Linarin and Luteolin Elicit Anti-Aβ Cytotoxicity and Inflammation Properties as Novel Treatments for Alzheimer's Disease

Abstract

Alzheimer's Disease (AD) is a dementia-associated neurodegenerative disease characterized by amyloid-beta (Aβ) aggregation and tau protein hyperphosphorylation. Synthetic AD treatments often have adverse side effects, prompting the need for natural treatments. This study examined linarin and luteolin, two plant-derived substances, as potential AD treatments. Linarin and luteolin, individually and combined, were tested on the survival rates of GT1-7 murine neuronal cells and RAW264.7 murine immune cells to investigate the cytotoxic and neuroprotective against Aβ effects, as well as potential synergistic effects. The effects of linarin and luteolin on SK-N-SH human neuroblastoma cell counts were investigated, in addition to the impacts on amyloid precursor protein (APP) concentration in SK-N-SH cells and interleukin-1-beta (IL-1β) concentration in RAW264.7 cells. Results demonstrated that while short-term treatments against Aβ-induced cytotoxicity significantly increased the survival of neuronal ($p<0.01$) but not immune cells, long-term treatments significantly increased survival of neuronal ($p<0.001$) and immune ($p<0.01$) cells. Combined treatments did not significantly increase neuroprotective effects from individual treatments, indicating that linarin and luteolin do not have synergistic effects. Treatments significantly increased the cell count of Aβ-treated cells ($p<0.05$), demonstrating neuroprotective properties against Aβ on human neuronal cells. Linarin and luteolin decreased the concentrations of APP and IL-1β in neuronal and immune cells treated with Aβ, respectively, indicating that they ameliorate Aβ-induced cytotoxicity by decreasing both the source of Aβ plaques and proinflammatory cytokines. Therefore, while both linarin and luteolin are neuroprotective against Aβ-induced cytotoxicity and inflammation, which makes them promising novel AD treatments, they have not demonstrated synergistic effects.

1. Introduction

Alzheimer's Disease (AD) is a progressive neurodegenerative disease associated with dementia, which is the loss of cognitive functions and behavioral abilities. AD currently accounts for 60-80% of dementia cases and ranks as the sixth leading cause of death in the United States ("What Is Alzheimer's Disease," n.d.). Age is the greatest known risk factor of AD, as symptoms first appear in the mid-60s in most cases and an estimated 5.5 million Americans over the age of 65 may have AD. While early AD may begin to show symptoms such as impaired reasoning and continue to progress into greater memory loss and confusion, those with severe AD become completely dependent on others for care and lose the ability to communicate ("Alzheimer's Disease Fact Sheet," n.d.). Currently, there is no cure for AD and treatments cannot stop the progression of this disease, however, treatments may slow the worsening of symptoms. There is widespread effort to develop better treatments for, delay the onset of, and prevent AD ("What Is Alzheimer's Disease," n.d.).

Physiological hallmarks of AD include acetylcholine (ACh) deficiency, inflammation, amyloid-β (Aβ) aggregation, tau (τ) protein hyperphosphorylation, senile plaques, and neurofibrillary tangles (NFT) (Pan et al., 2019). Aβ aggregation is associated with the formation of senile plaques. The Aβ peptides that help characterize AD are derived from an amyloid precursor protein (APP) via γ-secretase and β-secretase cleavage (Bhullar & Rupasinghe, 2013). Aβ monomers form oligomers and plaques through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which leads to the production of inflammatory cytokines with neurotoxic effects (Gu et al., 2018). Toll-like receptor 4 (TLR4) is a receptor for lipopolysaccharides (LPS) whose activation leads to the release of toxic substances to neurons. Aging and Aβ oligomers have been shown to enhance TLR4 expression, LPS-induced responses, and cell death (Calvo-Rodriguez et al., 2017).

