Research Article

Molecular Interactions of Cyanidin-3-glucoside with Bacterial Proteins Modulate the Virulence of Selected Pathogens in *Caenorhabditis elegans*

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ABSTRACT

Anthocyanins are plant pigments known for their radical scavenging activity. However, the current understanding of the effects of anthocyanins in bacterial infection is limited to crude extracts. Additionally, most studies about cyanidin-3-glucoside (C3G), an anthocyanin compound from the cyanidin class, were limited to *in vitro* experiments. This paper investigated the protective mechanism of C3G against selected pathogens, namely *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecalis* (*E. faecalis*), *Klebsiella pneumoniae* (*K. pneumoniae*), and *Proteus mirabilis* (*P. mirabilis*) using *Caenorhabditis elegans* (*C. elegans*) as the infection model. The nematodes were supplemented with varying sublethal concentrations of C3G every day after hatching. Besides, the concentrations of C3G used exhibit no bacterial growth suppression against the pathogens. The nematodes transferred to different plates were flooded with varying bacteria. The number of surviving worms were counted every day until all worms were deceased. To hypothesize the protective mechanism of C3G, its 3D structure was docked in the crystal structures of the different proteins produced by each pathogen. These protein molecules represent their virulence factors, which are available in the Protein Data Bank. The docked crystal structures were evaluated for their binding energies and binding interactions. Results show that C3G rescues *C. elegans* against *S. aureus* but not from *E. faecalis*, despite both are gram-positive. This observation is similar to the gram-negative pathogens where *C. elegans* were protected from *P. mirabilis* treatment but not against *K. pneumoniae*. Consistently, the docked crystal structures of C3G reveal to have a high binding affinity with amidohydrolase and exfoliative toxin A, which are virulence factors of *P. mirabilis* and *S. aureus*, respectively. These findings suggest that the protective mechanism of C3G against pathogens may influence the virulence factors of certain bacteria only.

Key words: Bacterial infection, Cyanidin-3-glucoside, Molecular docking, Virulence factors, *Caenorhabditis elegans*, *in silico*.

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INTRODUCTION

Recent studies have demonstrated that anthocyanin-rich plant extracts improve the longevity of *C. elegans*.[1-4] Other studies have demonstrated a life span extension in Drosophila.[5,6] Aside from longevity studies, evidence from previous experiments reported that anthocyanins show beneficial effects on cancer and neurodegenerative diseases. C3G is one of the common anthocyanin compounds found in fruits and vegetables. C3G is an anthocyanidin containing a carbohydrate moiety as shown in Figure 1. Longevity and stress tolerance was associated with crude extracts containing a high amount of C3G.[1] This flavonoid is popular for its numerous properties, such as anti-cancer, antioxidant, anti-inflammatory, and anti-angiogenic.[7,8] Likewise, studies have shown that plant extracts containing C3G attenuates bacterial growth.[9-11] The current

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understanding of anthocyanin in bacterial infection is limited to investigating crude extracts and bacterial growth suppression. Knowledge on the efficacy of pure anthocyanin compounds on bacterial infection in a multicellular organism is still scarce.

The single primed (') C backbone and R group represents those atoms found in the benzene ring linked to the furan ring via C2. Likewise, the double primed (") C atoms and R groups are those belonging to the carbohydrate moiety of the anthocyanin.

C. elegans has long been used as a model organism because of its simplicity, short lifespan, affordability, and available genome.[12] It is also an inexpensive large screening tool for antimicrobial drug discovery.[13] In this study, *C. elegans* was considered the infection model because of its simple innate immune response and the absence of adaptive immunity.[14]

