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Neuroprotection with Novel Anti-Oxidant and Cannabinoid-Based Small Molecules

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NEUROPROTECTION WITH NOVEL ANTI-OXIDANT AND CANNABINOID-BASED SMALL MOLECULES

By

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A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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ABSTRACT
JACQUELINE MCGRATH: NEUROPROTECTION WITH NOVEL ANTI-OXIDANT AND CANNABINOID-BASED SMALL MOLECULES
(Under the direction of Dr. Nicole Ashpole)

Neurodegenerative diseases are caused by various underlying mechanisms within the nervous system. Excitotoxicity is a hallmark of many neurodegenerative diseases, and is produced by over excitation of glutamate receptors on neurons, ultimately leading to cell death. Protein aggregates and oxidative stress have also induced neuronal cell death that lead to many neurodegenerative diseases as well. Many compounds isolated from plants have shown protective ability against these underlying toxicity mechanisms. In this study, we tested several cannabinoid and anthyocanin-based small molecules protective abilities against excitotoxicity. We found several compounds that protected against glutamate-induced excitotoxicity in a concentration-dependent manner. We also tested whether cannabinoid-based small molecules could protect against protein aggregate and oxidative stress toxicity, although our assays require further validation. We are now working on creating concentration-dependent response curves for the anti-oxidant based small molecules against excitotoxicity. Future application of these findings would include administering the neuroprotective compounds to animals to prevent neurodegeneration and its accompanying behavioral deficits.

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I. Background

As medicine advances, researchers are constantly searching for new drug therapies to combat disease. Many drug discoveries made utilize therapeutic extracts and compounds that already exist in nature because non-natural products often result in increased adverse side effects in treatment of disease (Lahlou 2013). Natural products are produced from plants, animals, or microorganisms and can be harvested and isolated for human use. Natural products include secondary metabolites produced by these types of organisms for defense within the organism or defense against a predator (Mushtaq 2018). For example, resveratrol is a strong anti-oxidant found in many fruits, such as red grapes, blueberries, and cranberries that is effective in preventing or fighting infections in the plant. It has also been shown to alleviate the pathologies of several diseases (Rehman et al 2018). Type 2 diabetes mellitus is a disorder that causes glucose intolerance and hyperglycemia. Resveratrol and vitamin E made mice with type 2 diabetes mellitus more glucose tolerant, and lowered their hyperglycemia (Rehman et al 2018). Alzheimer's disease is a neurodegenerative disease that is characterized by β-amyloid peptide aggregates surrounding neurons. When neurons were treated with both amyloid beta-peptide$_{1\text{-}42}$ and resveratrol, apoptosis and oxidative stress were significantly reduced in the neuronal cell lines (Wang et al 2018). Like resveratrol, there have been many other studies that emphasize the therapeutic effects of natural products and their constituents on a diverse range of human disease, including neurodegenerative diseases.

As the human life expectancy has slowly increased throughout all the years of medical advancements, there has also been a growing concern about the aging brain. With a larger population of older adults worldwide, there is a corresponding increase in the number of cases of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. It is estimated that about 5 million Americans are currently diagnosed with Alzheimer's disease and 500,000 are currently diagnosed with Parkinson's disease (NIEHS 2019). With the number of people affected by age-related neurodegenerative diseases, there has been research with natural product therapeutics that hope to prevent or delay the onset of neurodegenerative diseases and age-related cognitive impairment.

Many of the neurodegenerative diseases researchers are interested preventing neuronal death, also known as neurotoxicity, associated with neurodegenerative diseases. Neurotoxicity is when a certain exposure to natural or manmade toxic substances changes normal neuron activity, disrupts homeostasis, and leads to cell death (NINDS 2018). Excitotoxicity, protein aggregates, and oxidative stress are some of the known underlying mechanisms by which neurotoxicity is induced in neurons. Excitotoxicity is a neurodegenerative pathway in which the over-excitation of glutamate receptors causes too much calcium influx in neurons and ultimately leads to cellular necrosis and apoptosis (Choi 1985, Mattson 2019). Amyloid-beta peptide plaques, or other protein aggregates, are a characteristic found in Alzheimer's disease. The amyloid-beta$_{1\text{-}42}$ peptide acts as an agonist ligand to receptors that initiate apoptosis in neurons, decreasing neuronal cell viability in people diagnosed with Alzheimer's disease (Sotthibundhu 2008). In neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease,

and Amyotrophic Lateral Sclerosis, reactive oxygen species produced as by-products of cellular metabolism mediate neuronal apoptosis (Khan et al 2016, Ricart, Fiszman 2001). Excitotoxicity, amyloid-beta peptide aggregates, and reactive oxygen species all induce neurotoxicity through different pathways, yet they converge and mediate neuronal death in many well-known neurodegenerative diseases.

Many natural products are now being tested for their protective effects against these neurotoxic substances in order to find therapeutic discoveries for neurodegenerative diseases. Anthocyanins are compounds that provide the red, purple, and blue pigments in many fruits and vegetables. These compounds are polyphenols that belong to the flavonoid family, and are known for being strong anti-oxidants that protect against a diverse range of toxins within the body (Badshah et al 2014). Polyphenols are chemical structures widely produced by plants that protect against ultraviolet radiation and other toxins (Pandey 2009). Because of their protective properties, many anthocyanin-rich extracts are being tested for their protection against reactive oxygen species/oxidative stress in neurodegenerative diseases. Anthocyanin extracts are protective against neurodegeneration caused by oxidative stress and inflammation *in vitro* and *in vivo* (Khan et al 2016, Kim et al 2017, Vepsäläinen 2013). In a study performed by Badshah et. al., anthocyanin extracts also decreased neuronal cell death following exposure to amyloid-beta, suggesting they can protect against multiple pathways that cause neurodegeneration (Badshah et al 2014). Because of anthocyanin extracts' protective properties observed in common neurodegenerative pathways, the entire class of compounds and their derivatives are potential candidates for neuroprotection. Thus, in this study we explore the potential

of several anthocyanin derivatives for their ability to protect neurons from the various neurodegenerative mechanisms in vitro.

Another interesting set of natural products are those found in *Cannabis sativa.* Cannabis has been used for potential therapeutic purposes in humans for centuries, although we still understand very little about the protective compounds produced by cannabis. Its first origins in Central Asia, it resembles a weed-like plant and grows in wet environments. *Cannabis sativa* is thought to have been domesticated by humans early in history, with pre-civilization humans settling near bodies of water where the plant flourished. Because of its long-term domestication, it has been used by humans in the forms of textiles, antibacterial agents, and medicines (Andre et al 2016, Pollio 2016). While cannabis produces many classes of compounds, the two most prevalent are terpenes and cannabinoids. Cannabinoids found in cannabis are termed phytocannabinoids, translating to plant-derived cannabinoids. There are also synthetic and endogenously-produced cannabinoids (Campos 2016). The parent cannabinoids of interest in this study are two phytocannabinoids, cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC). CBD can be therapeutic and neuroprotective against neurodegenerative diseases because of its anti-inflammatory and anti-oxidant properties (Ruet et. al. 2013). Although THC is more psychoactive than CBD (Ruet et. al. 2013), a recent study has shown that a chronic low dose of THC can restore learning and memory in aged mice (Bilkei 2017). Because of previous findings on the protective effects of both CBD and THC, it makes their derivatives active candidates for this study.

Anthocyanins, CBD, and THC have all shown protective abilities in previous studies. Because of their known protective properties, it is worth examining potential

protective properties of the derivatives of these compounds. In this study, we hypothesize that anthocyanin and cannabinoid-based small molecules will protect neurons from excitotoxicity and other mechanisms of neuronal death. To explore this, we will culture neurons from rodents and treat them with novel anthocyanin and cannabinoid derivatives prior to treating them with excitotoxic insult. Cell death will then be measured. Ultimately, this study will highlight whether these compounds have protective potentials that should move into animal models of neurodegeneration.

II. Methods

Neuron Culture Preparation

Before neurons were cultured, the 96-well plates were prepared by coating them with 60 microliters of 0.01% Poly-L-Lysine, and placing them in the incubator for 24 hours. After 24 hours, the 96-well plates were washed twice with distilled water, and dried before use. The poly-lysine is added to give the neurons a surface to attach to on the plastic dishes.

