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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, Reactive oxygen species (ROS) signaling is activated to protect the cells from invading microorganisms. However, a high level of ROS may also damage the host tissue. The anthocyanin delphinidin is known to have a strong antioxidant activity that protects cells from oxidative damage. This study 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: *Caenorhabditis elegans* was used to study innate immune response because it lacks adaptive immunity. First, the sublethal concentration of *S. melongena* crude anthocyanin extract (SMCAE) and Delphinidin-3-glucoside (D3G) in *C. elegans* was determined. The sublethal concentration of SMCAE and D3G was used to supplement the nematodes during its exposure to *S. aureus* and *K. pneumoniae*. The survival rate was then observed until day five post-L4. SMCAE and D3G were also tested for probable antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae*.
Results and Conclusion: This study found 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, this study indicates 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, Staphpylococcus 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 commonly 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 that 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 products. *Solanum melongena* (Eggplant) is known for high content of flavonoids and other phenolic compounds which accounts for its high antioxidant activity [5]. Flavonoids such as anthocyanins were known to increase the 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 intercedes 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].

This study 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 afterward. 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 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 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 did 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 had 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 they moved 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 had 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 they moved 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 to measure the mid-lifespan for the survival assay. Mid-lifespan is the estimated day when 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 for the growth of *S. aureus* and *K. pneumoniae* with 1000 µg/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 had greater turbidity compared to the 0.5 McFarland which denotes bacterial growth.

Table 1. *Minimum Inhibitory Concentration of SMCAE and D3G*

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

Similarly, the highest concentration of D3G available, 1000 µg/mL, was also more turbid in contrast with 0.5 McFarland when treated with *S. aureus* and *K. pneumoniae*. These data suggest that ≤1000 µg/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 µg/ml, 100 µg/ml, and 10 µg/ml), the *C. elegans* consistently had a 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 µg/ml had a <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 due to D3G's innate enhanced efficacy – hence toxicity since there is lack of synergism.

SMCAE and D3G Protect C. elegans against S. aureus

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

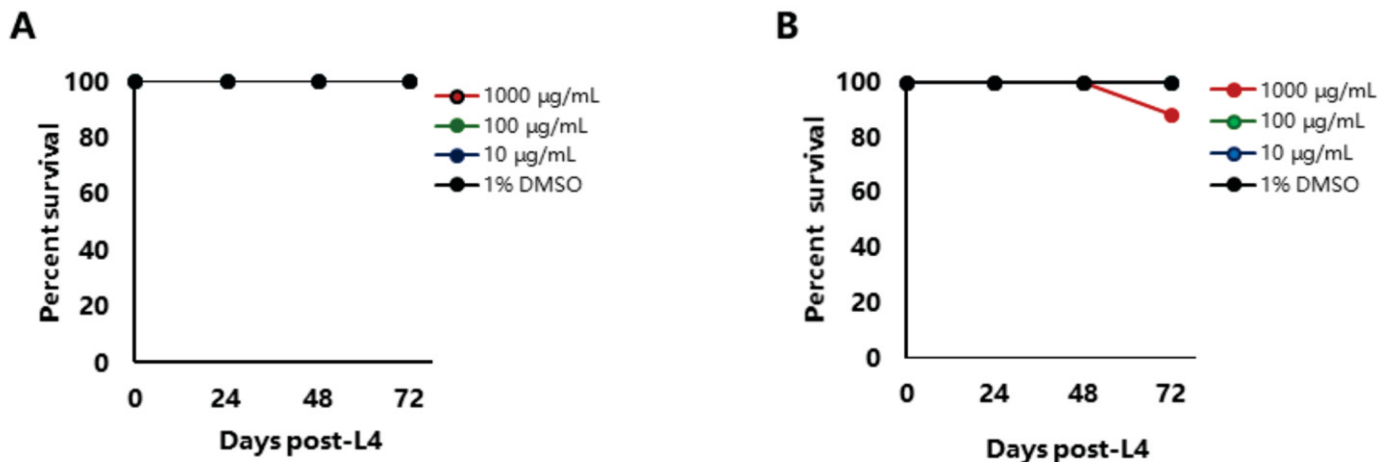

Figure 1. *SMCAE and D3G posed no chronic toxicity on C. elegans up to 1000 mg/mL and 100 g/mL, respectively. L4 nematodes were transferred to new NGM plates seeded with E. coli OP50 mixed with varying concentrations of A: SMCAE and B: D3G every day for 72 hours. The control groups which were not treated 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).*