In humans, pattern recognition molecules, hyaluronan breakdown products, and toll-like receptor ligands mediate pathogen recognition.[15,16] However, *C. elegans* lacks pattern recognition molecules and hyaluronic acid.[14] The known Toll-like receptor in *C. elegans*, toll-1, is associated with pathogen avoidance behavior.[17] Another study also supports that the Toll signaling pathway in *C. elegans* is responsible for its detection and avoidance of gram-negative bacteria.[18] Spätzle processing enzyme (SPE) mediates the toll-dependent antimicrobial response and development-related pathways.[19] Another study suggests that toll-1 participates in Mitogen Activates Protein Kinase (MAPK) pathway via the sek-1 and pmk-1. MAPK is also responsible for producing antimicrobial factors such as mlp-29, lys-1, and C-type lectins.[20] During an infection, different studies suggest that pathogens subdue the expression of pmk-1.[20,21] Thus, there is a decline in antimicrobial factor production. The daf-2/daf-16 pathway causes pathogen resistance.[22] This pathway produces various antimicrobial proteins such as lys-1, lys-2, spp3-3, and spp-18.[23]

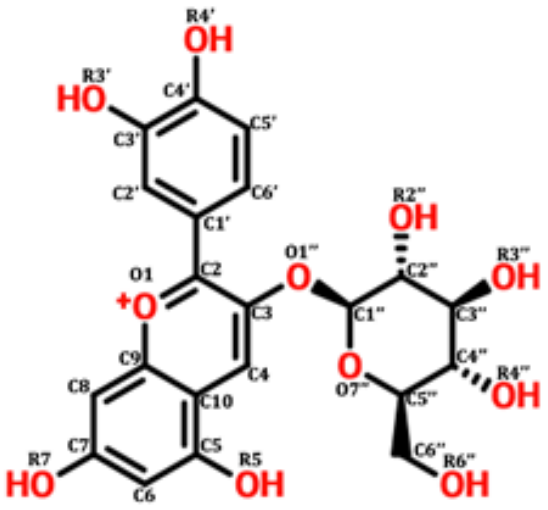

Figure 1: Structure of cyanidin-3-glucoside with assigned atom identification.

The different molecular pathogenesis in bacteria may directly or indirectly be affected by the stress response pathway.[24] Many gram-positive and gram-negative bacteria rely on their pore-forming toxins to inflict virulence in their host.[25] Bacterial infection may also cause oxidative damage to the host, as demonstrated by *S. aureus* and *P. mirabilis*.[26] The compensatory responses during oxidative stress activate the MAPK pathway.[27] Likewise, MAPK pathways defend *C. elegans* against pore-forming bacterial toxins.[25]

Aside from innate immune response, the MAPK pathway is also important in aging and stress response. Studies have also reported that anthocyanins influence this pathway.[28,29] Plant anthocyanin extracts were also reported to enhance longevity in *C. elegans* through the daf-16/FOXO transcription factor, MAPK, and NrF2 genes.[1,3]

This study investigates the protective mechanism of C3G against selected pathogens, namely *S. aureus*, *E. faecalis*, *K. pneumoniae,* and *P. mirabilis* using *C. elegans* as the infection model.

MATERIALS AND METHODS

Caenorhabditis elegans and bacterial strains handling procedures

Bristol N2 (wildtype) *C. elegans* and *Escherichia coli* (*E. coli*) OP50 were from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota (MN, USA). Nematodes were grown on a nematode growth medium (NGM) agar at 25°C.[4] The researchers incubated *E. coli* OP50 at 37°C and fed it to the worms. Worms were age synchronized by gathering eggs laid on NGM plates within 4 hr. These eggs were grown until L4. Different bacteria such as *S. aureus* (ATCC-25923), *E. faecalis* (ATCC 29212), *K. pneumoniae* (BAA-1705), *P. mirabilis* (ATCC 29906) were also used in the experiment, which was grown at 37°C.

Preparation of cyanidin-3-glucoside

Cyanidin-3-glucoside (C3G) (>97%) was obtained from AS Polyphenols (Norway). It was reconstituted with distilled water to 1 mg/mL and stored at 4°C until further use. The solution was placed in a dark room to protect it from direct contact with light. The compound was further diluted into 100, 10, and 1 μg/mL as used in the experiment.