Two petri dishes and five 15 mL conical tubes were placed in ice and cold HBSS was added to prepare for tissue isolation. The neurons that were to be cultured and used in the experiment were the cortical neurons of embryonic rats (day 16-18). After removing the embryo, the animals were decapitated and the brain was removed and placed in one of the petri dishes of HBSS for dissection. In order to harvest the cortical neurons, an incision first had to be made down the brain's midline and horizontally across the occipital bone of the skull. After removing the skull, the hemispheres were separated and the meninges of the brain were removed to access the cortex. The left and right hemisphere were separated, and the cortices of both were diced into fine pieces and placed in the 15 mL tubes of HBSS. After pieces of the cortex were added to the tubes, papain was also added. Papain enzymatically digested the tissue for 20-25 minutes at 37°C before being washed out twice with media. For mechanical digestion, the cells were subjected to successional pipetting. The cells were suspended in growth media and the cell count was recorded by placing the cells on a hemocytometer in order to ensure that

there would be the proper number of cells for plating. There were approximately 250,000 cells per well in this experiment. One-hundred microliter of growth media was plated on 96-well plates and stored in the incubator for twenty-four hours. The growth media consisted of neurobasal media, L-glutamine, fetal bovine serum (FBS), and penicillin-streptomycin. After incubating for 24 hours, the 100 microliter neurobasal media with serum was removed and replaced with 100 microliter fresh neurobasal media that did not include serum. The fresh neurobasal media without serum consisted of neurobasal media, L-glutamine, B27, and penstrep. After adding the fresh neurobasal media, the plates remained in the incubator until time of treatment (Ashpole, 2011).

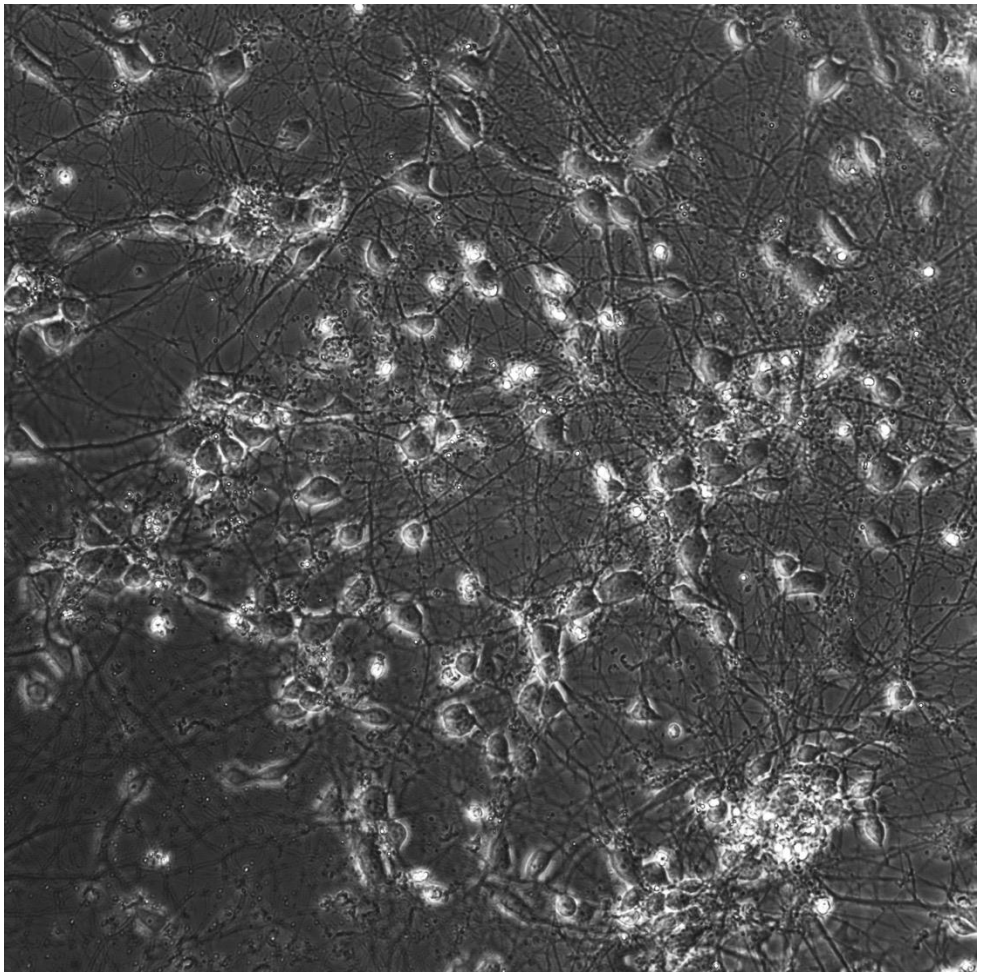

Figure 1: Healthy neuron culture grown under the neuron preparation stated above.

Excitotoxicity Treatment:

Because the compounds used in the experiment were testing for neuroprotection, the cultured neurons were exposed to glutamate and complete neurobasal media in order to induce excitotoxicity and reduce cell viability in the neuron cultures. Complete neurobasal media was warmed in a water bath at 37°C for 10 minutes. A 50 mL conical was used to collect the complete neurobasal media and glutamate. For every milliliter of complete neurobasal media used, one microliter of 200 millimolar glutamate was added to the conical to make a 200 micromolar glutamate media stock. This 50 mL conical was then labeled as "E-media." A pipette was used to thoroughly mix the newly made E-media. Using an automatic pipette, 1 mL of the E-media was added to each individual 1.5 mL microtube. The potential protective compounds of interest were then added to these 1.5mL tubes prior to application on the cells (see anthocyanin and cannabinoid sections below).

Amyloid-Beta Treatment

Amyloid-Beta was used as another method to induce neurotoxicity on the cultured neurons in order to test the CBD derivatives' neuroprotective abilities. Complete neurobasal media was removed from the refrigerator and placed in a water bath at 37°C for 10 minutes. β-Amyloid Peptide$_{1\text{-}42}$ was removed from the freezer at -20°C and thawed under the fume hood. Once thawed, 7.5 microliters of 5 millimolar A$\beta_{1\text{-}42}$ were pipetted into 15 mL of complete neurobasal media in a 15 mL conical giving a final concentration of 2.5 micromolar. Twenty-six microtubes were then filled with 400 microliters each of

the AB/media solution so we could add the potential neuroprotective compounds prior to application on the neurons.

Hydrogen Peroxide Treatment

Hydrogen peroxide was used as a method to induce neurotoxicity through oxidative stress on the cultured neurons in order to test the CBD derivatives' neuroprotective abilities. Hydrogen peroxide is a reactive oxygen species that can often be produced as a by-product of oxygen metabolism in cells. When there is an imbalance and large production of hydrogen peroxide, it is called oxidative stress and it induces cell death (Ricart, Fiszman 2001). Complete neurobasal media was removed from the refrigerator and placed in a water bath at 37°C for 10 minutes, and 1.5 microliters of 9.8 molar peroxide was diluted into 1.5 mL of distilled water to make a 9.8 millimolar peroxide stock. A 50 mL conical was filled with 26 mL of neurobasal complete media, and 408 microliters of the 9.8 millimolar peroxide was pipetted into the neurobasal complete media to give a final stimulation media of 150 micromolar. Peroxide media (1 mL) was pipetted into each of the 26 microtubes and the compounds of interest were then added (see below).

Anthocyanin Derivatives Treatment:

Twenty-three compounds based on anthocyanins found in blueberries were tested for neuroprotection. The compounds were suspended in a solution of dimethyl sulfoxide (DMSO) at 10 millimolar concentrations. The compounds and extracts were received from Dr. Colby in conjunction with the Department of BioMolecular Sciences. Due to

intellectual property rights, the compounds are labeled as numbers throughout this report, rather than their chemical name.

Using a pipette, 1 mL of the E-media was pipetted into each of the 23 microtubes. Each microtube received 2 microliters of a specific compound so that the final concentration of the test compound was 5 micromolar. After the compound was added, the microtube lid was labeled with that compound's corresponding number. After all of the 23 tubes contained E-media and one compound, three 96-well plates were removed from the incubator. Each column on each plate was designated to a specific compound, and all of the wells of that column received 100 microliters of the compound/E-media solution contained in the specific microtube. Control wells received 100 microliters of complete neurobasal media, and two columns contained 100 microliters of E-media in their wells. The plates were placed back in the incubator for 1-2 hours. The same process as stated previously was repeated but with concentrations of 4 microliters of compound and 8 microliters of compound in E-media solution so that the final concentration of the test compound was 20 micromolar and 40 micromolar, respectively. After the incubation, the plates were then subjected to several washes. One-hundred fifty microliters were removed from all of the wells of the plate, and discarded. Then, 100 microliters of fresh neurobasal complete media were added to all of the wells of the plates, then immediately the 100 microliters of the fresh complete neurobasal media were then removed and discarded. Finally, 50 microliters of fresh complete neurobasal media were added to all of the wells on all of the plates, and placed in the incubator for 24 hours.