τ protein alteration contributes to the pathology of AD through the abnormal phosphorylation and aggregation of τ proteins, which lead to neural dysfunction (Bhullar & Rupasinghe, 2013). Abnormal hyperphosphorylated τ protein is deposited within neurons as NFT in AD (Silveyra et al., 2012). Glycogen synthase kinase-3 (GSK3) is an important τ-kinase and key mediator of apoptosis that is involved in mechanisms of learning and memory, the hyperphosphorylation of τ, the increased production of Aβ from APP, and local cerebral inflammatory responses. The inhibition of GSK3 activity is able to reverse some effects of the overexpression of mutated APP and τ in AD models (Hooper et al., 2007).

While both Aβ peptides and τ proteins are mediators of AD, the neurodegenerative disease is initiated and enhanced by oxidative stress. The production of reactive oxygen species (ROS), which can react with molecules to affect tissues and organs, in mitochondria increases under stressful conditions. The mechanisms of neuron degeneration through mitochondrial dysfunction in AD are associated with ROS generation, activation of mitochondrial permeability transition, and excitotoxicity (Huang et al.,

2016). Nuclear factor erythroid 2-related factor 2 (NRF2) is a major regulator of homeostatic responses, including adapting ROS signaling, inflammation, and metabolism. The absence of NRF2 in mice leads to the early onset of AD with more severe amyloidopathy and tauopathy, as well as increased sensitivity to oxidative stress and inflammation (Rojo et al., 2017). The activity of the phosphatidylinositol 3-kinase (PI3K) /protein kinase B (Akt) pathway is crucial for synaptic activity and normal function, as it suppresses the autophagic process, and disruption of this pathway may lead to imbalances and cell death (Heras-Sandoval et al., 2014).

The early increased acetylcholinesterase (AChE), a substrate specific enzyme that degrades ACh in nerve synapses, in AD may be triggered by either Aβ or τ. τ protein expression may be an important regulator of AChE levels by causing increased levels of AChE activity and AChE protein, which may contribute to the early neurological disturbances in AD. While disturbed τ phosphorylation may cause the early increase of AChE around the NFT in AD, it is unknown what the effect of Aβ is on AChE levels (Silveyra et al., 2012). Since the most prominent abnormality in AD brains is associated with the cholinergic system, which is comprised of neurons that are activated by ACh, a popular therapeutic approach for treatment is to enhance cholinergic neurotransmission, which can be done by increasing the neurotransmitter ACh through inhibiting AChE (Feng et al., 2015).

Since AD is such a complex disease with many physiological implications as well as cognitive and behavioral symptoms, it is likely that multiple interventions are needed to effectively treat the disease. Current approaches are meant to help those with AD maintain mental function, manage behavioral symptoms, and slow the progression of the disease ("How Is Alzheimer's Disease Treated," n.d.). Currently, the United States Food and Drug Administration (FDA) has approved two types of medications to treat the cognitive symptoms of AD: cholinesterase inhibitors and memantine. Cholinesterase inhibitors, such as Donepezil, Galantamine, and Rivastigmine, treat cognitive symptoms by preventing the breakdown of Ach and delaying the worsening of symptoms in the early to moderate stages of AD. However, these medications have side effects that commonly include nausea, vomiting, loss of appetite, and increased bowel movements. Memantine is prescribed to improve basic cognitive abilities by regulating the activity of glutamate. However, it can also cause side effects that commonly include headaches, constipation, confusions, and dizziness ("Medications for Memory," n.d.). While there are a few synthetic drugs in the market commonly used to treat AD, they have several adverse effects (Feng et al., 2015). It is thus necessary to develop a natural drug to potentially help treat AD without the adverse effects of synthetic drugs.