Broth Macro-dilution Qualitative Assay

The broth was prepared by mixing 2.8 g of Trypticase Soy Broth (TSB) with 100 mL of distilled water and transferred it into screw-top tubes before sterilizing. The screw-top tubes were cooled down to room temperature before use. *S. aureus, P. mirabilis, K. pneumoniae,* and *E. faecalis* were grown in the TSB tubes following a 0.5 McFarland standard. Varying concentrations of C3G (100, 10, 1, and 0 µg/mL) were added in each screw-top tubes. The bacterial suspensions in each broth were qualitatively evaluated by inspecting the presence of turbidity against a white background.

Caenorhabditis elegans sublethal assay

Different concentrations (100 µg/mL, 10 µg/mL, 1 µg/mL, and 0 µg/mL) of C3G were prepared. The negative control 0 µg/mL of C3G was prepared by using distilled water only. 50 µl of the C3G and *E. coli* OP50 mixture were dispensed on a new Nematode Growth Media (NGM) plate. Different NGM plates were used for each treatment, which contains 30 worms. The number of live, dead, and missing worms were counted for 0, 24, 48 hr. Worms were considered dead when they do not respond to fine touch.

Caenorhabditis elegans lifespan assay without pathogens

Fifty µl freshly prepared *E. coli* OP50 suspensions were dispensed into each NGM plate daily. Starting with 30 L4 nematodes for each treatment, the number of live, dead, and missing worms were counted every day until all worms are dead.

Caenorhabditis elegans lifespan assay with pathogens

Following the previously mentioned protocol, fresh *S. aureus*, *E. faecalis*, *K. pneumoniae*, and *P. mirabilis* suspensions instead of *E. coli* were prepared every day. They mixed each suspension with varying concentrations of C3G solutions. Fifty µl of each freshly prepared C3G-bacterial mixture was dispensed on different NGM plates. Thirty L4 nematodes were placed on each plate at the start of the experiment, and the number of living, dead, and missing nematodes were counted daily until all worms are deceased.

Statistical Analysis

In the study, each nematode was considered as a replicate. The experiments were repeated twice. Kaplan-Meier curve was used to determine the mean lifespan of the nematode. Furthermore, a log-rank test was used for post-hoc analysis in determining significant differences between the treatment groups. All statistical analysis was carried out through OASIS version 2 (Korea). The significant difference was set at $p<0.05$.

Molecular docking of C3G to different virulence factor-associated proteins

Representative virulence factor-associated proteins of *S. aureus, E. faecalis, K. pneumoniae,* and *P. mirabilis* were searched in the Protein Data Bank (www.rcsb.org). Only the crystal structures of the representative protein molecules present in the Protein Data Bank were downloaded and evaluated further. There were five virulence factors of *S. aureus* with corresponding proteins available in PDB found, namely gamma-hemolysin (PDB: 3B07), leukocidin F (PDB: LKF), enterotoxin C2 (PDB: 1STE), exfoliative toxin A (PDB: 1EXF), and EsxA (PDB: 2VS0). In *E. faecalis*, there were three representative proteins found in the database, which includes PrgA (PDB: 6Z9K), hemolysin (PDB: 2R2Z), and CylR2 (PDB: 1UTX). For *K. pneumoniae*, there were five proteins evaluated, such as KacT (PDB: 5XUN), LucC (PDB: 6CN7), MrkD1P (PDB: 3U4K), LptDE (PDB: 5IV8), and GspL (PDB: 5HL8). Lastly, the three proteins from *P. mirabilis* identified were AtfE (PDB: 6H1X), amidohydrolase (PDB: 3RHG), and hemolysin A (PDB: 5SZ8).

The different proteins' crystal structures were prepared by adding hydrogen and Gasteiger charges; merging the charges; and removing non-polar hydrogens, lone pairs, water molecules, and non-standard residues. cyanidin-3-glucoside (CID: 197081) was docked to the crystal structure of the protein molecules. The protein molecule preparation and the docking of the ligand were carried out using Mcule (Mcule Inc., USA) and Autodock Tools (The Scripps Research Institute, USA).