Cannabinoid Derivative Treatment:

Twenty-six cannabinoid compounds were used in this experiment. The compounds were suspended in a solution of dimethyl sulfoxide (DMSO) at 10 millimolar concentrations. The compounds and extracts were received from Dr. Majumdar and Dr. ElSohly in conjunction with the National Center for Natural Product Research. Due to intellectual property rights, the compounds are labeled as numbers throughout this report, rather than their chemical name.

Fifteen of the compounds were first tested on neuron cultures. Using a pipette, 1 mL of the E-media was pipetted into each of the 15 microtubes. Two microliters of a specific CBD compound were then added to one of the 15 microtubes so that the final concentration of compound was 10 micromolar. Then the lid of that microtube was labeled with the corresponding compound's labeling number. Once all of the microtubes contained 2 microliters of a certain CBD compound, 96-well plates were removed from the incubator. Each microtube's contents were pipetted in a specific column of wells, with each well receiving 100 microliters. Two control columns received 100 microliters of E-media in each of their wells, and one control column received 100 microliters of fresh neurobasal complete media in each of its wells. Once all plates were treated with the 2 microliters of compound solution, they were placed back in the incubator for 1-2 hours. Using the same steps above and the same compounds, this process was repeated three different times with three different sets of plates with 4 microliters of compounds, 6 microliters of compounds, and 8 microliters of drug in the microtubes for a final concentration of 20 micromolar, 30 micromolar, and 40 micromolar, respectively. All of these plates were also placed in the incubator for 1-2 hours. After incubation, all plates

were removed subjected to washings. One-hundred fifty microliters from all of the wells were removed and discarded. One-hundred microliters of fresh complete neurobasal media were added, and then the 100 microliters of fresh complete neurobasal media were removed and discarded. Finally, 50 microliters of fresh complete neurobasal media was added and all plates were placed in the incubator for 24 hours.

All 26 compounds were then tested for their neuroprotection against glutamate on neuron cultures. Using a pipette, 1 mL of the E-media was pipetted into each of the 26 microtubes. Three point five microliters of a specific CBD compound were then added to one of the 26 microtubes, so that the final concentration was 17.5 micromolar. The lid of that microtube was then labeled with the corresponding compound's number. Once all of the microtubes contained 3.5 microliters of a certain CBD compound, 96-well plates were removed from the incubator. Each microtube's contents were pipetted in a specific column of wells, with each well receiving 100 microliters. One column received 100 microliters of E-media in each of its wells, and one column received 100 microliters of fresh neurobasal complete media in each of its wells. Once all plates were treated with the 17.5 micromolar compound solution, they were placed back in the incubator for 24 hours. The following procedures described above were repeated with derivative concentrations of 5 micromolar, 10 micromolar, and 40 micromolar.

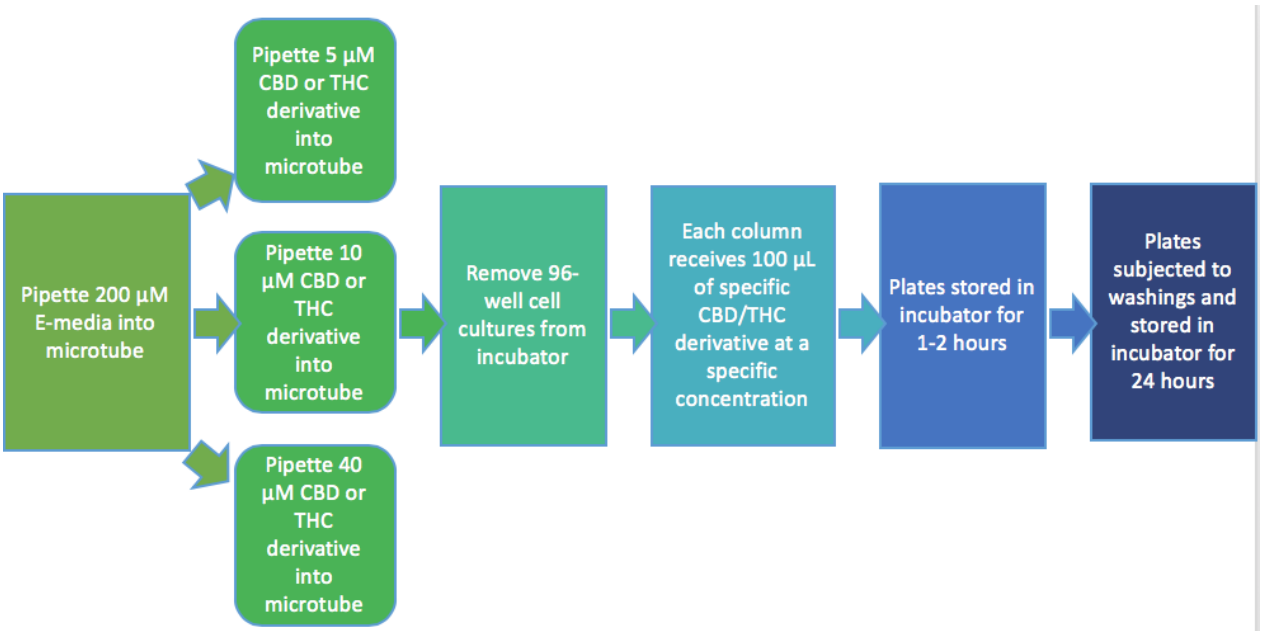

Figure 2: The workflow of concentration-response compound treatment on glutamate insulted neurons.

All 26 compounds were then tested for their neuroprotection against amyloid-Beta (AB) on the neuron cultures. Using a pipette, 1 mL of the AB-media was pipetted into each of the 26 microtubes. Each microtube then received 1.6 microliters of a specific compound so the final concentration of compound was 20 micromolar, and that microtube was labeled with the compound's corresponding number. Three 96-well plates of cultured neurons were then removed from the incubator to be treated. Fifty microliters of media were removed from all of the wells on the plate before the plates were treated with any of the compound and AB solution. Once the 50 microliters of media were removed, 50 microliters of a specific compound and AB media were pipetted into each well of a specific column. This was repeated until each compound had its own column of treated cells. One column received 100 microliters of AB media in each of its wells, and

one column received 100 microliters of fresh neurobasal complete media in each of its wells. The plates were then placed in the incubator for 24 hours.

All 26 compounds were then tested for their neuroprotection against peroxide (H_2O_2) on neuron cultures. Using a pipette, 1 mL of the H_2O_2-media was pipetted into each of the 26 microtubes. Three point five microliters of a specific compound was pipetted into each microtube so the final concentration was 17.5 micromolar, and then the microtube was labeled with the compound's corresponding number. Once all of the microtubes contained 3.5 microliters of a certain CBD compound, 96-well plates were removed from the incubator. Each microtube's contents were pipetted in a specific column of wells, with each well receiving 100 microliters. One column received 100 microliters of peroxide and media in each of its wells, and one column received 100 microliters of fresh neurobasal complete media in each of its wells. Once all plates were treated with the 3.5 microliters of compound solution, they were placed back in the incubator for 24 hours.

Eight of the CBD compounds underwent an individual concentration curve using the 200 micromolar E-media stock for excitotoxicity insult. Each of the compounds had their own 96-well plate. For each of the 8 compounds, a 10-fold dilution in microtubes was done. The first microtube contained 16 microliters of one of the compounds into 1.5 mL of the E-media, so the final concentration was 100 micromolar. Seven-hundred fifty microliters of the first microtube was then pipetted into the second microtube containing 750 microliters of E-media, so its final concentration was 50 micromolar. This was repeated 8 times, until a final concentration of 0.195 was reached in the last microtube. Each concentration had a specific column on the 96-well plate, with each well receiving

90 microliters of that specific concentration. Every plate contained one column of fresh media and one column untouched. After all of the 8 compounds had their concentrations plated, they were placed in the incubator for 24 hours.

