Exploring Exogenous Cannabinoids as a Potential Therapeutic for the Reduction of Oxidative Stress in *C. Elegans* Model Systems

A Major Qualifying Project Report submitted to the Faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science.

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Abstract

This project aimed to study the relationship between cannabidiol (CBD) and levels of reactive oxygen species (ROS) present in N2 strain *C. elegans*. It was found that six-hour treatments of two different concentrations of CBD produced inconclusive data regarding the impact of CBD on ROS. Due to the exceedingly high variability in the data, no conclusions could be drawn, suggesting the necessity of further experimentation. Interestingly though, the CBD treatment appeared to increase worm longevity as the CBD samples yielded a higher count of live worms relative to the control samples.

Acknowledgements

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Introduction

This project aimed to explore the use of cannabidiol (CBD) as an agent for the reduction of oxidative stress in *C. elegans* model systems. By looking into the ability of CBD to reduce the levels of reactive oxygen species (ROS), conclusions regarding CBD's potential as a therapeutic can begin to be made. These conclusions can then begin to broaden not only the understanding of CBD's healing abilities, but also the understanding of how to treat issues pertaining to oxidative stress and neurodegeneration.

In the wake of increased research and acceptance of the use of CBD as a therapeutic, claims regarding the scope of its healing properties have expanded to the realm of neurodegenerative diseases. These claims are rooted in CBD's proposed interactions with both the endocannabinoid system and the one-carbon metabolism (OCM) pathway (Martínez-Reyes & Chandel, 2015). Thus far, research pertaining to the effect of exogenous cannabinoids on longevity and stress response has been performed on *C. elegans* models, suggesting that CBD has a positive effect on these organisms in these areas (Land et al., 2021). There has also been limited research into the effect of CBD on the regulation of the OCM, more specifically, how CBD impacts the levels of methionine within this system in *C. elegans*. However, there has been no research into the implications of CBD as a therapeutic for neurodegenerative diseases aimed toward the reduction of oxidative stress.

Caenorhabditis elegans are a species of nematode that has been used in a wide range of scientific research. Due to their rapid growth, small size, and ease of maintenance, *C. elegans* have been used in different studies pertaining to longevity, aging, animal behavior, and development. Their

genomes and transcriptomes have been fully characterized, in addition to their nervous system which consists of 302 neurons (Estrada-Valencia et al., 2021). With this, multiple conserved molecular pathways (homologous to those found in humans) have been found throughout their genome, making this model advantageous at several levels (Estrada-Valencia et al., 2021). Despite their wide advantages, limited studies on cannabinoids employ the use of these nematodes. Regardless, an endocannabinoid system in *C. elegans* was proposed due to evidence showing their ability to synthesize endocannabinoids such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in 2008 (Lehtonen et al., 2008). Due to this, studies pertaining to CBD have utilized these organisms as *in vitro* models.

Cannabidiol (CBD) is a highly lipophilic molecule that is the non-psychoactive component of *Cannabis sativa* (Millar et al., 2018). The pharmacological effects of CBD are mediated via the endocannabinoid system which contains G-protein coupled receptors (CB_1 and CB_2). CB_1 receptors are located throughout most of the central nervous system (CNS) while CB_2 receptors exist in lymphatic and immune tissues (Boyaji et al., 2020). The endocannabinoid system in the human body is responsible for regulating metabolism, food behavior, mood, anxiety, and pain perception and modulation (Boyaji et al., 2020). This endocannabinoid system, at the biochemical level, primarily exists to mediate endogenous cannabinoids (2-arachidonoylglycerol and N-arachidonoylethanolamine), but exogenous cannabinoids (cannabidiol and tetrahydrocannabinol) are also known to interact with the system, albeit a bit differently (Chye et al., 2019). Based upon recent research, CBD has been described as a non-competitive antagonist of CB_1 with a low affinity for CB_1's primary ligand binding site (Chye et al., 2019). It is believed that CBD inhibits the signaling of endocannabinoids most likely by binding the

allosteric site of CB_1 and altering the potency of endocannabinoids (Chye et al., 2019). This effect has been observed to be dose-dependent and has been characterized as negative allosteric modulation of CB_1 (Laprairie et al., 2015). It is still uncertain how this mechanism of action promotes the therapeutic effect that CBD is known for.