The ligand's docking to the protein molecules was validated by redocking a known ligand to the protein. The re-docked crystal structure was downloaded and superimposed to the original crystal structure through Superpose v.1.0 (Wishart Lab, University of Alberta, Edmonton, Canada). The root means square deviation (RMSD) of the superimposed structures' atomic position was evaluated to be less than 1.2 Å. RMSD value lower than 1.2 Å indicates that the re-docked crystal structure's crystal structures are similar to the original structure.[28]

The best-docked crystal structures with binding energies ranging between -8.0 and -11.71 kcal/mol were visualized through PLIP (BIOTEC TU Dresden, Saxony, Germany).[30] The different binding interactions of the ligand with the protein were identified and compared with the known ligand.

RESULTS

C3G does not inhibit bacterial growth up to 100 µg/mL

There were five varying concentrations of C3G tested on the growth of *S. aureus*, *K. pneumoniae*, *E. faecalis*, and *P. mirabilis*. They used 100 µg/mL as the highest limit since this is the concentration that does not affect the suspensions' color. All the treatments shown in Table 1 have shown comparable turbidity to 0.5 McFarland. These data show that ≤100 µg/ml of C3G may not be enough to suppress bacterial growth.

Sublethal Assay

There were thirty L4 nematodes placed on each NGM plate with *E. coli* OP50. Each plate received varying concentrations of C3G ranging from 100 to 0 µg/ml. The highest concentration with ≥ 90% survival rate after 48 hr was considered as the sublethal concentration.[31] All C3G concentrations tested have a ≥ 90% survival rate, as shown in Figure 2. Suggestively, 100 µg/ml of cyanidin does not pose chronic toxicity to *C. elegans*.

A total of 30 L4 nematodes were given various concentrations (0, 1, 10, 100 µg/mL) of C3G suspended in *E. coli* OP50 every day for 48 hr. Surviving worms were counted at 0, 24, and 48 hr. Worms that did not respond to light touch were considered dead. Sublethal concentration was set at the concentration where ≥90% of worms survived.

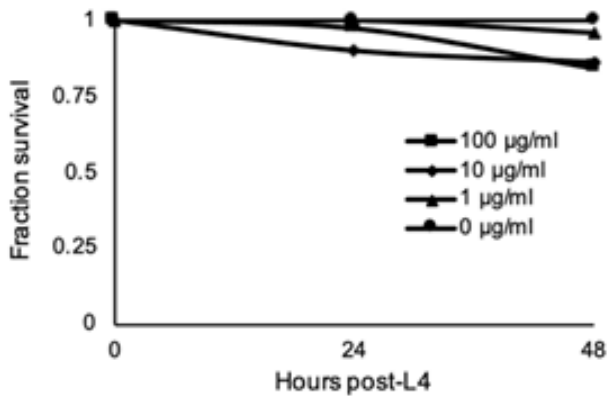

Figure 2: C3G shows no chronic toxicity on *C. elegans* up to 100 µg/ml.

Varying protective effect of C3G in *C. elegans* against various bacteria

In Figure 3A, *C. elegans* fed were exposed to *E. coli* OP50, which is a non-pathogenic bacterium to *C. elegans*. Nematodes fed with 100 µg/ml C3G mixed with *E. coli* OP50 suspension were observed to have a 19% longer lifespan. However, the nematodes' lifespan who received 1 and 10 µg/ml were comparable with those who did not receive C3G.

Treatments with varying concentrations of C3G exhibit an increase in the survival rate of *C. elegans* when exposed to gram-positive bacteria *S. aureus* (Figure 3B). The highest concentration 100 µg/ml exhibited 34% lifespan extension (Figure 3C). This data suggest that C3G affects the defense system of *C. elegans* during its exposure to *S. aureus*. Meanwhile, no improvement in the mean lifespan was observed in treatments exposed to *E. faecalis*. These data imply that C3G may not protect *C. elegans* against all gram-positive bacteria.

To investigate this result further, the effects of C3G on two gram-negative bacteria were determined. For the nematodes fed with varying C3G concentrations mixed with *K. pneumoniae*, the mean lifespans of the treatments were comparable with the untreated. Conversely, about 42% lifespan extension was observed in the nematodes given 100 µg/ml C3G mixed with *P. mirabilis*. These observations were similar to the gram-positive bacteria, indicating that C3G may not warrant protection against all kinds of pathogens.