MTS Assay

After the neurons were treated with the compounds and the neurotoxicity insults, the plates were placed in the incubator for 24 hours. In order to determine the number of viable cells left in the plates, the MTS assay was performed on the plates. The solution used in the experiments to perform the MTS assay was CellTiter 96® AQ_{ueous} One Solution Reagent. This reagent includes tetrazolium salt and an electron coupling reagent. MTS is used to detect viability because viable cells with active metabolisms can change MTS into formazan. When MTS is changed to formazan, a color change from a pale yellow to dark purple is seen in the wells. The tetrazolium detects viable cells, and is reduced by the electron coupling aspect of the reagent to form the colored formazan that is seen (Riss et al 2013). The MTS AQ_{ueous} One Solution Reagent was kept in the freezer at -20°C, and thawed out for 10 minutes before use. Ninety-six well plates with treated neurons were removed from the incubator, and 5 microliters of MTS was added to each of the wells for the anthocyanin compounds, and 2.5 microliters of MTS was added each of wells for the CBD compounds. Once the MTS AQ_{ueous} One Solution Reagent was added to each of the wells, the plates were placed back in the incubator for 1-2 hours. After the plates were removed, a color change from yellow to dark purple was observed for certain wells. The plate was then put in the plate reader, and the wells were at an absorbance at 490 nm.

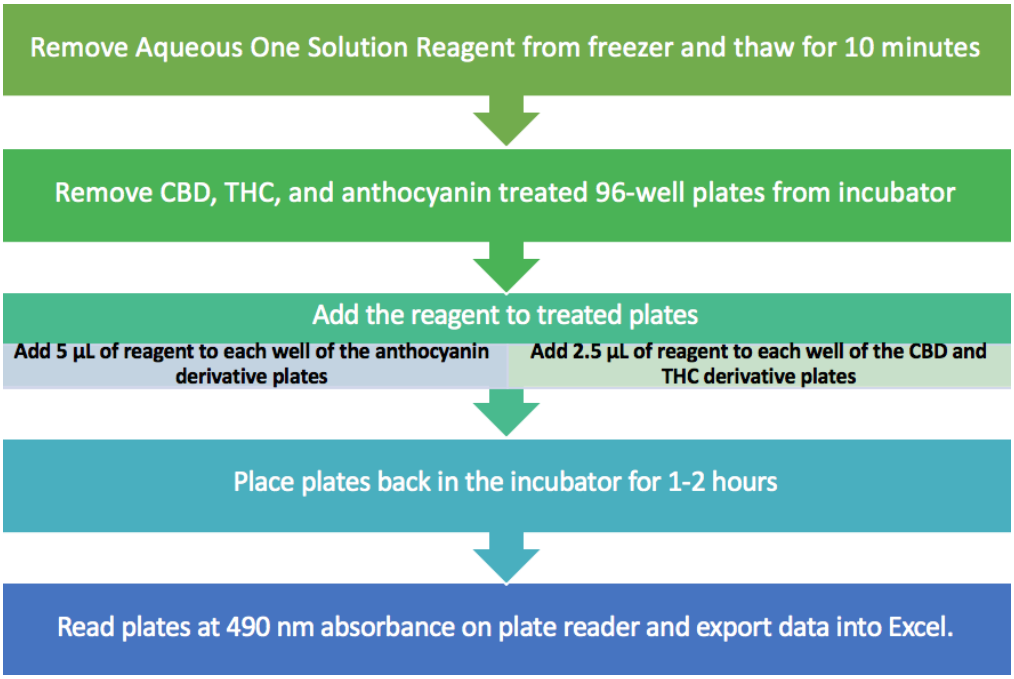

Figure 3: Workflow of MTS Assay.

Live/Dead Viability/Cytotoxicity Assay

After MTS was ran, Live/Dead Viability/Cytotoxicity was then run on the plates of different concentrations for the 8 specific cannabinoid compounds. The Live/Dead assay uses two fluorescent stains simultaneously to mark live and dead cells. Intracellular esterase activity and an intact plasma membrane are strong indicators for live cells. The Live/Dead Assay uses calcein-AM to mark intracellular esterase activity, and ethidium homodimer-1 to mark cells without intact plasma membranes. When examined under fluorescent microscopy, the calcein-AM fluoresces green and the ethidium homodimer-1 fluoresces red. Per manufacturer recommendation (L3224), the calcein and ethidium homodimer-1 were stored in the freezer at -20°C and protected from light. To make the solution, 160 microliters of 2 millimolar ethidium homodimer-1 was added into 80

microliters of PBS. Then, 40 microliters of calcein was added to 80 milliliters of the ethidium homodimer-1 and PBS solution. The 80 milliliter was then vortexed, yielding a 2 micromolar calcein AM and 4 micromolar ethidium homodimer-1 working solution. 100 microliters of working solution is then added directly to each well. The solution remained in each well for 30-45 minutes at room temperature protected from light, then were taken to be visualized on the fluorescent microscope.

Data Analysis

All data from the plate reader was exported and saved in Excel. The absorbance data from each compound's column was averaged. These averages of the absorbances were then exported into SigmaPlot, where graphs of average cell viability were constructed and the variance was analyzed. All data was normalized to the control in both Excel and SigmaPlot. SigmaPlot was used to create the graphs of the data, and to run analysis of variances in the data. In the bar graphs, data was shown as averages of the data and error bars represent plus/minus standard error measurement (± SEM). A one-way ANOVA using multiple groups was used to examine statistical analyses and variance between the cannabinoid derivatives and anthocyanin derivatives. After the ANOVA analysis was ran, a Dunnett's post hoc test assessed significant variance against the control at $p<.05$ for each derivative in the cannabinoid and anthocyanin derivative excitotoxicity insult bar graphs. For the CBD and THC derivative concentration viability scatter plots, scatter points represented means and the bars represented plus/minus one standard error measurement. The scatter plot data is plotted in linear form.

III. Results

We established neuron cultures from rat cortices and grew cells in vitro for 8-10 days prior to treatment. After the neurons were developed in culture, the neurons were treated with an excitotoxic level of glutamate, in combination with the cannabinoid and anti-oxidant small molecules. These prodrugs and derivatives were provided by Dr. David Colby, Dr. Mahmoud ElSohly, Dr. Soumyajit Majumdar, and the National Center for Natural Product Research.

All cells were assessed for viability using MTS assay 24 hours after treatment. Because each column of a 96-well plate was treated with the insult and derivative, all data on the graphs represent the viability average of 8 wells of treated neurons. All averages were normalized to control. DMSO labels in all bar graphs reflect neuron cultures that were treated with just the toxic treatment (glutamate, amyloid-beta, or peroxide), and no protective compounds.

To first assess the amount of glutamate needed to induce excitotoxicity, cultured neurons were treated with increasing concentrations of glutamate (**Figures 4-5**). As the concentrations of glutamate on neurons increased, there was a significant decrease in cell-viability at the 100 and 200 micromolar concentrations of glutamate. This set the baseline for a 200 micromolar glutamate concentration to be used in the experiment to induce neurotoxicity and reduce cell viability.