Within the one-carbon metabolism (OCM), CBD has been observed to act as a regulator by interacting with methionine levels. One-carbon metabolism consists of interlinking metabolic pathways that are central to cellular function and by providing methyl groups for the synthesis of DNA, polyamines, amino acids, creatine, and phospholipids. It is also a key element in maintaining balance of cellular redox states and regulation of reactive oxygen species (Martínez-Reyes & Chandel, 2015). An integral molecule to the OCM is methionine, which, due to its importance to the OCM in conjunction with its interactions with CBD, acts as the focus for this project. Based upon recent research, it has been found that CBD reduces methionine levels in a glycine cleavage system-dependent manner (Perry et al., 2020). It has been observed that methionine restriction reduces oxidative stress, so CBD's potential mechanism in regard to reducing methionine levels, may translate to a reduction in oxidative stress (Kitada et al., 2021).

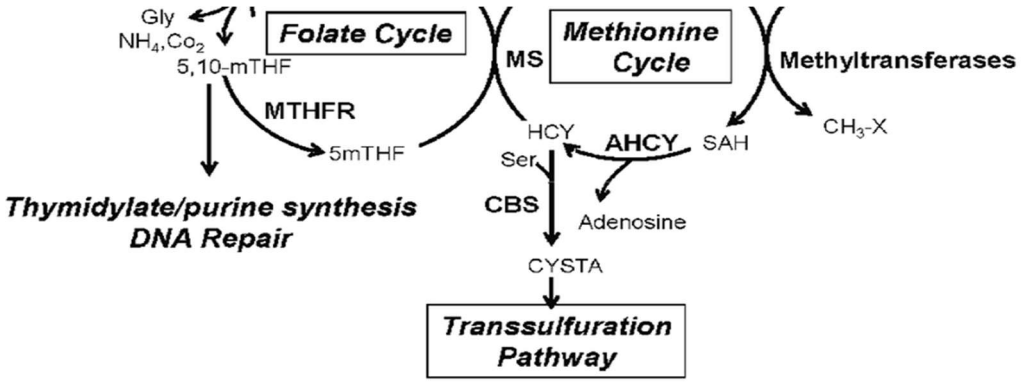

Figure 1. Illustration of the one carbon metabolism pathway (Corbin & Ruiz-Echevarria, 2016).

Oxidative stress occurs when there is an intracellular imbalance of reactive oxygen species (ROS). Under normal circumstances these ROS are regarded as regulatory elements that coordinate cell signaling, protein phosphorylation, differentiation, and other essential cellular functions. However, when levels of ROS within the cell elevate beyond typical amounts, these ROS can lead to damage in lipid, protein, and DNA molecules (Pizzino et al., 2017). In terms of neurodegenerative diseases, it is known that oxidative stress is responsible for initiating and enhancing the symptoms of these diseases (Singh et al., 2019). The causes of diseases such as Parkinson's disease, Huntington's disease, Alzheimer's Disease, and amyotrophic lateral sclerosis (ASL) have been found to have major links to oxidative stress (Singh et al., 2019).

One particularly devastating neurodegenerative disease is Alzheimer's Disease. In 2020, an estimated 5.8 million Americans were living with Alzheimer's, making it the most common form of dementia (CDC, 2020). In humans, this disease is characterized by the abnormal accumulation of amyloid β (Aβ) peptide and neurofibrillary tangles consisting of hyperphosphorylated τ protein. The Aβ peptide in conjunction with the τ protein act as mediators of the neurodegeneration experienced by those with Alzheimer's Disease. It is known that the build-up of both of these materials is mainly induced and exacerbated by oxidative stress (Huang et al., 2016), therefore, the exploration of CBD's effect on oxidative stress is imperative.

In order to monitor the levels of ROS, the nematodes would need to be exposed to a marker for oxidative stress. In this project, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was employed as the ROS indicator. H_2DCFDA is a reduced form fluorescein, a widely used fluorescence marker. Once exposed to reactive oxygen species, it is deacetylated and oxidized, a

fluorescent signal is produced. H_2DCFDA can easily penetrate the cuticle of the *C. elegans*, making it a useful marker for these nematodes in particular (Yoon et al., 2018).