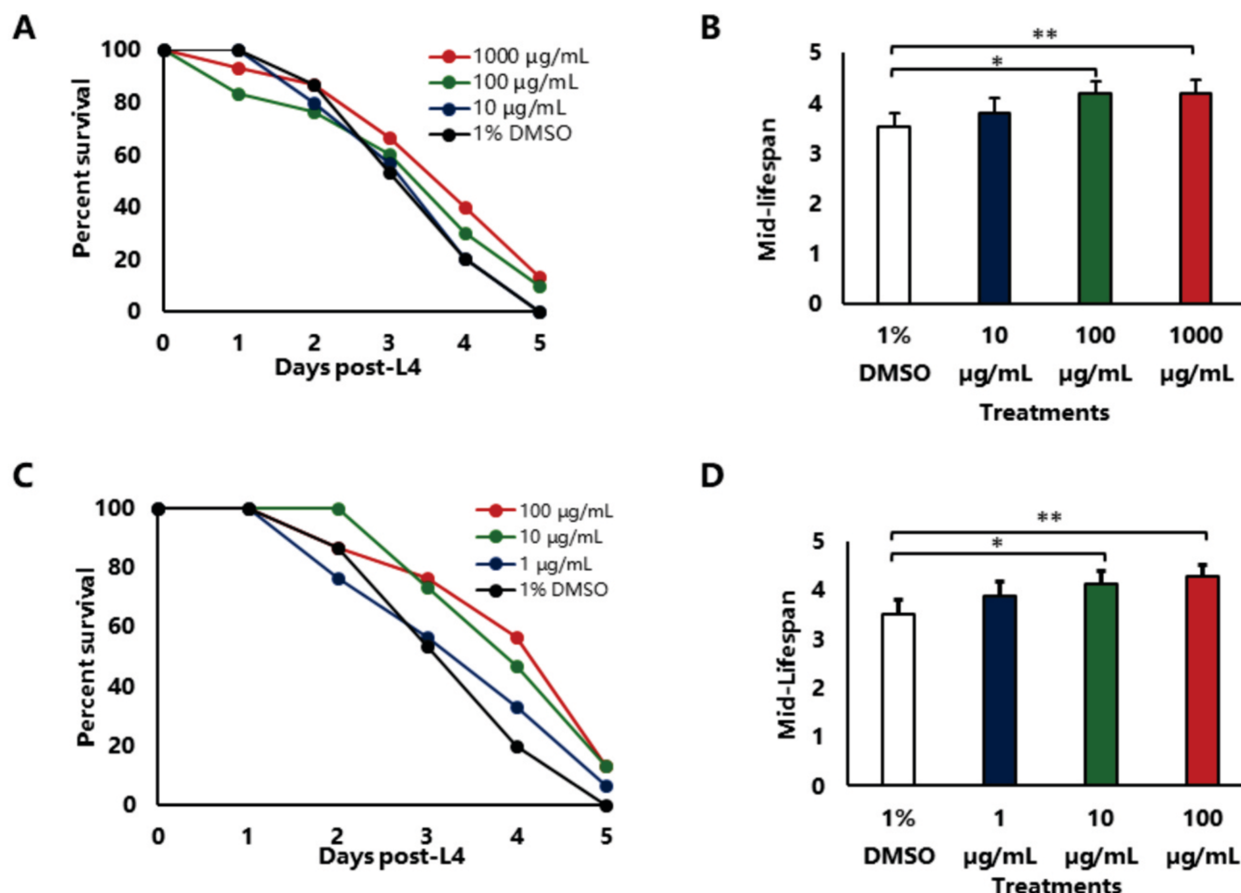

Figure 2. *SMCAE and D3G protect C. elegans against S. aureus. L4 nematodes were transferred to new NGM plates seeded with S. aureus mixed with varying concentrations of SMCAE and D3G every day for 5 days post-L4. The median lifespan was determined by days where 50% of worms survived. A. Survival plot 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.*