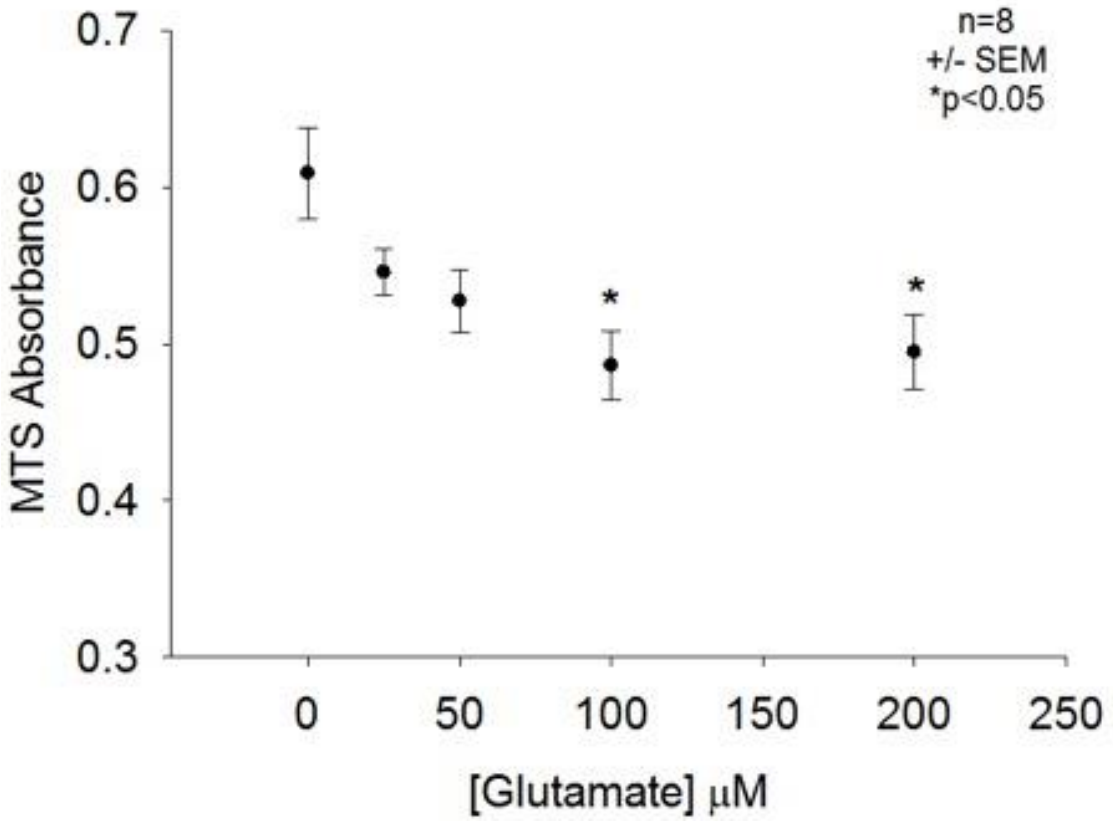

Figure 4: Increasing concentrations of glutamate reduce neuronal viability. The 100 and 200 micromolar concentrations significantly decreased cell viability, as indicated by the asterisks. This significance was verified using a One-Way ANOVA with a Dunnett's post-hoc test.

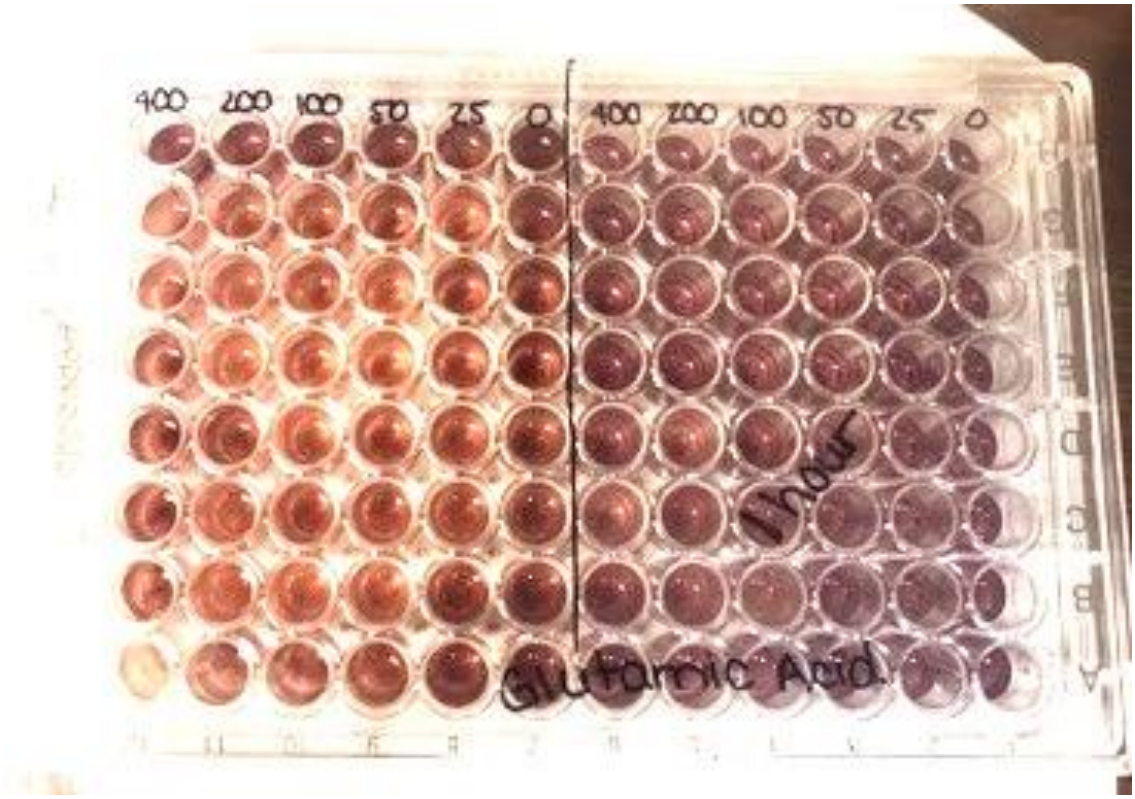

Figure 5: Visualization of MTS assay after glutamate concentration curve treatment. A pale color well is indicative of a low cell density, meaning cells have died. A dark purple color well is indicative of a higher cell density, because more cells are alive and actively metabolizing to convert the tetrazolium salt into formazan. The left side of the plate received the excitotoxic insult for a 24-hour insult period, while the right side of the plate received a limited insult time for 1 hour. The 24-hour insult killed more cells than anticipated, and it would be difficult to find a drug that would protect against the 24 hour excitotoxic insult. This established that our excitotoxic insult needed to be 1 hour.

The protective ability of the cannabinoid and anthocyanin small molecules against the 200 micromolar insult was then assessed using a 10 micromolar concentration of each of the compounds (**Figures 6-7**). For both sets of these compounds, an ANOVA statistical analysis with a Dunnett's post-hoc test was run to evaluate significant differences from control. There were many protective hits on several compounds in both the cannabinoid and anthocyanin-based experiment. CBD, CBD derivative 1, CBD derivative 3, CBD derivative 4, THC derivative 2, and THC derivative 3 were all significantly protective against the excitotoxic insult. Anthocyanin-based compounds 1, 2, 3, 6, 10, 11, and 12 were all significantly protective against the excitotoxic insult. After testing all cannabinoid derivatives against the glutamate insult, CBD, CBD derivative 1, CBD derivative 2, CBD derivative 3, CBD derivative 4, CBD derivative 5, CBD derivative 6, THC derivative 2, THC derivative 3, THC derivative 4, and THC derivative 5 were all of interest for concentration-response curves (**Figures 8-9**). Increasing concentrations of 5, 10, and 40 micromolar of the derivatives were applied to the glutamate induced cultured neurons, and viability was assessed 24 hours later. In both **Figure 8 and Figure 9**, a concentration-dependent response can be seen for both the CBD derivatives and THC derivatives. The compounds' protective ability against glutamate-induced toxicity increases as the compound concentration increases. Between the 10 micromolar and 40 micromolar concentrations, protective abilities of the derivatives begin to plateau, seen in **Figure 8** and **Figure 9.**