L4 nematodes were transferred to new NGM plates daily. Various concentrations of C3G reconstituted with either A– *E. coli* OP50; B– *S. aureus*; C– *E. faecalis*; D– *K. pneumoniae*; or E– *P. mirabilis* suspension were distributed to each plate each day until all worms are deceased. * significant difference at $p<0.05$.

Molecular interactions of C3G with the virulence factors of the selected pathogens

To hypothesize a possible mechanism of the protective property of C3G, its 3D structure was docked with

Table 1: The minimum inhibitory concentration of cyanidin-3-glucoside.

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

+/- indicates presence or absence of turbidity

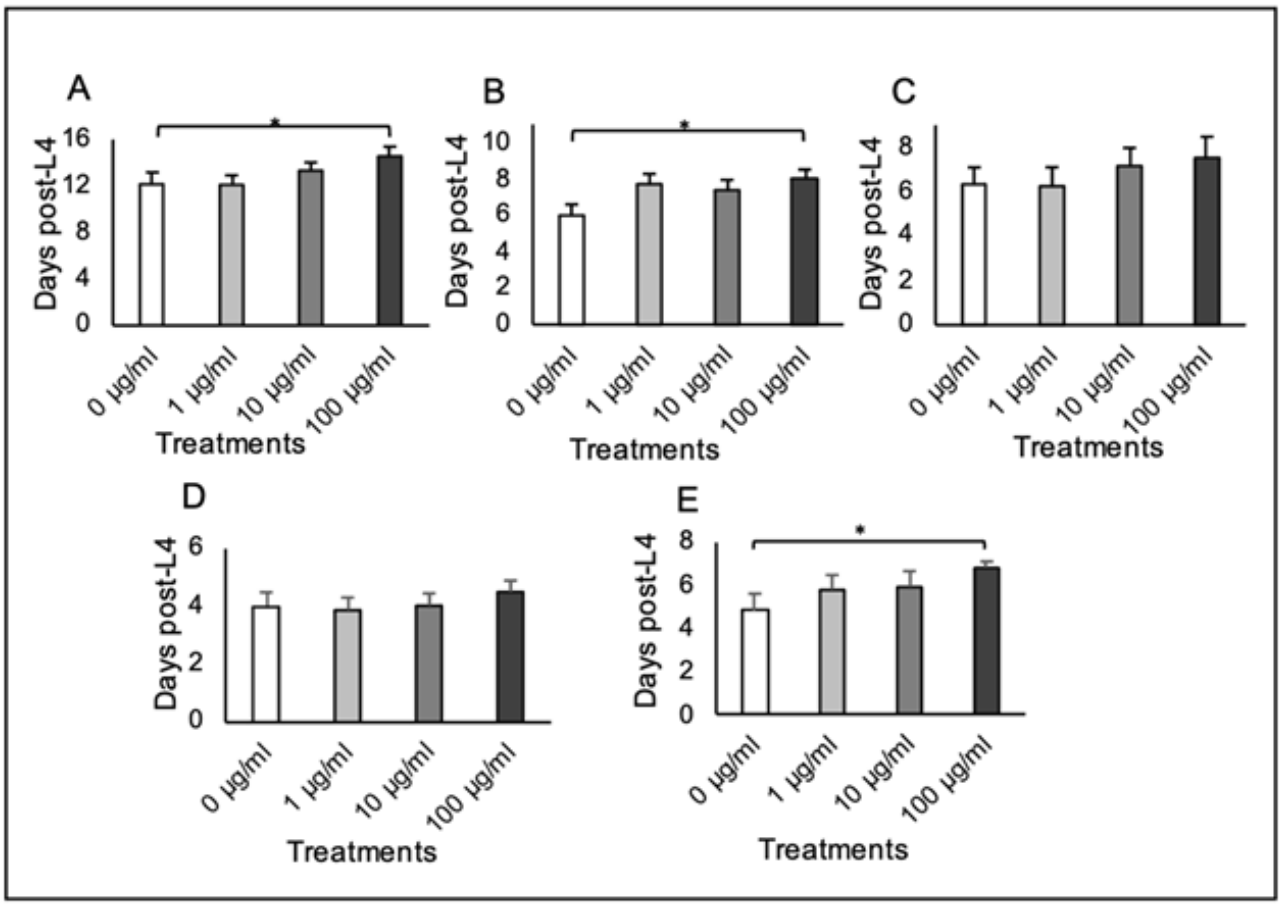

Figure 3: Mean lifespan of *C. elegans* infected with different bacteria.