Linarin is a naturally occurring flavanol glycoside that is derived from medicinal plants, such as *Buddleja davidii*, *Buddleja officinalis*, *Cirsium setosum*, *Flos chrysanthemi indici*, and *Mentha arvensis* (Feng et al., 2015). Linarin has been known to have antibacterial, anti-inflammatory, sedative,

coagulative, and anticancer effects. Its anti-inflammatory, AChE inhibitory, and neuroprotective properties make it a promising AD treatment (Pan et al., 2019). Linarin has been shown to inhibit AChE in a dose dependent manner by binding to the AChE sites in mice brains (Feng et al., 2015). Linarin was able to inhibit inflammation by inactivating NF-κB, mitogen-activated protein kinases (MAPKs), tumor necrosis factor (TNF), and PI3K/Akt pathways (Pan et al., 2019). In addition, t has been shown to be a selective AChE inhibitor in PC12 cells that increases cell viability against Aβ-induced toxicity, decreases apoptotic cell death and mitochondrial damage, and has neuroprotective effects through increased Akt phosphorylation and suppressed GSK-3β activation. Linarin also suppressed the Aβ associated activation of GSK-3β, which leads to neuron death and τ phosphorylation, via the PI3K/Akt pathway through GSK-3β phosphorylation (Lou et al., 2011).

Luteolin is an important flavone with antioxidative, anticancerous, anti-inflammatory, and neuroprotective abilities that is naturally found in a glycosylated form in various plants. It has been shown to be present in many common edible plants, such as carrots, celery, peppermint, thyme, and oregano, as well as traditional medicinal plants, such as *Terminalia chebula*, *Salvia tomentosa*, and *Senna petersiana* (Nabavi et al., 2015). The antioxidative and neuroprotective effects of luteolin were associated with the NRF2 pathway and the upregulation of the NRF2 protein levels (Ashaari et al., 2018). Due to the increased cell viability and inhibited ROS production in cells with induced oxidation, it was indicated that luteolin is antioxidative through its anti-apoptotic behavior and the upregulation of an antioxidant enzyme expression (Kim et al., 2014). It exhibited its neuroprotective abilities by inhibiting LPS-induced inflammatory activation in BV2 cells (Kwon, 2017). It has also exhibited its ability to inhibit cytokine expression, NF-κB signalling, and TLR4 signaling at micromolar concentrations in immune cells (Nabavi et al., 2015). Luteolin ameliorated the neurotoxicity of Aβ fragments due to its antioxidative behavior (Choi et al., 2014). It has also been shown to reduce zinc-induced τ phosphorylation in SH-SY5Y cells (Ashaari et al., 2018). Luteolin has been able to reduce cerebral Aβ accumulation in Tg2576 mice, as well as abolish τ phosphorylation and microbial-induced inflammatory cytokines release, which indicates the potential of luteolin as AD treatment (Kwon, 2017).

Linarin and luteolin are two plant-derived substances with anti-inflammatory, antioxidative, and neuroprotective properties, which make them promising potential natural options for AD treatment. Although both have been shown to inactivate the NF-κB pathway to reduce inflammation, these two substances generally affect different pathways to cause anti-inflammatory, anti-oxidative, and Aβ toxicity ameliorating effects. The effects of luteolin against Aβ-induced cytotoxicity and inflammation has been studied much more in-depth and on a wider spectrum of cells and organisms than the effects of linarin. While linarin has been studied on PC12 rat adrenal cells, which is considered an AD model, the effects of linarin on neuronal and immune cells have yet to be investigated directly (Lou et al., 2011). Therefore,

this current study will investigate the effects of linarin and luteolin on the survival rates of GT1-7 mouse hypothalamus cells and RAW264.7 mouse immune cells *in vitro*, as well as on the cell counts of SK-N-SH human neuroblastoma cells. The mechanisms of the effects of linarin and luteolin will be studied by investigating their effects on APP concentration in SK-N-SH cells and IL-1β concentration in RAW264.7 cells. Linarin (**Fig. 1**) and luteolin (**Fig. 2**) have similar molecular structures as plant-derived substances. These molecular similarities may indicate that these substances affect similar pathways, however, the slight variation between the two may also indicate that they can affect slightly different mechanisms. Similar effects of these substances through varying pathways would demonstrate that linarin and luteolin may induce synergistic effects when combined. However, the effects of these two substances as potential AD treatments have yet to be studied in tandem. It is thus hypothesized in this study that linarin and luteolin both have Aβ-toxicity ameliorating and anti-inflammatory properties, as well as have synergistic neuroprotective effects against Aβ-induced cytotoxicity when combined.

