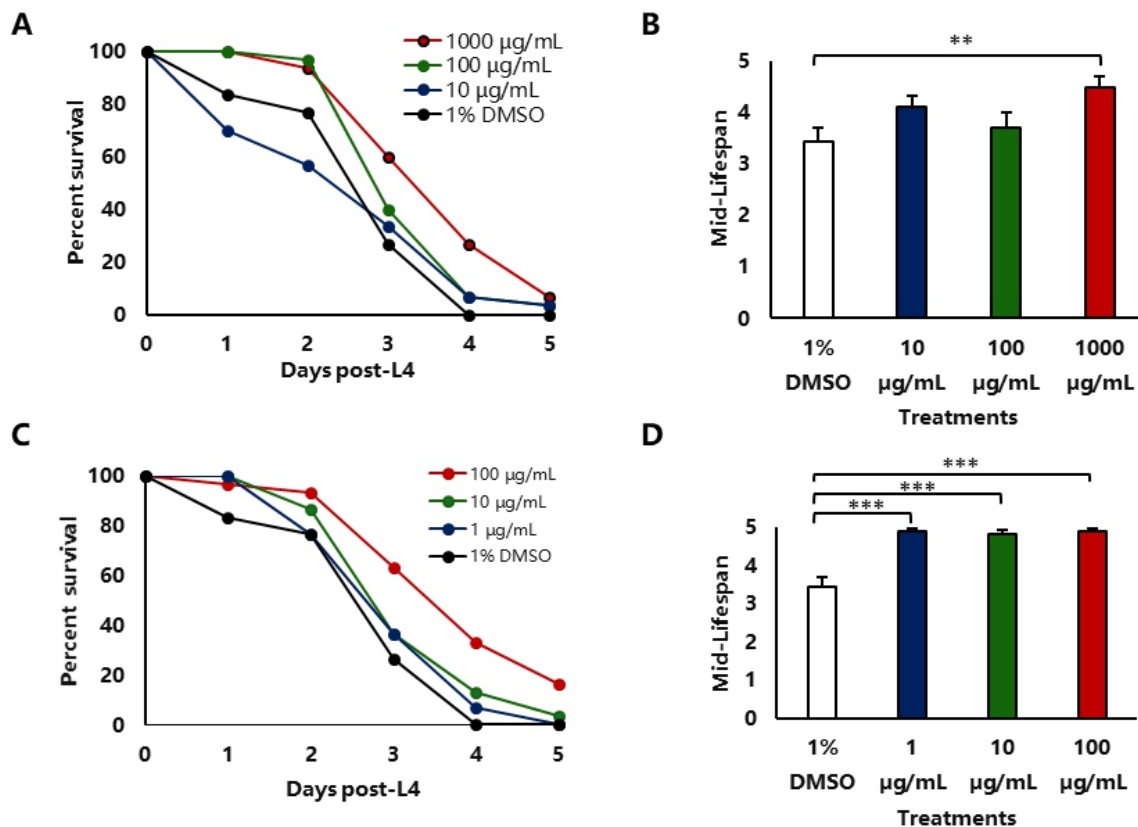


Figure 3. SMCAE and D3G protect *C. elegans* against *K. pneumoniae*. L4 nematodes were transferred to new NGM plates seeded with *K. pneumoniae* mixed with varying concentrations of SMCAE and D3G every day for 5 days post-L4. The control groups which were not treated with SMCAE and D3G were given 1% DMSO. The median lifespan was determined by days where 50% worms survived. A. Survival plot of *C. elegans* treated with varying concentrations of SMCAE. B. Median lifespan of *C. elegans* treated with varying concentrations of SMCAE. C. Survival plot of *C. elegans* treated with varying concentrations of D3G. D. Median lifespan of *C. elegans* treated with varying concentrations of D3G ($n=30$, $p<0.05$, 2 trials).

Discussion

Crude anthocyanin extracts from various plant sources are widely studied in the past decade not only for its antioxidant activity but mostly for aging and longevity. Example of which are anthocyanin-rich purple wheat, Acai berry, and Mulberry Anthocyanin Extract (MAE), which prolong the life span of *C. elegans* by 2 days, 1 day and 0.45 day, respectively [11,12,13]. Moreover, different studies claim that anthocyanin also have antimicrobial properties [14,15,16]. Conversely, our data fail to show antimicrobial property in low concentrations of both the pure compound and crude extract. This may be due to the low level of the anthocyanin necessary to inhibit the growth of the bacteria tested.

We were able to show that the concentrations of SMCAE and D3G given to the worms were not able to inhibit the growth of *S. aureus* and *K. pneumoniae*. Thus, the worms were exposed to substantial amount of pathogens daily. This resulted to a lower lifespan in *C. elegans* compared when fed

with non-pathogenic strain [17]. Even when our results compared to a previous study in *E. coli* OP50, there has been a relative rapid decline in the lifespan of *C. elegans* [6,11,12,13]. This further supported previous studies that establish *S. aureus* and *K. pneumoniae* pathological consequences to the health of *C. elegans*, as well as, in humans [18].