various proteins. These molecules represent the different virulence factors of the bacteria, which may be limited to those available in the Protein Data Bank. As shown in Table 2, two proteins notably exceeded -8 kcal/mol, namely exfoliative toxin A and amidohydrolase. Exfoliative toxin A, a toxin produced by *S. aureus*, scored -8.2 kcal/mol. Additionally, amidohydrolase, a deaminase elicited by *P. mirabilis*, exhibited -11.1 kcal/mol binding energy.

The crystal structure of exfoliative toxin A was docked with the C3G and compared with the re-docked structure of small ligand GLY (CID: 750), whereas the docking of C3G with amidohydrolase was compared with the known inhibitor BEZ (CID: 243). The ligands' molecular docking was validated by superimposing the re-docked structure of the ligand inhibitor with the original crystal structure. The RMSD value of the superimposed structure crystal structures was lower than 1.2 Å, which indicates that the redocked structure is similar to the original crystal structure.[32]

The small ligand GLY interacts with lys21, glu24, asn71 and gly300 in the chain A of exfoliative toxin A through hydrogen bonding, as shown in Figure 4A. Also, a salt bridge was observed with lys21. The C3G-exfoliative toxin A docked crystal structure's hydrophobic interactions and hydrogen bonding were observed, as shown in Figure 4B. The hydrophobic interactions were found in lys16 (C4) and lys21 (C2'). Meanwhile, seven hydrogen bonds were formed with leu14 (R2"), leu14 (R3"), ser20 (R6), asp74 (R4'), his145 (R6"), lys151 (R6"), and his156 (R6").

In the docked crystal structure of BEZ with amidohydrolase, hydrophobic interactions were observed in leu257, phe261, phe296, whereas water bridges were formed with tyr63 and gly202, as shown in Figure 4C. Other non-covalent interactions, such as pi-stacking and salt-bridges, were found in his229 and his 199, respectively. Similarly, hydrophobic interaction, salt bridges, ad pi-stacking were also observed in the C3G-amidohydrolase docked structure, as shown in Figure 4D. However, this crystal structure does not have water bridges but instead forms hydrogen bonds. The hydrophobic interaction was seen in leu30 (C8). Meanwhile, nine hydrogen bonds were observed in asn28 (R7), asp35 (R4'), a double H bond in lys129 (R3'), met200 (R4"), gly202 (R4"), his229 (R2"), asp294 (R2"), and asp294 (R5). There were pi-stacking interactions