Due to unforeseen circumstances, time constraints, and uncooperative nematodes, this project has evolved over the course of the academic year. The original goal of this project was to understand CBD's ability to reduce oxidative stress and regulate the one carbon metabolism via the attenuation of methionine. As time went on, it was determined that the use of methionine was supplying more issues than answers to the experimental question, so it was decided that the effect of varying CBD doses on the presence of reactive oxygen species in N2 nematodes would be explored. Doses of CBD found in the literature (Land et al., 2021) were applied to the nematodes to assess whether exogenous cannabinoids had any impact on the reactive oxygen species present in nematode samples. Despite the shift in focus, difficulties were still encountered, resulting in this project consisting of troubleshooting and problem solving within the procedures rather than consisting of data and conclusions related to our experimental question.

Materials and Methods

NGM Plate Preparation

Nematode Growth Medium (NGM) was prepared by adding 970 mL of diH_2O, 3 g of NaCl, 17 g difco agar, and 2.5 g of peptone to a 1 L media storage bottle. The solution was mixed until all dry reagents were fully dissolved. The agar was autoclaved for 35 minutes and cooled to 50℃. Once cooled, 1 mL of 1 M $CaCl_2$, 1 mL of 0.5% cholesterol (dissolved in ethanol), 25 mL of KPO_4, and 1 mL of 1 M MgSO4 was aseptically added to the bottle. Each 60 mm petri plate had 10 mL of agar solution poured into them and were left to solidify for up to 24 hours at room temperature. Plates were then placed in a 4℃ refrigerator for storage.

Prepared NGM agar plates were seeded with 100 µL of liquid OP_{50} strain *E. coli* with a micropipette, making sure to keep the bacterial lawn in the middle of the plate. Freshly spotted plates were left to dry at room temperature for up to 48 hours and then transferred to the refrigerator until needed for nematode husbandry or experimental procedures.

10X M9 Buffer Preparation

M9 salt buffer was used to wash nematodes off of the NGM plates and to suspend them during washing. A liter of 10X M9 salt buffer was prepared by combining 70 g of Na_2HPO_4, 30 g of KH_2PO_4, 5 g of NaCl, and 10 g of NH_4Cl in a 1 L media bottle with 1 L of diH_2O. The solution was mixed until all dry reagents were completely dissolved and then autoclaved. Portions of this solution were diluted to a 1X concentration using more diH_2O prior to use.

N2 C. elegans Maintenance & Husbandry

N2 *C. elegans* were seeded onto spotted NGM plates by two different methods: picking with a small wire hook or "chunking". When picking the nematodes, the platinum wire was washed in ethanol and sterilized by passing it through a flame. After allowing the pick to cool for a few seconds, the end of the wire was used to scrape a small amount of OP_{50} *E. coli*, and a single nematode was picked from a plate, gently to avoid piercing the agar, and placed onto a new spotted NGM plate. When chunking the plate, a larger number of *C. elegans* can be transferred to at a time. A small spatula was sterilized with ethanol and flamed before cutting a small chunk into the agar. This chunk (about 1-3 mm wide) was transferred to a new spotted plate, allowing the nematodes within to crawl out. Plates were stored in a 20℃ incubator until the bacterial lawn was gone, or until the density of *C. elegans* grew too high (as pictured in *Figure 2* below). *C. elegans* were transferred to new plates, on average, every 5 days.

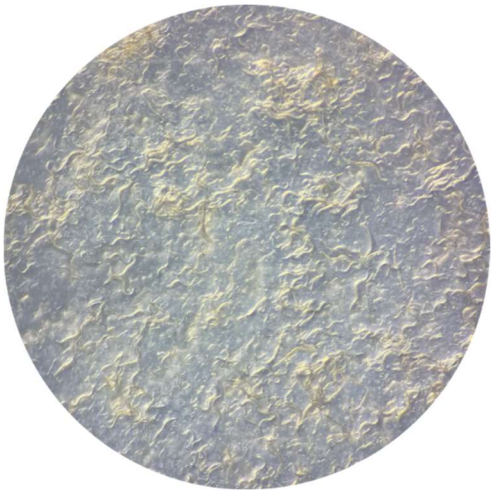

Figure 2. Image of nematode plate with a density that would require nematodes to be transferred to a new plate.