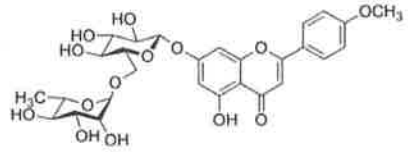

Figure 1. The molecular structure of linarin. Linarin is a flavanol glycoside that has been known to have antibacterial, anti-inflammatory, anticancer, sedative, coagulative, AChE inhibitory, and neuroprotective effects (Lou et al., 2011).

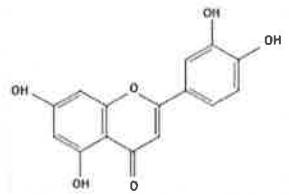

Figure 2. The molecular structure of luteolin. Luteolin is a flavone that has been known to have antioxidative, anticancer, anti-inflammatory, and neuroprotective properties (Ashaari et al., 2018).

2. Materials & Methods

2.1 Cell Cultures and Well Plate Preparations

GT1-7 (Sigma-Aldrich) is an immortalized mouse hypothalamic Gonadotropin-releasing hormone (GnRH) neuronal cell line. RAW264.7 (ATCC) is an immortalized mouse Abelson murine leukemia virus transformed macrophage cell line. SK-N-SH (ATCC) is an immortalized human metastatic site derived neuroblastoma cell line.

The cells were cultured *in vitro* in flasks with minimum essential media (MEM) at 37.0°C in a humidified incubator with 5.0% CO_2 concentration. The cells were transferred by detaching the cells from the bottom of the flask with the protease trypsin. The waste media was then discarded, and the cells were placed in new MEM. The cell solutions were dispensed into well plates at 100μL per well in a 96-well plate and at 2000μL per well in a six-well plate. The cells were allowed to attach to the bottoms of the wells in an incubator at 37.0°C with 5.0% CO_2 concentration before treatment.

2.2 Reagents and Dilutions

Linarin (Cayman Chemical Company) was dissolved using dimethyl sulfoxide (DMSO) to create a 1mM concentration stock solution and dilutions were prepared using phosphate-buffered saline (PBS).

Luteolin (Sigma-Aldrich) was dissolved using DMSO to create a 1mM concentration stock solution and dilutions were prepared using 50% DMSO and 50% distilled water.

Dilutions of linarin and luteolin were prepared at 100μM, 10μM, and 1μM concentrations from the stock solutions and were used to treat the well plates. The final concentrations of the treatments were calculated and presented based on the concentration of the solution, the amount of solution added, and the amount of cell solution added to each well.

2.3 MTT Assay

Treatments of linarin, luteolin, and combined linarin and luteolin at 0.5μM and 0.05μM concentrations were added to GT1-7 cells, GT1-7 cells treated with 5μM concentration Aβ, RAW264.7 cells, and RAW264.7 cells treated with Aβ in 96-well plates for 24 hours and 72 hours to test the short term and long term cytotoxic effects and neuroprotective effects against Aβ-induced cytotoxicity, respectively, of these substances on neuronal cells and immune cells. The cells were treated with linarin, luteolin, and combined linarin and luteolin directly before the addition of Aβ. The control cells were left untreated to provide a negative control for cytotoxic effects of the substances, while cells were also treated with 0.5μM Aβ to provide a positive control of Aβ-induced cytotoxicity for the neuroprotective effects of the substances.

The cell plates were left to incubate at 37.0°C and 5.0% CO_2 concentration for their designated treatment times. The yellow MTT dye was then added to the 96-well plates at 10.00μL per well. The plates were left to incubate for two hours to allow the MTT to undergo the reaction. After incubation, 70μL of DMSO were added to each well. The DMSO was allowed to dissolve the purple formazan crystals that were formed for 10 minutes, in order to properly measure the color density of the results. The results on the cell plate were then analyzed using an iMark™ Microplate Absorbance Reader set to a wavelength of 595nm and data was collected with the Microplate Manager 6 Software. The collected data was then transferred to an Excel spreadsheet.