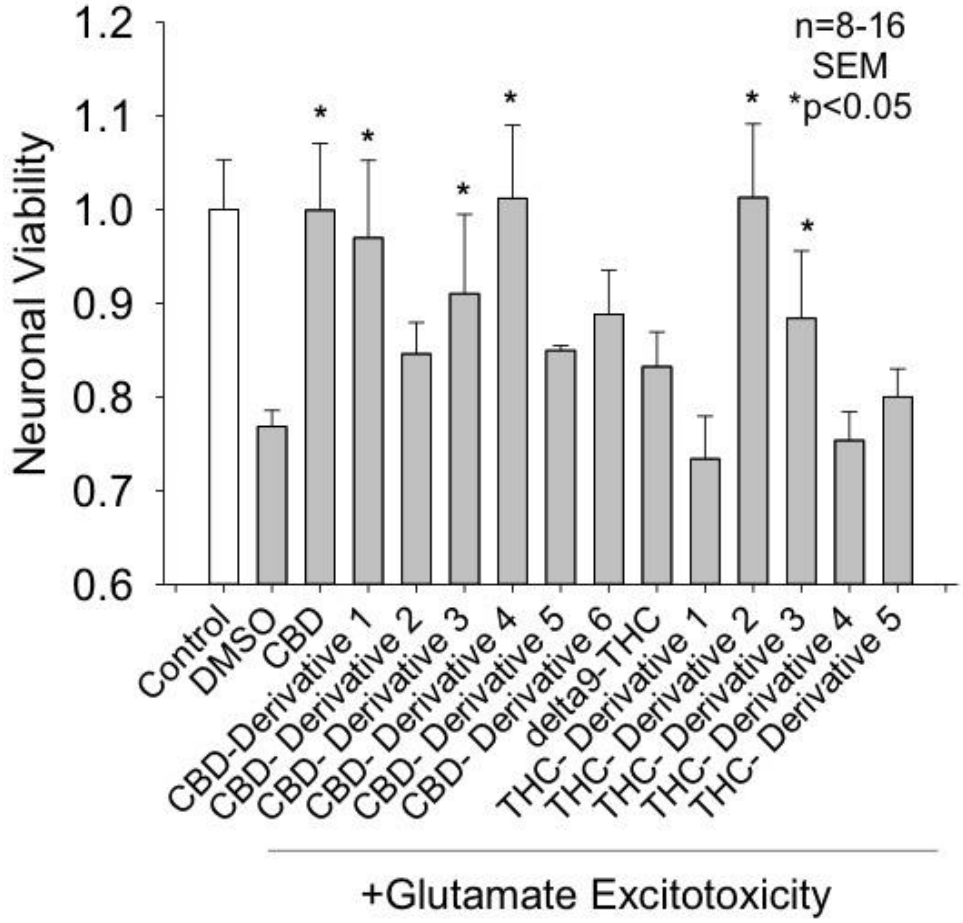

Figure 6: Several of the CBD and THC derivatives when treated at a 10 micromolar concentration were protective against the 200 micromolar glutamate insult, indicated by asterisks. DMSO reflects wells that received only the excitotoxic insult. The control bar is reflective of wells that were unstimulated, and received no treatments. The compounds were compared to the DMSO control, and significance was verified using a One-Way ANOVA with a Dunnett's post-hoc test.

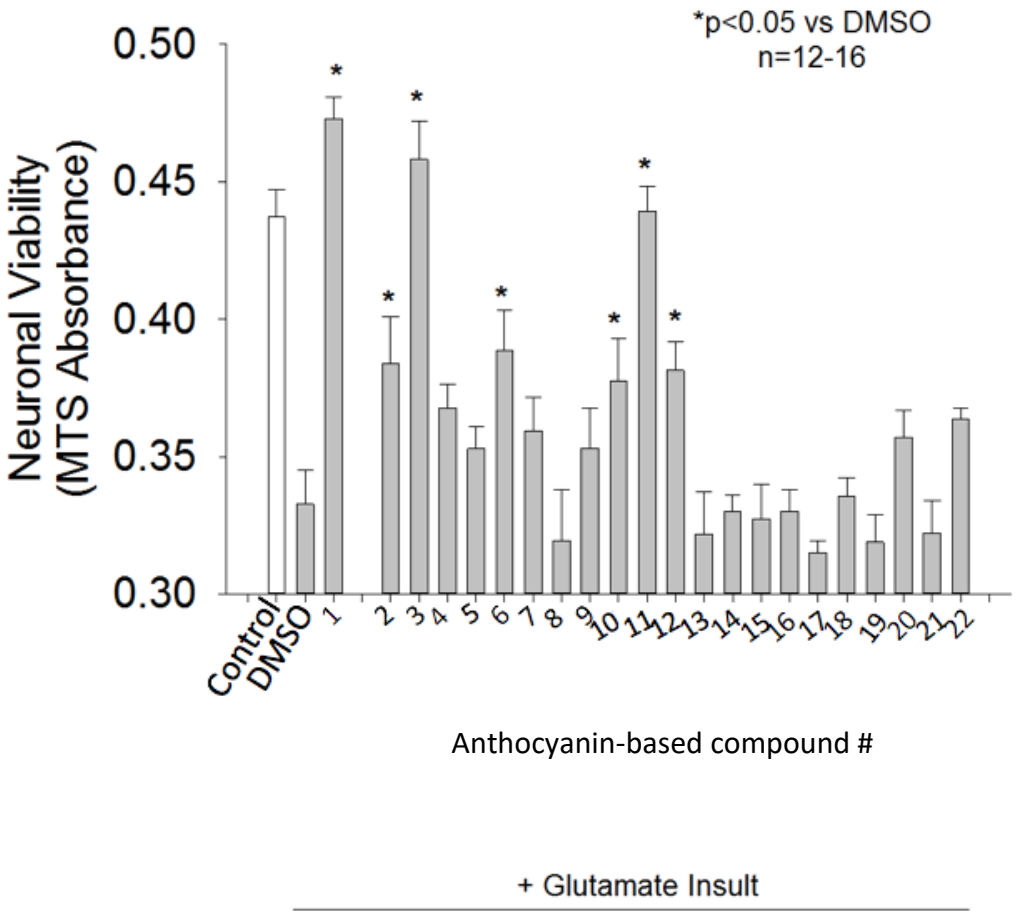

Figure 7: Several of the anthocyanin derivatives when treated at a 10 micromolar concentration were protective against the 200 micromolar glutamate insult, indicated by asterisks. DMSO reflects wells that received only the excitotoxic insult. The control bar is reflective of wells that were unstimulated, and received no treatments The compounds were compared to the DMSO control, and significance was verified using a One-Way ANOVA with a Dunnett's post-hoc test.

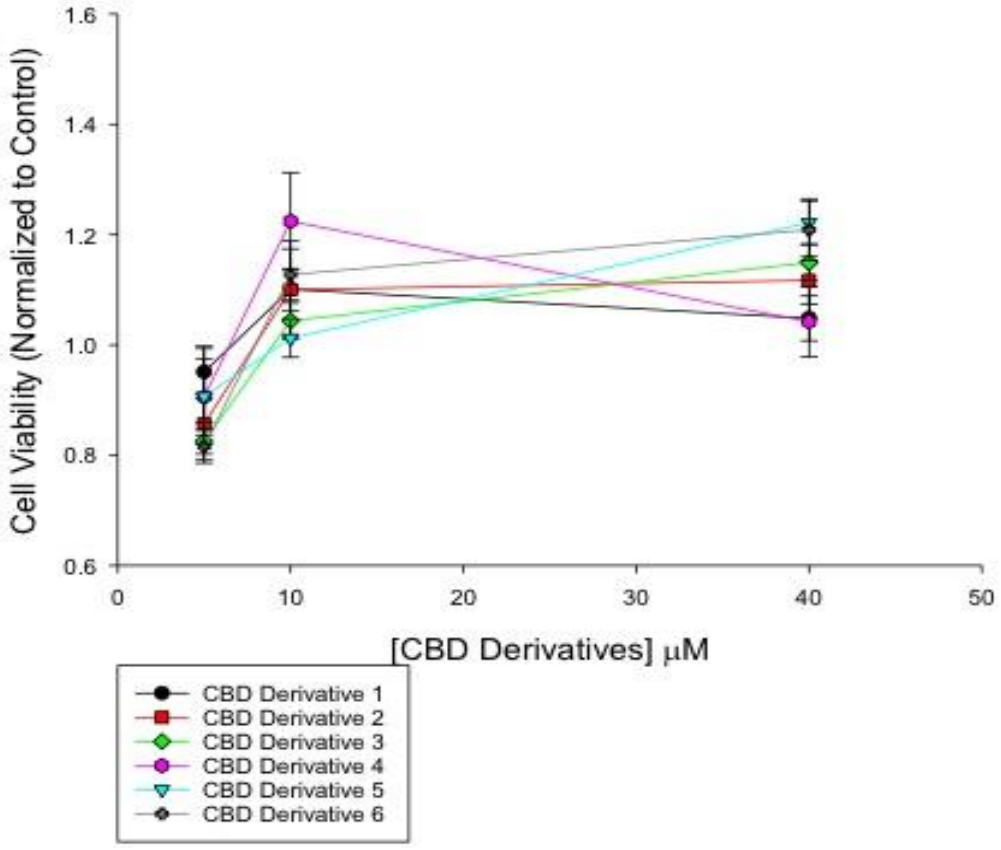

Figure 8: The CBD derivatives showed a concentration-dependent response in protection. Their protection increases up to the 10 micromolar concentration, then the compounds were maximally protective.