CBD Dosing

Two concentrations of liquid CBD solution were used throughout the tests. To make the 40 μM solution, 99% CBD isolate was dissolved in 100% cell culture grade DMSO to produce a 4mM CBD/DMSO solution. This was then diluted further with 1X M9 to produce the final concentration of 40 μM CBD. This procedure was repeated for the 100 μM solution, but a 0.1 M CBD/DMSO solution was prepared instead of 4 mM. Once prepared, the solutions were stored at room temperature in sealed containers until needed.

To dose the *C. elegans*, a plate of nematodes was washed using 1 mL of 1X M9 buffer. The solution was aspirated from the plate and transferred to a microcentrifuge tube. To wash the nematodes, the tube was centrifuged at 1000 rpm for 1 minute and the supernatant disposed. This was repeated three times to wash all nematodes. Nematode samples being dosed with CBD were resuspended in 1 mL of the prepared liquid CBD solutions while control groups were resuspended in M9. All groups were sat in liquid culture for six hours at 20°C. After six hours, the tubes with CBD solutions were washed three times with M9 (this should be done shortly before conducting the ROS assay to avoid the potential weakening of the effect of the CBD). The amount of nematodes within each solution were counted by vortexing the microcentrifuge tube, spotting some solution onto a microscope slide, and counting the nematodes present. Solutions were diluted with M9 buffer until all groups contained ~50 nematodes per 10 μL.

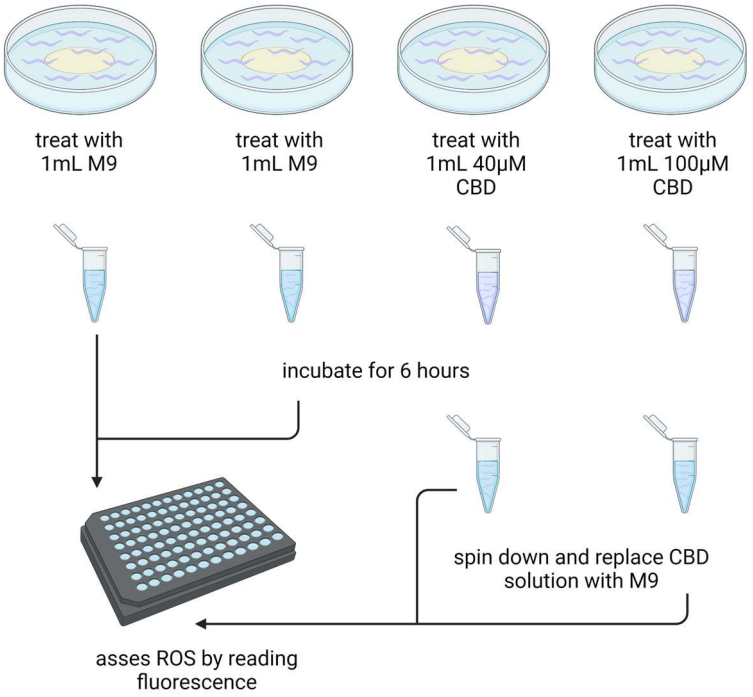

Figure 3. Method design for CBD dosing prior to ROS assay.

ROS Assay using H_2DCFDA

The protocol used for this assay closely follows the protocol from the Yoon et al., 2018 paper, "Measurement of Intracellular ROS in *Caenorhabditis elegans* Using 2',7'-Dichlorodihydrofluorescein Diacetate".

To prepare the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) reaction solution, a 50 mM stock of H_2DCFDA was made by dissolving the compound in cell culture grade DMSO. Before conducting the assay, aliquots of the stock were diluted to 50 µM with 1X M9 buffer. The diluted solution was used immediately after preparation.

In a black, clear bottom 96-well plate, M9 buffer, nematode solution, and the 50 μM H_2DCFDA solution were added to each well. A blank was prepared by adding M9 buffer and the H_2DCFDA reaction solution to a separate well. For the positive control, 0.5% H_2O_2 was substituted for M9 . Immediately after adding the H_2DCFDA reaction solution, the plate was placed in the fluorophotometer (Perkin Elmer Victor 3 1420 Multilabel Plate Counter). The fluorescence signal was checked every hour for 4 hours. Resulting blank subtracted RFU's were subjected to an ANOVA test to uncover any significant fluorescence differences between the groups.

ANOVA

To analyze the data, the blank-subtracted fluorescence values from the eight biological data sets were subjected to a single-factor ANOVA. The P-value from the ANOVA would allow for conclusions to be made regarding the significance of the data.