2.4 Image Analysis

Treatments of linarin and luteolin at 0.1μM concentration were added to SK-N-SH cells and SK-N-SH cells treated with 0.5μM concentration Aβ in a six-well plate for 24 hours to test the effects of these substances on healthy neuronal cells and neuronal cells with Aβ-induced cytotoxicity. The cells were treated with linarin and luteolin directly before the addition of Aβ. The control cells were left untreated to provide a negative control for cytotoxic effects of the substances, while cells were also treated with 0.5μM Aβ to provide a positive control of Aβ-induced cytotoxicity for the neuroprotective effects of the substances.

The cell plate was left to incubate at 37.0°C and 5.0% CO_2 concentration for its designated treatment time of 24 hours. The cell media was then removed from the well plate before adding 1.000mL

of Hema 3 fixation to each well and allowing the fixation to harden the cell cultures for one minute. The fixation was then removed from the well plate before adding 1.000mL of Hematoxylin staining solution to each well and allowing the solution to stain the cells for two minutes. The staining solution was subsequently removed from the wells and distilled water was used to gently rinse the wells of excess staining solution. The well plate was placed under an electron light microscope set to 100x magnification and linked to a computer. The ScopeImage 9.0 application was used to take pictures of the cell cultures, which were then analyzed with the ImageJ computer application. Cell counts and cell densities were analyzed with the ImageJ plug-in Image-based Tool for Counting Nuclei (ITCN) set to a width of 40 pixels and a minimum distance of 20.0 pixels.

2.5 ELISA

Treatments of linarin and luteolin at 0.1μM concentration were added to SK-N-SH cells, SK-N-SH cells Aβ treated with 0.5μM concentration Aβ, RAW264.7 cells, and RAW264.7 cells treated with in a six-well plate for 24 hours to test the effects of these substances on healthy neuronal cells, neuronal cells treated with Aβ, immune cells, and immune cells treated with Aβ. The cells were treated with linarin and luteolin directly before the addition of Aβ. The control cells were left untreated to provide a negative control for cytotoxic effects of the substances, while cells were also treated with 0.5μM Aβ to provide a positive control of Aβ-induced cytotoxicity for the neuroprotective effects of the substances. The SK-N-SH cells were analyzed for APP, the precursor molecule that creates Aβ plaques, through a Human APP ELISA, while the RAW264.7 cells were analyzed for IL-1β, a prominent microglia-derived proinflammatory cytokine, through a Human IL-1β ELISA.

The cell plates were left to incubate at 37.0°C and 5.0% CO_2 concentration for its designated treatment time of 24 hours. The cell media was then removed from the wells and 1.000mL of distilled water was added to each well before the cell cultures were put through four freeze-thaw cycles to break up the cell membranes and release the peptides and molecules from within the cells. The cell solutions were transferred to tubes to be centrifuged in order to separate the cell debris from the solution containing the antigens. The supernatants containing the antigens were then added to the 96-well plates coated with antibodies of the ELISA kits and the instructions of the ELISA kits were followed for several washes to remove any unbound material. Following the procedures, the 96-well plates were analyzed with and iMark™ Microplate Absorbance Reader and data was collected with the Microplate Manager 6 Software. The collected data was then transferred to an Excel spreadsheet, where the collected data was analyzed in comparison to a standard curve of optical density versus concentration to calculate the concentrations of the antigens in the samples, thus gaining quantitative results.

2.6 Statistical Analysis

Percent change was used to compare the results of the treatments to the baselines of the untreated control and Aβ-treated cells, in order to compare the effects of the treatments. Data was presented as mean ± standard error (SEM). Statistical analysis was performed with two-tailed student's t-tests with p-value <0.05 indicating the threshold for significance.