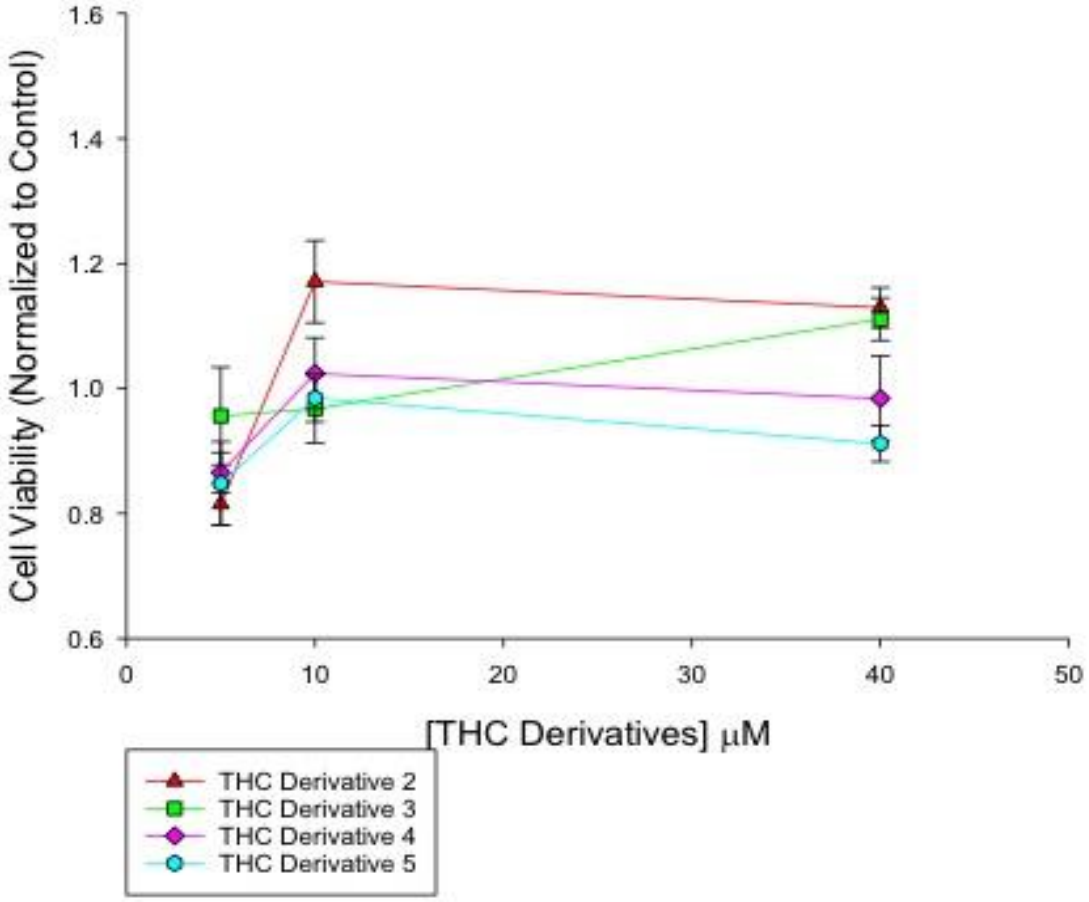

Figure 9: The CBD derivatives show a concentration-dependent response in protection. Their protection increases up the 10 micromolar compound concentration, then the compounds are maximally protective.

Because of the protection seen in the CBD and THC derivative treated excitotoxicity neuron cultures, their protection was tested in amyloid-beta and peroxide treated neuron cultures. In the amyloid-beta treated cell cultures, amyloid-beta statistically significantly reduced cell viability, but none of the compounds protected against its toxicity (**Figure 10**). In the peroxide treated cell cultures, the peroxide did not effectively induce toxicity or decrease neuronal viability, so there was not a window to show neuroprotection with the compounds of interest (**Figure 11**). A full concentration-response curve for H_2O_2 needs to be performed to identify a level of H_2O_2 that kills our cultured neurons.

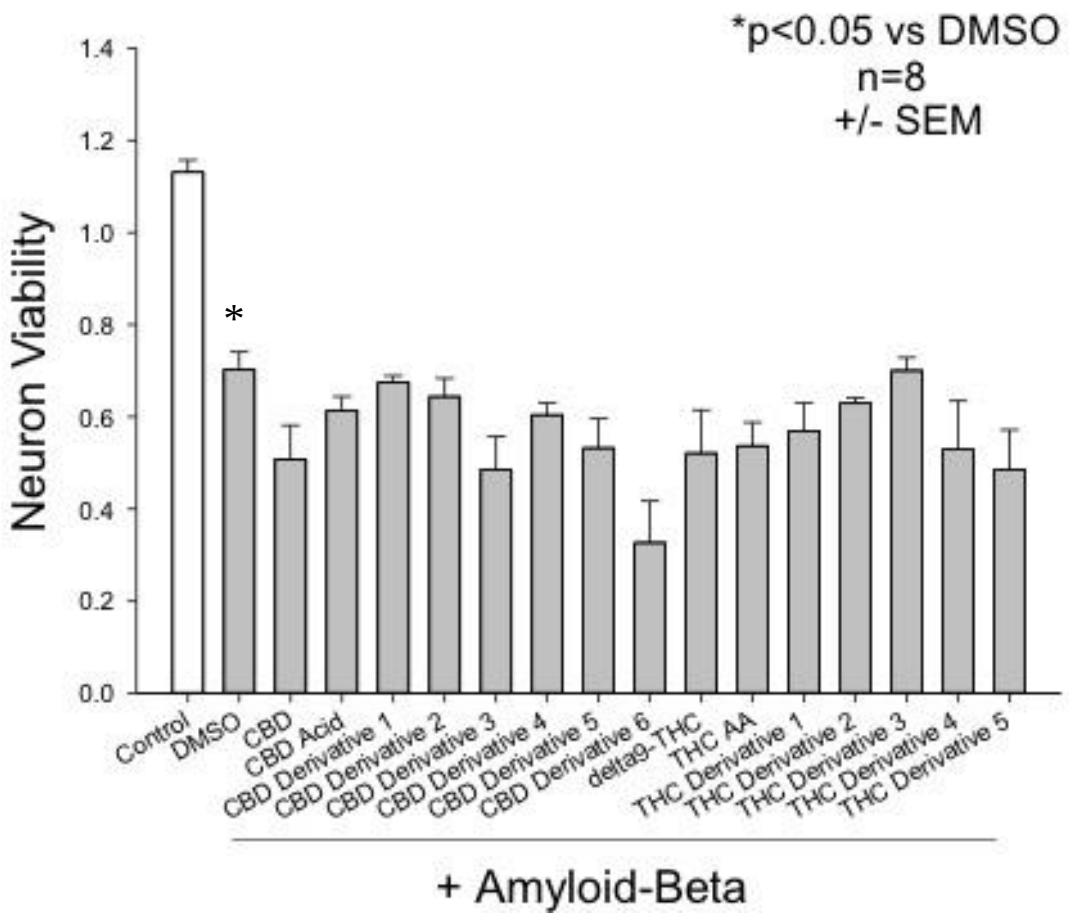

Figure 10: The CBD and THC derivatives were not protective against a 2.5 micromolar concentration of amyloid-beta. The control bar reflects wells of cells that were unstimulated. The DMSO bar reflects wells of cells that just received the amyloid-beta insult.