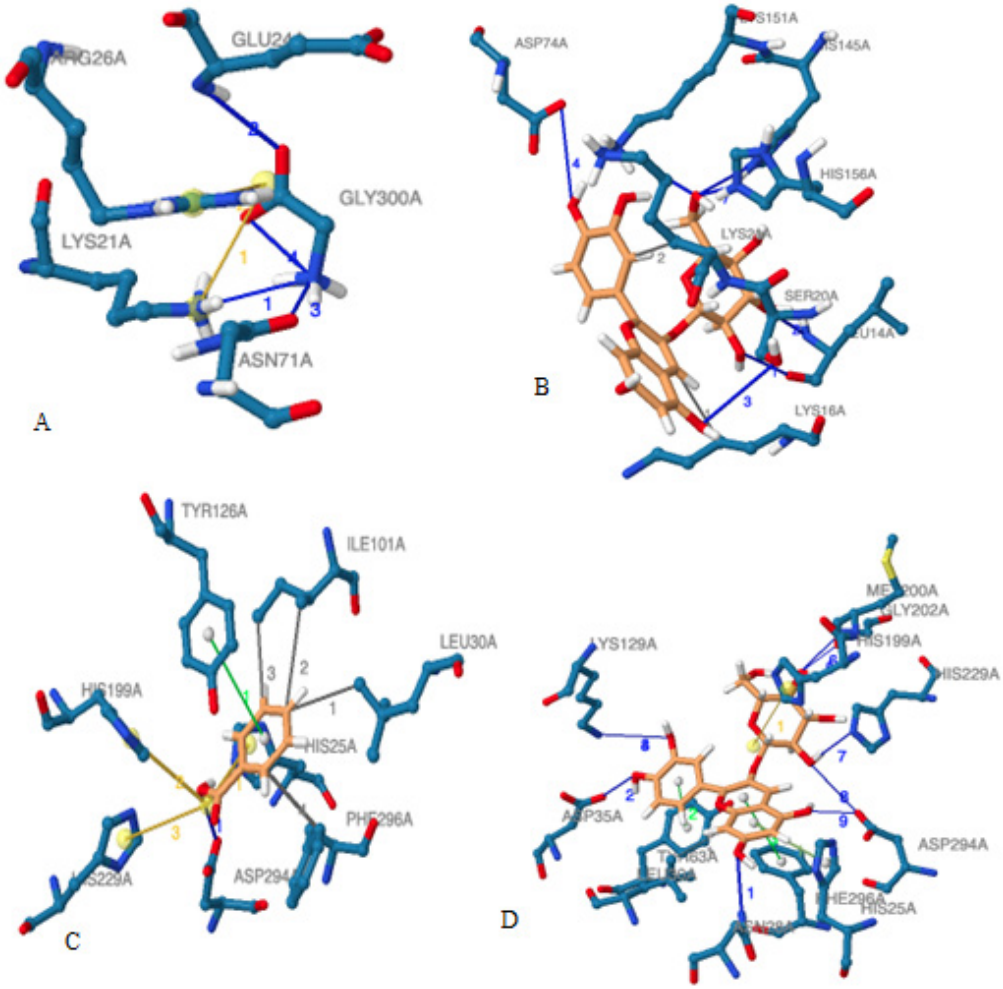

Figure 4: Binding interactions of exfoliative toxin A (PDB: 1EXF) with ligands A– GLY and B– C3G and amidohydrolase (PDB: 3RHG) with ligands C– BEZ and D– C3G.

observed in his25, tyr63, phe296, whereas a salt bridge was formed with his 199.

DISCUSSION

Previous studies that used crude extract containing anthocyanins, such as cranberry and thyme, exhibited antibacterial property.[33,34] In this case, there may be a synergistic effect among the crude extract compounds, which leads to an antibacterial effect. Another study about *Myrcianthes hallii* anthocyanin extract shows minimal antibacterial activity.[35] In this experiment, cyanidin-3-glucoside (C3G) concentration may be low enough to elicit an antibacterial activity.

Similarly, a recent study in delphinidin that used the same amount demonstrates no bacterial growth inhibition in *S. aureus* and *K. pneumoniae*.[4] This evidence may indicate that the amount of anthocyanin influences its antibacterial property. However, this warrants further investigation due to certain limitations in a qualitative procedure.

Recent studies with anthocyanin-rich plant extracts reported improvement in longevity of *C. elegans*.[1-4] Likewise, a study on a different class of anthocyanin, delphinidin-3-glucoside, was also reported to lengthen the lifespan of *C. elegans*.[4] Interestingly, even though C3G protected *C. elegans* from *S. aureus,* this was not evident against *E. faecalis*. Similarly, C3G-nourished nematodes did not guarantee protection from *K. pneumoniae*, whereas those who were exposed to *P. mirabilis* were protected. These findings suggest that the protective mechanism of C3G against pathogens may not only be involved in enhancing the innate immune system of the *C. elegans*. Otherwise, the nematode might have been protected from both gram-positive and gram-negative bacteria. In this case, the researchers hypothesize that C3G may have influenced the bacteria's virulence factors.

C3G was docked to the crystal structure of the different virulence factors-associated proteins of the different

Table 2: Docking score of C3G with the protein crystal structure of the virulence factor of the different pathogens.