3. Results

3.1 MTT Assay with GT1-7 Mouse Hypothalamic Cells

3.1.1 The Effects of Individual Linarin and Luteolin on the Survival Rates of GT1-7 Cells and GT1-7 Cells Treated with Aβ

Treatment of 0.5μM linarin on GT1-7 cells for 24 hours caused the cell survival to significantly decrease 37.22% from the 100.00% survival control baseline ($p<0.001$), while treatment on GT1-7 cells treated with Aβ caused the survival to insignificantly increase 24.98% from the 53.42% survival baseline of GT1-7 cells treated with 5μM Aβ (**Fig. 3**). Treatment of 0.5μM luteolin on GT1-7 cells caused the cell survival to significantly decrease 38.53% ($p<0.001$), while treatment on GT1-7 cells treated with Aβ caused the survival to significantly increase 28.33% ($p<0.01$) (**Fig. 4**).

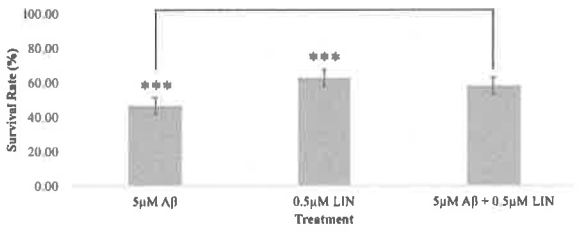

Figure 3. Survival rates of GT1-7 cells after 24 hour treatments of linarin. GT1-7 cells and GT1-7 cells treated with Aβ were treated with linarin at 0.5μM for 24 hours to evaluate the potential cytotoxic and neuroprotective effects of linarin on neuronal cells individually ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

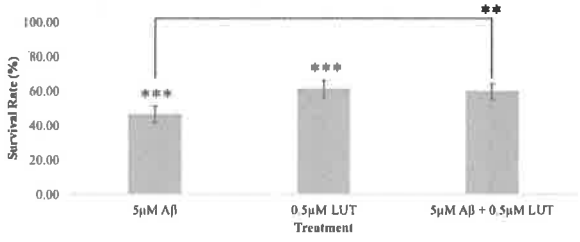

Figure 4. Survival rates of GT1-7 cells after 24 hour treatments of luteolin. GT1-7 cells and GT1-7 cells treated with Aβ were treated with luteolin at 0.5μM for 24 hours to evaluate the potential cytotoxic and neuroprotective effects of luteolin on neuronal cells individually ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

Treatment of 0.5μM linarin on GT1-7 cells at 72 hours caused the cell survival to significantly decrease 22.57% from the 100.00% survival control baseline ($p<0.001$), while treatment on GT1-7 cells treated with Aβ caused the survival to significantly increase 51.40% from the 42.21% survival baseline of GT1-7 cells treated with 5μM Aβ ($p<0.001$) (**Fig. 5**). Treatment of 0.5μM luteolin on GT1-7 cells caused the cell survival to significantly decrease 23.92% ($p<0.001$), while treatment on GT1-7 cells treated with Aβ caused the survival to significantly increase 12.23% ($p<0.001$) (**Fig. 6**).

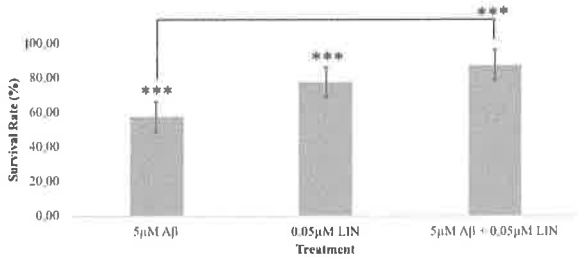

Figure 5. Survival rates of GT1-7 cells after 72 hour treatments of linarin. GT1-7 cells and GT1-7 cells treated with Aβ were treated with linarin at 0.05μM for 72 hours to evaluate the potential cytotoxic and neuroprotective long term effects of linarin on neuronal cells individually ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