Unlike in human, *C. elegans* lacks adaptive immune system, macrophages, and cytokine and chemokine signaling pathways [19]. Instead, it utilizes its epithelial cells to secrete antimicrobial peptides and enzymes distinct to a specific pathogen [20,21,22]. We are interested to determine whether anthocyanins can improve survival of the nematode independent of adaptive immunity. To test this, we exposed the worms to various concentrations of SMCAE and D3G that is unable to inhibit the growth of the pathogens, which is also sublethal to the worms.

As previously mentioned, we found out that both *S. aureus* and *K. pneumoniae* were infectious to the worm.

Interestingly, there are various responses of the nematode to different pathogens. For instance, in *Pseudomonas aeruginosa*, the worm only reacts after detection of pathogen-associated damage, while it was able to elicit response by detecting the molecular pattern of *S. aureus* [18]. This may be the reason behind the difference in the mid-lifespan survival of *C. elegans* exposed to different pathogens like *S. aureus* and *K. pneumoniae*. During infection, the epithelial cell of the nematode acts as the first line of defense [21]. Inside the cell, the mitochondria amplifies its cytotoxic activity against microbial infection [23,24]. Even though little is known in the cytopathology in *C. elegans*, previous studies have determined that various signalling pathways are responsible for the host defense such as daf-16, hsf-1, pmk-1, and skn-1 [21,22,25,26].

Lifespan modulation is affected by various pathways involving daf-16, pmk-1, and skn-1 [27,28]. These genes play pivotal roles in oxidative response in in vitro and in vivo studies [11]. Reactive oxygen species promote c-Jun-N-terminal kinase (JNK) that would impair insulin signal transduction pathway activating daf-16 [29]. A recent study shows that anthocyanin extract is unable to extend lifespan under oxidative stress in daf-16 mutants suggesting that anthocyanin requires daf-16 for their antioxidant activity [13]. Damage repair pathways in aged tissues are impaired, likewise, cellular clearance pathways are impaired due to accumulated damage [30].

During oxidative damage, percent survival of the *C. elegans* treated with crude anthocyanin extract was prolonged [11]. In vivo studies identify anthocyanin to be a powerful antioxidant and have potential to alleviate cellular damage caused by oxidative stress, thus prolonging lifespan of *C. elegans* [12]. It is confirmed by in vitro studies that anthocyanin extract mitigates H₂O₂-induced cytotoxicity [11].

The cytotoxic activity of the mitochondria is a response of the host to eliminate severely damaged cell caused by pathogens. Difference in the response of the nematode to different pathogens may be attributed to the structure of the bacteria [18]. We were able to observe that in *S. aureus* only the high and the mid-concentrations of both SMCAE and D3G was able to extend the mid-lifespan of the nematode. This result is likely to be attributed to the effect of anthocyanin, delphinidin, in recruiting lysozymes to degrade the peptidoglycan of gram-positive bacteria [18]. Meanwhile, *K. pneumoniae* produces enterotoxins that increases the level of cGMP [31]. cGMP activates the ROS signalling cascade which may inhibit the worms feeding activity [32,33,34]. It is

possible that the high ROS level and poor nutrition shortened the lifespan of *C. elegans*. Conversely, a recent study reported that anthocyanins from bilberries activate nitric oxide(NO)-cGMP pathway to enhance insulin-like signaling pathway [35]. Thus, it is possible that a certain pathway intercedes the NO-cGMP pathway in such a way that it regulates the production of cGMP and prolong lifespan through the daf-16 signalling pathway.

Conclusion

In this study, we showed that *S. melongena* crude anthocyanin extract and delphinidin-3-glucoside at 1000 µg/mL allow growth of *S. aureus* and *K. pneumoniae*. However, our results indicated that *S. melongena* crude anthocyanin extract and delphinidin-3-glucoside have comparable influence in protecting *C. elegans* from *S. aureus*. Likewise, delphinidin-3-glucoside enhances the nematode's protection against *K. pneumoniae* relative to *S. melongena* crude anthocyanin extract. Overall, delphinidin a pure anthocyanin, benefits the *C. elegans* significantly from pathogens. Both the pure compound and the crude extract have potential protective effect against pathogenic bacteria which supports previous studies on anthocyanin's effect on longevity. In the light of these findings, we recommend further validation in higher organisms.

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Author Contribution

JSBN, CKFR, RRG, JPK, ALPG designed the experiment. CKFR, RRG, JPK, ALPG, ACC, JAR, and AQS performed the experiment. CKFR performed the statistical analysis with the supervision of JSBN. JSBN and CKFR analyzed and validated the results. JSBN wrote the paper.

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