Troubleshooting and Method Development

Methionine

Initially, it was assumed that methionine would be dissolved in water and added to the worm plates in varied concentrations (0 μM, 1 μM, 5 μM, 10 μM, 15 μM, and 20 μM). A 2 M stock solution of methionine was planned to be made before discovering that methionine has very low solubility in water. In order to dose the nematodes with methionine, it was determined that the individual amounts of methionine could be dissolved in the agar and poured into the plates that the nematodes would live on. Unfortunately, there was no sterile way to do this, and the methionine plates were quickly found to be contaminated. With no effective way to dose the nematodes with methionine, this branch of the project was abandoned.

CBD

Similar to the methionine, issues were encountered regarding the solubility of CBD in water. Because the solution needed to be applied to the nematodes, common non-polar solvents could not be used. It was found that CBD could be dissolved in DMSO, but the DMSO would need to be diluted down to less than a 1% solution using M9 in order for the nematodes to have a chance at survival. This would then present the issue of the CBD being extremely dilute in the final CBD solution. The capacity of the DMSO and M9 to hold the CBD in solution was limited, so only low doses of CBD could be dissolved and later used through experimentation. Other than this specific method, no methods were developed that allowed for CBD to be dissolved in higher concentrations than 100 μM.

ROS Assay

When running the ROS assay for the first time, the exact procedure from the Yoon et al. paper was followed. This procedure used only 10 nematodes per well in the 96-well plate. It was quickly found that 10 nematodes was too low, as no signal was picked up by the plate reader. After deciding to wash down a plate and use 10 μL of a solution of all of the worms, the fluorescence signal was found to be satisfactory. It was then decided that a positive control was needed, so peroxide was added to an experimental group to see how the assay would react under conditions that had guaranteed oxidative stress. The peroxide was originally diluted down to 1%, but it was later decided that 0.5% would be a more suitable concentration. It was found in some trials that the fluorescence emitted by the samples (most often the peroxide sample) would exceed the threshold of the plate reader. This issue was not resolved, but it may have been useful to dilute the peroxide even further.

Results and Discussion

After incubation in liquid culture and measurement via fluorophotometer, the fluorescence recorded for each experimental group was analyzed to further assess the presence and level of reactive oxygen species in each sample. The fluorescence emitted by each sample correlates to the amount of reactive oxygen species present. After subtracting the fluorescence measurements of the blank, the resulting RFU values showed little differences between the CBD dosed samples and the control samples (*Table 1*). While there are differences present, the values do not differ by a large enough margin (between experimental groups) to constitute any sort of mentionable result. Of course, the peroxide trials displayed significantly increased RFU values, due to their nature as the positive control, but these values are ultimately only useful as a validation that the assay works properly. It was observed, however, in the final two trials that the peroxide samples displayed results that were drastically lower than expected. It is unknown why this occurred, but this data suggests that even the positive controls display significant variability. The standard deviation for each experimental group also suggests large variability within the trials of each experimental condition. This variability between each test (experimental groups and both control groups) indicates that the ROS assay may need further validation and development before it is used in future studies.

Table 1. Resulting blank-subtracted RFUs for each of the eight trials conducted. The averages are highlighted in dark purple and little change is observed between them. The standard deviations are displayed as well (in light purple) and indicate a high variability between the trials of each variable.

	dosage of treatment			
	40uM	**100uM**	**0.5% peroxide**	**none**
blank subtracted fluorescence (RFUs)	153048	36476	1918897	112805
	377907	238289	1967949	368126
	287631	470257	1848530	56747
	475328	770855	1931280	356742
	192375	74921	1901989	157003
	-96938	4303	1889148	50739
	-18533	80218	905832	189302
	260955	188746	601350	439217
average	203972	233008	1620622	216335
st. dev	191579	263715	542372	151323

In order to confirm that the results were insignificant, an ANOVA was run on all data excluding the peroxide trials (as the addition of the peroxide was only for validation of the assay). The P-value (0.961382) indicates that the variations within the data in the experimental groups are not a result of the CBD doses (*Table 2*). The huge variability in the data is likely the cause of the high P-value. Due to the variability, no true conclusions can be made as the data does not inform the ability to accept or reject the hypothesis. Based on the above findings, it cannot be determined whether CBD does or does not have an effect on the levels of oxidative stress in *C. elegans.*

Table 2. A single-factor ANOVA test was conducted to examine any significant differences in oxidative stress across the groups. The resulting P-value (highlighted in purple) implies that the data cannot be used to make conclusions that either accept or reject the hypothesis.