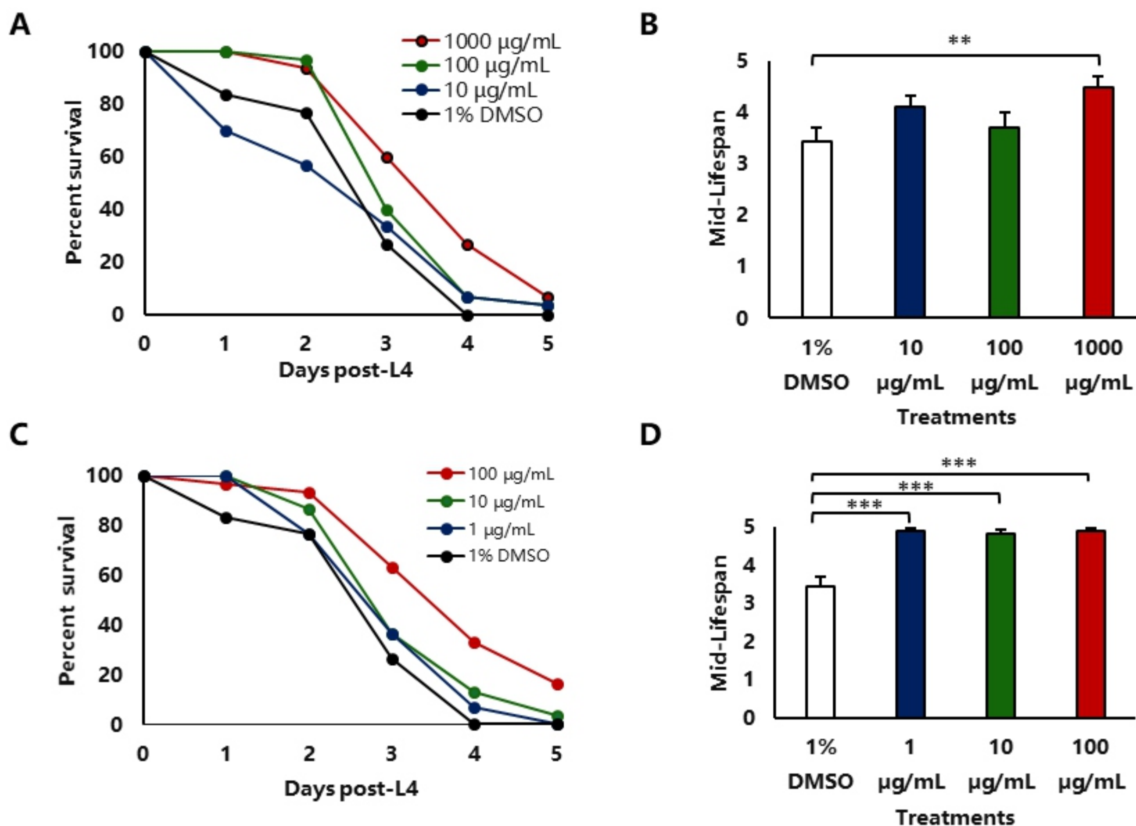

Figure 3. *SMCAE and D3G protect C. elegans against K. pneumoniae. L4 nematodes were transferred to new NGM plates seeded with K. pneumoniae mixed with varying concentrations of SMCAE and D3G every day for 5 days post-L4. The control groups which were not treated with SMCAE and D3G were given 1% DMSO. 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 their antioxidant activity but mostly for aging and longevity. Examples 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 has antimicrobial properties [14,15,16]. Conversely, data from this study 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.

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

Unlike in humans, *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]. To determine whether anthocyanins can improve the survival of the nematode independent of adaptive immunity, the worms were exposed to various concentrations of SMCAE and D3G that were unable to inhibit the growth of the pathogens, which were also sublethal to the worms.

As previously mentioned, it was found 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 detecting a pathogen-associated damage, while it was able to elicit a 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 amplify their 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 were 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 (ROS) promote c-Jun-N-terminal kinase (JNK) that would impair insulin signal transduction pathway activating daf-16 [29]. A recent study showed that anthocyanin extract was unable to extend lifespan under oxidative stress in daf-16 mutants suggesting that anthocyanin requires daf-16 for its antioxidant activity [13]. Damage repair pathways in aged tissues were impaired, likewise, cellular clearance pathways were impaired due to accumulated damage [30].

During oxidative damage, the survival of the *C. elegans* treated with crude anthocyanin extract was prolonged [11]. *In vivo* studies identify anthocyanin to be a powerful antioxidant with the potential to alleviate cellular damage caused by oxidative stress, thus prolonging the 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 cells caused by pathogens. The difference in the response of the nematode to different pathogens may be attributed to the structure of the bacteria [18]. It was observed that in *S. aureus,* only the high and the mid-concentrations of both SMCAE and D3G were able to extend the mid-lifespan of the nematode. This result is likely to be attributed to the effect of anthocyanin and delphinidin in recruiting lysozymes to degrade the peptidoglycan of gram-positive bacteria [18]. Meanwhile, *K. pneumoniae* produces enterotoxins that increase the level of cGMP [31]. cGMP activates the ROS signaling 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 activated a 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 prolongs the lifespan through the daf-16 signalling pathway.

Conclusion

This study showed that *S. melongena* crude anthocyanin extract and delphinidin-3-glucoside at 1000 µg/mL allow the growth of *S. aureus* and *K. pneumoniae*. However, results indicate 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 light of these findings, further validation in higher organisms is recommended.

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Author Contribution

JSBN, CKFR, RRGA, JPK, ALPG designed the experiment. CKFR, RRGA, 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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