Organism	Virulence factor	Protein	PDB ID	Docking Score (kcal/mol)
S. aureus	Hemolysins	Gamma-hemolysin	3B07	-6.9
	Leukocidins	Leukocidin F	3LKF	-6.9
	Enterotoxins	Enterotoxin C2	1STE	-6.4
	Exfoliative toxin	Exfoliative toxin A	1EXF	-8.2
	Cell invasion	EsxA	2VS0	-5.8
K. pneumoniae	Toxin	KacT	5XUN	-7.2
	Siderophores	LucC	6CN7	-6.5
	Adhesin	MrkD1P	3U4K	-5.9
	LPS	LptDE	5IV8	-5.8
	LPS	GspL	5HL8	-5.6
P. mirabilis	Adhesin	AtfE	6H1X	-5.2
	Deaminase	Amidohydrolase	3RHG	-11.1
	Hemolysin	Hemolysin A	5SZ8	-5.8
E. faecalis	Adhesin	PrgA	6Z9K	-7.1
	Hemolysin	Hemolysin	2R2Z	-5.7
	Cytolysin	CylR2	1UTX	-7.5

bacteria. The more negative docking score is predicted to have a higher binding affinity between the ligand to protein.[28] Generally, C3G has a higher binding affinity to amidohydrolase and exfoliative toxin A than the other proteins. This finding suggests that the lifespan extension observed in *C. elegans* treated with *S. aureus* and *P. mirabilis* may be attributed to the modulation of their protein-associated virulence factors, exfoliative toxin A and amidohydrolase, respectively.

Additionally, exfoliative toxin A is associated with the intraepidermal separation of skin layers.[36] The high binding affinity of C3G to exfoliative toxin A suggests that it may have inhibited the toxin from causing insult in the nematode's skin. In *C. elegans*, the epidermis is its first line of defense against pathogens, similar to humans.[37] Moreover, amidohydrolase hydrolyzes urea to ammonia and carbon dioxide. A study shows that this catalytic reaction leads to an increased pH due to the precipitation of Ca^{2+} and Mg^{2+} ions.[38] In humans, the urinary tract's increased pH leads to crystal formation due to struvite and hydroxyapatite.[38] Previous studies reported that *C. elegans* excrete a higher amount of ammonia than urea.[39] Additionally, it was observed that urea protects *C. elegans* from hypertonic stress due to an increase in the impermeable ions.[40] The high binding affinity of C3G amidohydrolase may have inhibited the enzyme activity, which results in preventing hypertonic stress in *C. elegans*.

The high binding affinity of C3G with exfoliative toxin A and amidohydrolase may be attributed to the high number of hydrogen bonds formed. The number of hydrogen bonds directly influences the binding affinity of the ligand.[41] Consequently, other protein-ligand interactions present in C3G-amidohydrolase interaction, namely pi-stacking and salt-bridges, may have contributed to the increase in its binding affinity compared to C3G-exfoliative toxin A docked crystal structure. Pi-stacking and salt-bridges also stabilize the docking of a ligand to the protein.[28]

CONCLUSION

The protective property of C3G against various bacteria was investigated in this research using *C. elegans* as the infection model. The ability of C3G to rescue *C. elegans* only from particular pathogens, such as *S. aureus* and *P. mirabilis*, was demonstrated since the mean lifespan of the nematode was significantly higher compared to the untreated. The molecular docking experiment supports this observation, which shows that C3G may have influenced the virulence factors of *S. aureus* and *P. mirabilis*. Therefore, these findings suggest that the

virulence factor plays a pivotal role in the protective mechanism of C3G. Likewise, further investigations are needed to augment these findings.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

C3G: cyanidin-3-glucoside; **NGM:** nematode growth medium; **MAPK:** Mitogen Activates Protein Kinase; **SPE:** Spätzle processing enzyme; **L4:** larval stage 4; **RMSD:** root mean square deviation.

SUMMARY

The current study demonstrated that C3G protects *C. elegans* against specific pathogens only. Hence, the mechanism of action of C3G in *C. elegans* was hypothesized to be independent of the innate immune response of the nematode. Moreover, this protective mechanism appears to be associated with modulating a particular virulence factor of the pathogen.

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