ANOVA

Source of Variation	*df*	*F*	*P-value*	*F crit*
Between Groups	2	0.039458	0.961382	3.4668
Within Groups	21			
Total	23			

Despite the lack of significant difference between the experimental groups, it was observed that there were more live worms in the liquid cultures containing CBD following the six hour incubation period. While this was not a focus of the study, this incidence, which occurred for each trial, suggests that CBD may play a role in longevity. This occurrence has been mentioned in other studies involving CBD and *C. elegans* (Land et al. 2021), but the mechanism by which this occurs and the reason for these observations are outside the scope of this project. Despite this, CBD's role in nematode longevity and survival may act as an interesting and positive note on the impact of CBD on *C. elegans* in general.

Conclusions

Main Findings & Implications

Unfortunately, any existence (or lack) of a link between CBD and oxidative stress in *C. elegans* could not be determined from the data. The lack of change between the average fluorescence may be due to the extremely low CBD dosages or the length of incubation time, while the variability between each trial could be the result of an underdeveloped assay protocol. These findings could be strengthened by further experimentation and development of the methods employed. In addition to the data from the ROS assays, it is important to note that increased longevity was observed when the nematodes were incubated in CBD versus M9. While this observation does not serve the purpose of this project, it is consistent with some literature findings and could be further explored and validated through future projects.

Limitations

Before the start of this project, methionine and its downstream effects on the one-carbon metabolism pathway was a driving factor in the development of the original experimental design. Originally, some *C. elegans* would be dosed with a fixed amount of methionine in order to create an imbalance in the redox state, while others would be given a combination of methionine and CBD to see if the CBD could balance the ROS levels that would have been skewed due to the presence of methionine. Unfortunately, methionine is a non-polar amino acid, meaning the solubility of the compound is dramatically decreased in water (the most viable solvent for nematode experimentation). Because of this, dissolving the methionine into a nematode-safe solution was unrealistic. In addition, there was no sterile way to introduce the powdered methionine into the nematode cultures since autoclaving would potentially disrupt the structure

of the methionine. Melting the methionine in the NGM agar directly resulted in contaminated plates that were unfit for nematode husbandry. If a new method for methionine dosage is developed, this could be a beneficial addition to this research.

On a similar note, the CBD used throughout these experiments also came in a powdered form. This form of CBD is not soluble in water and in order to use this in our dosage, cell culture grade DMSO was necessary for dissolving the CBD in the M9. While this was achievable, DMSO is generally harmful unless extremely dilute and may have a negative effect on the *C. elegans* despite being properly diluted. DMSO has the potential to cause developmental decay in *C. elegans*, negatively affect fertility levels, and decrease their overall lifespan (AlOkda & Van Raamsdonk, 2022). This method also only allowed for small amounts of CBD to be dissolved, resulting in low dosage concentrations.

Future Considerations and Recommendations

Due to the results (or lack thereof) of this project, there are quite a few recommendations and future considerations to be made. Firstly, it would be extremely beneficial to perform a dose response experiment using the nematodes and CBD to find the best possible dose of CBD for experimentation. It would also be helpful to increase the doses as the doses found in literature were extremely small and appeared to make very little impact (whether that be negative or positive). Future projects should also verify the identity of the CBD (to ensure the substance is indeed CBD). A more sensitive or different ROS assay may also be explored as the fluorescence values exceeded the threshold of the plate reader in some cases. Additionally, the ROS assay might need to be validated through dilution of the positive control to create a standard curve.

Other than these more technical recommendations, we would highly recommend looking into the potential relationship between CBD and methionine further as this aspect of the project needed to be abandoned due to solubility issues and time constraints. If there was a way that the solubility issues could be effectively resolved, the exploration of the link between methionine, CBD, and oxidative stress would be incredibly interesting and may aid in validating the unsupported claims from literature. Finally, we recommend assessing oxidative stress reduction in Alzheimer's Disease model worms (CL2355). This would allow for further validation of oxidative stress reduction via CBD as a therapeutic for neurodegenerative diseases.