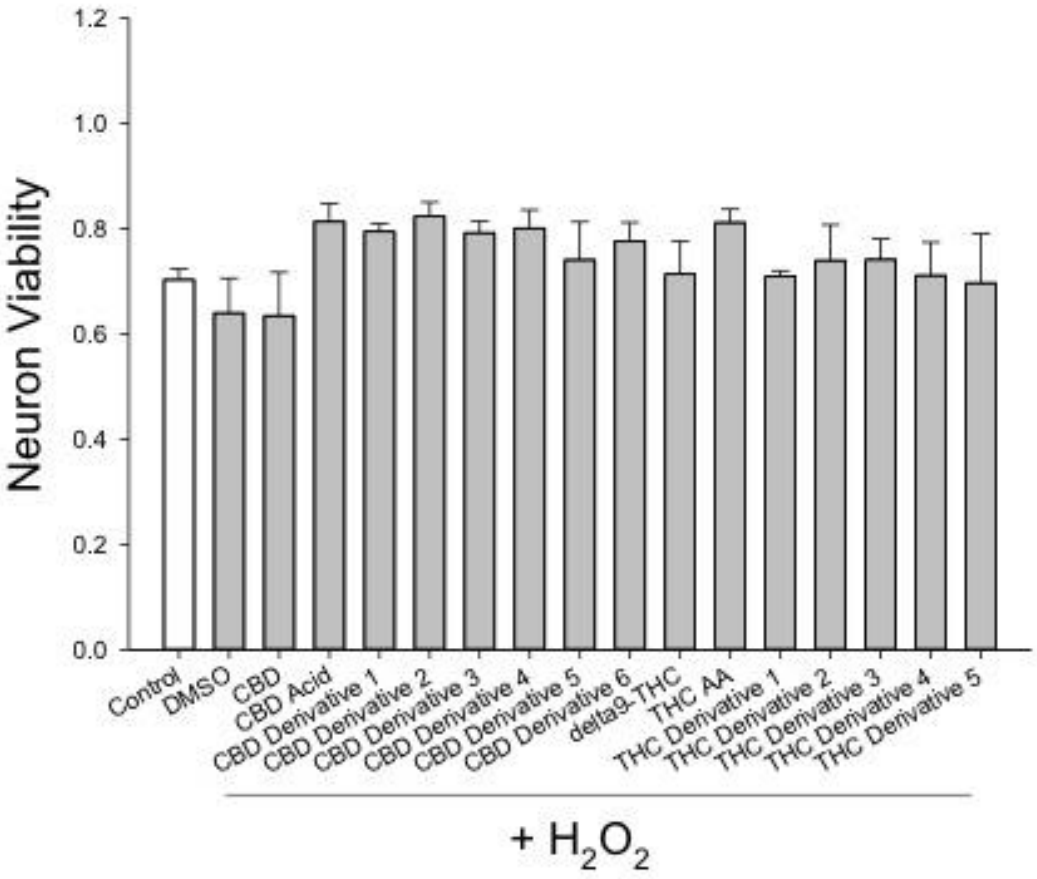

Figure 11: The peroxide did not reduce cell viability significantly, so the protective abilities of the CBD and THC derivatives could not be assessed. The control bar reflects wells of cells that were unstimulated. The DMSO bar reflects wells of cells that just received the peroxide insult.

IV. Discussion

We observed that several of the cannabinoid and anthocyanin small molecules showed neuroprotective capabilities *in vitro*. At the 10 micromolar concentration, both sets of cannabinoid and anthocyanin-based compounds had specific hits that were protective against glutamate-induced excitotoxicity. Although the anthocyanin derivatives were only tested at one concentration against one stressor (excitotoxicity), these data show the protective capabilities of the anthocyanin-derivatives *in vitro*. In previous research, the protective abilities of anthocyanins against amyloid-beta induced toxicity have been reported. Anthocyanins from black soybeans treated in combination with amyloid-beta increased cell viability and reduced cell apoptosis (Badshah et. al. 2014). We did not observe any neuroprotection against amyloid-beta with the compounds of interest. Examining the effects of the anthocyanin derivatives in this study against amyloid-beta toxicity will be an interesting future study, with potential clinical applications. Thus, it is important to repeat this study.

After treating neurons with increasing concentrations of CBD and THC derivatives of interest, there was a concentration-dependent response of protection in our excitotoxic glutamate assay. Because of their protection against excitoxocity, CBD

derivatives 1-6 and THC derivatives 2-5 could be used as potential therapeutic agents against neurodegenerative diseases such as Alzheimer's disease, epilepsy, and amyotrophic lateral sclerosis. Some of the previous CBD and THC-based drugs have been removed from the commercial market because of their adverse side effects (Badal et al 2017), so it will be important to assess whether these derivatives could cause neuroprotection in animals without inducing adverse effects.

It would also be interesting to investigate how the cannabinoid derivatives' antioxidant properties would affect inflammation that accompanies many of these neurodegenerative diseases. The inflammation and microglial activation causes indirect decreases in neuronal viability in Alzheimer's disease (Ramírez et al 2005). In a study by Ramírez et al., cannabinoid analogs *in vitro* were also unable to prevent toxicity directly induced by amyloid-beta in cortical neuron cultures, but some analogs were protective against the indirect toxicity caused by inflammation and microglia activation (Ramírez 2005). The cannabinoid agonist WIN55,212-2 used *in vivo* in the 2005 study also prevented cognitive impairment in amyloid-beta treated rats without adverse side effects (Ramírez 2005). Although the cannabinoid analogs did not prevent toxicity induced by amyloid-beta directly, the research with other cannabinoid analogs against amyloid-beta toxicity provides implications for some of the protective abilities of the cannabinoid derivatives in our study.

In June of 2018, the first CBD-based medication, Epidiolex®, was approved by the FDA to treat people diagnosed with Lennox-Gastaut syndrome and Dravet syndrome. Both of these syndromes cause seizures beginning in early childhood (FDA 2018). Although the medication reduced frequency of seizures, side effects of elevated liver

enzymes, insomnia, diarrhea, and infections were also noted in the study (FDA 2018). An interesting clinical application would be to see the effects of the CBD-derivatives that showed protection against excitotoxicity on these seizures. Perhaps, because of slight differences between the CBD derivatives and CBD, the derivatives would reduce side effects. This is a speculation, but an interesting area of further study.

The CBD and THC derivatives in this study also could have potential clinical applications in delaying the onset of amyotrophic lateral sclerosis (ALS). ALS is a neurodegenerative diseases of motor neurons which are extremely susceptible to oxidative stress, inflammation, and excitotoxicity (Ludolph et al 2000, Robberecht 2000). In a study by Raman et al, the progression of ALS in mice was delayed with the administration of THC after diseases symptoms had already began in the mice (2004). Although protective, it is unknown what the effects of THC would be in a human model of ALS because of THC's known psychoactive properties (Ruet et. al. 2013). Therefore, there are potential applications of the THC derivatives protection against ALS induced degeneration, while minimizing psychoactive side effects. Another study has shown how cannabinoid agonist AM1241 significantly delays progression of ALS in male mice, but not in female mice (Kim, 2006). It is interesting that the cannabinoid agonist used did not protect in female mice with ALS. This leads to another potential application of the protective cannabinoid derivatives in this study. Replicating this experiment with some of the CBD derivatives to test their protection in animal models, and to analyze if they are significantly protective in both sexes to find a universal therapeutic agent.

Limitations of Experiment and Future Directions

There are several experiments that need to be done to improve and advance our current findings. First, a concentration-response curve for the anthocyanin-based compounds is needed. While we attempted this, the assay failed as glutamate did not kill neurons. We are unsure why glutamate did not kill, thus the assay needs repeating. Because there is not a concentration-response curve, it is unknown how protection changes with increasing concentrations of the compounds. Another limitation is the fact that the hydrogen peroxide did not reduce neuronal viability. This assay requires further validation before making conclusions about the cannabinoid based derivatives effectiveness in protecting against oxidative stress. The amyloid beta assay also needs to be validated using a positive control before making conclusions about the effectiveness of the test compounds.

Because these studies were only conducted *in vitro*, it is also unknown whether these compounds can protect neurons in the full, living system. It is important to now test whether these drugs can be effective within animal models of neurodegeneration. Moreover, *in vivo* tests are critical for determining the possible side effects these derivatives have on other aspects of the human body and its metabolism.

Many future directions can be taken after this study. A concentration-response curve of the anthocyanin-derivatives should be constructed. This will enable us to see how the anthocyanin-derivatives protection against excitotoxicity changes with increasing applications of the derivatives. Anthocyanin-derivatives will also be tested for their protection against amyloid-beta toxicity and oxidative stress. Because the peroxide did not reduce neuronal viability in the oxidative stress test in this study, the oxidative

stress study will also be repeated for the cannabinoid-derivatives in order to accurately assess their protection against oxidative stress and reactive oxygen species. Live-dead assays will also be accompanied by future protective assays, in order to accurately quantify the protective extent of the derivatives. If future *in vitro* studies show significant protective abilities of the anthocyanin and cannabinoid-derivatives, future animal models of learning and memory will be of interest.

Overall Conclusions

In conclusion, there were many significantly protective hits against neurotoxicity from multiple CBD, THC, and anthocyanin derivatives. We believe that the derivatives of interest have potential to protect neurons. There is selectivity in the compounds used in this research, as some were protective against glutamate excitotoxicity, and others were not. The mechanisms of action of these compounds are unknown, and require further research. There is also potential in the protective compounds to use their structural information to create other derivatives that could potentially be more effective in neuroprotection. We hope that these compounds' neuroprotective capabilities can be used and seen in both animal and human models.

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