Investigation of the Neuroprotective effects of Haskap Berry *(Lonicera caerulea)* extract on *Caenorhabditis elegans*

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Introduction

- Alzheimer's Disease (AD) is the most common form of neurodegenerative dementia in Canada[1].
- The pathogenesis of AD is characterized by the biochemical event of amyloid-β (Aβ) plaque accumulation in the brain[2].
- Many researchers are investigating therapeutic options for the prevention and treatments of AD using natural plant-based sources i.e. superfoods.
- Haskap berries (HBs) (Fig. 1) are originally native to Siberia and northeastern Asia but have recently expanded to the Canadian market[3].
- The compounds found in HBs, such as the anthocyanin cyanidin-3-glucoside (C3G), have demonstrated in purified *in vitro* studies to have beneficial effects on lifespan, development, and neuronal growth[4].

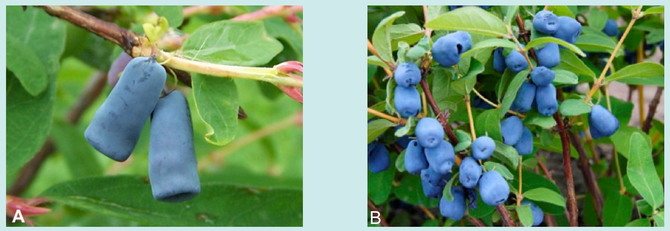
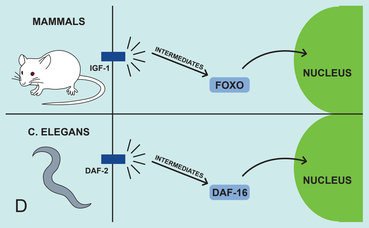


Fig. 1: (A) Close-up HBs morphology[5]. (B) HBs growing morphology[6].

- Due to the presence of C3G in HBs, there is the potential for the fruit to have neuroprotective effects against the accumulation of amyloid-β (Aβ) plaques.
- HBE is known to increase expression and nuclear translocation of DAF-16 transcription factor (TF) (Fig. 2C) which is involved in the stress response pathway[7].
- In addition, DAF-16 upregulation is associated with delayed Aβ toxicity[8].
- This research supports a causative link between the known upregulation of DAF-16 by HBE and the effects of DAF-16 on delayed Aβ toxicity.



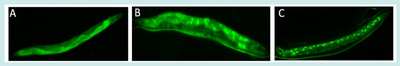


Fig 2: (A) Three designations of DAF-16 localization. Represents cytoplasmic DAF-16 localization (B) Represents intermediate DAF-16 localization, (C) Represents nuclear DAF-16 localization. (D)DAF-16 pathway and the homologous pathway found in mammals.

Research Goals

- Continue previous work into the investigation of the effects of Haskap Berry Extract (HBE) on *C. elegans.*
- Determine if HBE has neuroprotective effects in *C. elegans,* in terms of delaying the onset of phenotypic symptoms, and reducing the accumulation of Aβ plaques.

Materials and Methods

Model Organism

- *C. elegans* were chosen for this experiment due to our vast knowledge of cellular pathways, the availability of inducible amyloid (Aβ) transgenic strains, and the ease of care within the laboratory[9].

Neuroprotection Assay

- A transgenic *C.elegans* strain CL4176 was used as they produce β-amyloid protein under a temperature sensitive (23°C) repressible mRNA surveillance system.
- Accumulation of toxic levels of Aβ protein in the *C.elegans* results in total paralysis.
- CL4176 strain also has a characteristic rolling phenotype to differentiate the worms, (Fig. 3) from regular N2 worms that have S-shaped movement (Fig. 4).
- Age-synchronous worms were grown on experimental (HBE) and control (NGM) conditions and then β-amyloid protein production was initiated at L3 life stage by temperature upshift from 16°C to 23°C.
- Onset of paralysis between the conditions was recorded every 2 hours from 24-40 hours after temperature upshift.
- Analysis of the paralysis phenotype was determined by the ability of the worm to move their posterior segment, complete a full body-wave movement, and respond to manual stimulus.
- Statistical analysis was conducted by OASIS 2, a surviorship analysis tool[10].

Fig. 3: Phenotypic movement patterns of strains. Scan QR.

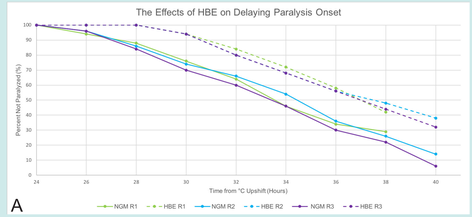
Fig. 4: (A) N2 strain showing S-like movement (B) CL2006/CL4176 showing rolling movement (C) Paralyzed CL2006/CL4176.