References

Martínez-Reyes, I., & Chandel, N. S. (2020). Mitochondrial TCA cycle metabolites control physiology and disease. Nature communications, 11(1), 102. https://doi.org/10.1038/s41467-019-13668-3

Land, M. H., Toth, M. L., MacNair, L., Vanapalli, S. A., Lefever, T. W., Peters, E. N., & Bonn-Miller, M. O. (2021). Effect of Cannabidiol on the Long-Term Toxicity and Lifespan in the Preclinical Model *Caenorhabditis elegans*. *Cannabis and cannabinoid research*, *6*(6), 522–527. https://doi.org/10.1089/can.2020.0103

Estrada-Valencia, R., de Lima, M. E., Colonnello, A., Rangel-López, E., Saraiva, N. R., de Ávila, D. S., Aschner, M., & Santamaría, A. (2021). The endocannabinoid system in Caenorhabditis elegans. *Reviews of Physiology, Biochemistry and Pharmacology*, 1–31. https://doi.org/10.1007/112_2021_64

Lehtonen, M., Reisner, K., Auriola, S., Wong, G., & Callaway, J. C. (2008). Mass-spectrometric identification of anandamide and 2-arachidonoylglycerol in nematodes. Chemistry & Biodiversity, 5(11), 2431–2441. https://doi.org/10.1002/cbdv.200890208

Millar, S. A., Stone, N. L., Yates, A. S., & O'Sullivan, S. E. (2018). A systematic review on the pharmacokinetics of cannabidiol in humans. Frontiers in Pharmacology, 9. https://doi.org/10.3389/fphar.2018.01365

Boyaji, S., Merkow, J., Elman, R. N., Kaye, A. D., Yong, R. J., & Urman, R. D. (2020). The role of Cannabidiol (CBD) in Chronic pain management: An assessment of current evidence. Current Pain and Headache Reports, 24(2). https://doi.org/10.1007/s11916-020-0835-4

Chye, Y., Christensen, E., Solowij, N., & Yücel, M. (2019). The endocannabinoid system and Cannabidiol's promise for the treatment of substance use disorder. *Frontiers in Psychiatry*, *10*. https://doi.org/10.3389/fpsyt.2019.00063

Laprairie, R. B., Bagher, A. M., Kelly, M. E., & Denovan-Wright, E. M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. British Journal of Pharmacology, 172(20), 4790–4805. https://doi.org/10.1111/bph.13250

Kitada, M., Ogura, Y., Monno, I., Xu, J., & Koya, D. (2021). Effect of methionine restriction on aging: Its relationship to oxidative stress. Biomedicines, 9(2), 130. https://doi.org/10.3390/biomedicines9020130

Corbin, J., & Ruiz-Echevarría, M. (2016). One-carbon metabolism in prostate cancer: The role of Androgen Signaling. *International Journal of Molecular Sciences*, *17*(8), 1208. https://doi.org/10.3390/ijms17081208

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., & Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. Oxidative medicine and cellular longevity, 2017, 8416763. https://doi.org/10.1155/2017/8416763

Singh, A., Kukreti, R., Saso, L., & Kukreti, S. (2019). Oxidative Stress: A Key Modulator in Neurodegenerative Diseases. Molecules (Basel, Switzerland), 24(8), 1583. https://doi.org/10.3390/molecules24081583

Centers for Disease Control and Prevention. (2020, October 26). What is Alzheimer's disease? Centers for Disease Control and Prevention. Retrieved October 20, 2022, from https://www.cdc.gov/aging/aginginfo/alzheimers.htm

Huang, W. J., Zhang, X., & Chen, W. W. (2016). Role of oxidative stress in Alzheimer's disease. *Biomedical reports*, *4*(5), 519–522. https://doi.org/10.3892/br.2016.630

Yoon, D., Lee, M.-H., & Cha, D. (2018). Measurement of intracellular ROS in Caenorhabditis elegans using 2',7'-dichlorodihydrofluorescein diacetate. *BIO-PROTOCOL*, *8*(6). https://doi.org/10.21769/bioprotoc.2774

AlOkda, A., & Van Raamsdonk, J. M. (2022). Effect of DMSO on lifespan and physiology in C. elegans : Implications for use of DMSO as a solvent for compound delivery. microPublication biology, 2022, 10.17912/micropub.biology.000634.