β-Amyloid Accumulation X-34 Assay

- Transgenic *C.elegans* strain CL2006 was used, which constitutively produce β-amyloid protein, and toxic accumulation of β-amyloid protein in the *C.elegans* result in total paralysis.
- CL2006 strain also has the characteristic rolling phenotype (Fig. 4B).
- Age-synchronous worms were grown on experimental and control conditions and then stained with β-amyloid plaque sensitive X-34 dye.
- Qualitative β-amyloid plaque accumulations were recorded via fluorescence microscopy. Quantitative normalized fluorescence was compared between the groups via ImageJ, a image analysis software, and statistically analyzed by t-tests.

Results

Neuroprotection Assay

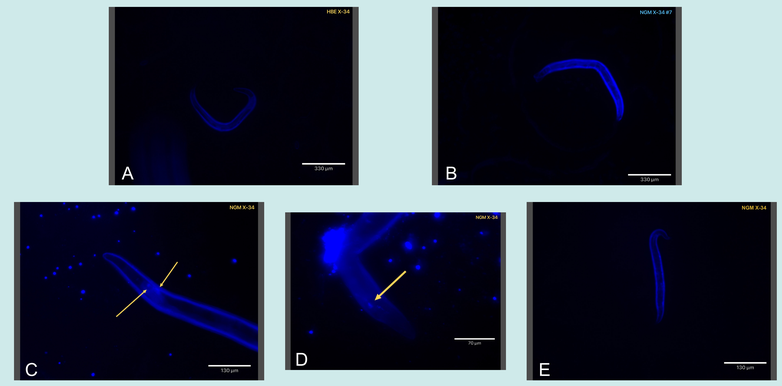


Experimental Conditions	N Value	P-value
NGM vs. HBE (replicate one)	100	0.0035
NGM vs. HBE (replicate two)	100	0.0042
NGM vs. HBE (replicate three)	100	0.0004

B

Fig. 5 (A). Survivorship curve that demonstrates the percentage of HBE and NGM worms affected by paralysis. The x-axis shows the number of hours since temperature upshift, and the y-axis shows the percent of each treatment not paralyzed at that time. Data was processed using OASIS 2[10]. (B) Statistical analysis of the data of three replicates of the neuroprotection assay analyzed by OASIS 2[10]. Significance is found at p<0.05.

β-Amyloid Accumulation X-34 Assay



Experimental Conditions	N-value	Mean Normalized Fluorescence	P-value
NGM	21	27.24 a.u.	2.58653E-05
HBE		9.52 a.u.	

F

Fig. 6: (A). A qualitative look at HBE worms stained with X-34. (B). A qualitative look at NGM control worms stained with X-34. All photos were taken at the 565ms exposure. (C) A dead NGM control worm's internal accumulation (arrows) of β-amyloid. (D)(E) An NGM control worm's internal accumulation of β-amyloid. (F) The mean normalized fluorescence and p-values for the accumulation assay as analyzed by one tailed t-test. Data was replicated in triplicate studies and demonstrated consistency.

Discussion

- In the first assay, triplicate studies were conducted as to determine the effect of HBE on delaying the paralysis brought on by the accumulation of β-amyloid protein *in vivo.*
- All three replicates resulted in consistent results and p-values <0.05 via statistical analysis by OASIS 2 (Fig. 5B)[10].
- Interestingly, onset of paralysis in both control and experimental conditions was delayed by 4 hours across all replicates as compared to literature expectations.
- The delay in paralysis could be due to inconsistent upshift temperature, developmental stage, or differing lawn size shifting the kinetics of paralysis.
- The β-amyloid accumulation X-34 assay was conducted in triplicate to determine if the presence of HBE would significantly reduce the accumulation of the Aβ protein *in vivo.*
- Due to the lack of available procedures for the use of X-34 dye, a procedure for live staining was designed and carried out to effectively stain the β-amyloid in the live worms without sacrificing.
- The analysis via ImageJ excluded the body walls to isolate fluorescence by the protein accumulation.
- The three replicates resulted in consistent results and p-values <0.05 via statistical analysis by one tailed t-test assuming equal variance (Fig. 6F).

Conclusion

- Results of the neuroprotective assay demonstrate that HBE significantly delays the effects of β-amyloid protein toxicity *in vivo*.
- Results of the β-amyloid accumulation X-34 assay demonstrate that HBE significantly reduced the amount of β-amyloid protein accumulation *in vivo.*

Future Directions

- Future studies using larger sample size studies of the β-amyloid accumulation X-34 assay is necessary to ensure replicability of the study.
- Integration of RT-PCR work would allow us to elucidate if DAF-16 is the gene responsible for the neuroprotective effects seen in the study.

Acknowledgments

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