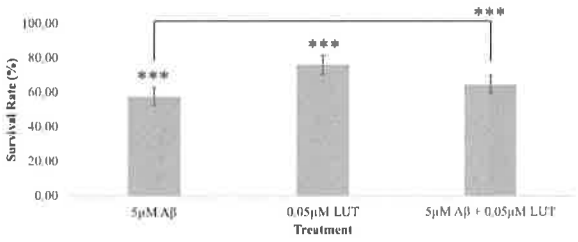

Figure 6. Survival rates of GT1-7 cells after 72 hour treatments of luteolin. GT1-7 cells and GT1-7 cells treated with Aβ were treated with luteolin at 0.05μM for 72 hours to evaluate the potential cytotoxic and neuroprotective long term effects of luteolin on neuronal cells individually ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

Both linarin and luteolin had significant short term and long term cytotoxic effects on GT1-7 cells, although neither was significantly more cytotoxic. While only luteolin significantly increased survival against Aβ-induced cytotoxicity in GT1-7 cells at 24 hours and both substances significantly increased survival against Aβ-induced cytotoxicity at 72 hours, linarin significantly increased cell survival more than luteolin ($p<0.001$) at 72 hours.

3.1.2 The Effects of Concentration on Cytotoxicity Induced by Linarin, Luteolin, and Combined Linarin and Luteolin on the Survival Rates of GT1-7 Cells

Treatment of combined linarin and luteolin at 0.5μM significantly decreased cell survival 39.52% from the 100.00% survival control baseline ($p<0.001$). However, combined linarin and luteolin did not have significantly more cytotoxic effects than linarin or luteolin individually at 0.5μM (**Fig. 7**). Treatment of combined linarin and luteolin at 0.05μM significantly decreased cell survival 22.17% from the control ($p<0.001$). While combined linarin and luteolin at 0.05μM was significantly less cytotoxic than linarin ($p<0.001$), it was not significantly less cytotoxic than luteolin (**Fig. 8**). While linarin decreased cell survival more at 0.05μM and luteolin decreased survival more at 0.5μM, neither difference in survival decrease was significant. Combined linarin and luteolin significantly decreased cell survival at both concentrations, however, it significantly decreased cell survival rate more at 0.5μM ($p<0.01$).

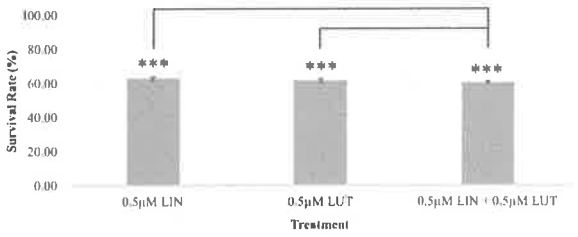

Figure 7. Survival rates of GT1-7 cells after 24 hour treatments of 0.5μM. GT1-7 cells were treated with linarin, luteolin, and combined linarin and luteolin at 0.5μM for 24 hours to evaluate the potential cytotoxic effects of linarin, luteolin, and combined linarin and luteolin at 0.5μM concentration on neuronal cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

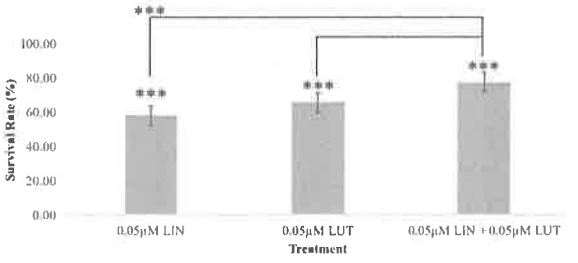

Figure 8. Survival rates of GT1-7 cells after 24 hour treatments of 0.05μM. GT1-7 cells were treated with linarin, luteolin, and combined linarin and luteolin at 0.05μM for 24 hours to evaluate the potential cytotoxic effects of linarin, luteolin, and combined linarin and luteolin at 0.05μM concentration on neuronal cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

3.1.3 The Effects of Linarin, Luteolin, and Combined Linarin and Luteolin on the Survival Rates of GT1-7 Cells Treated with Aβ

GT1-7 cells were treated with 5μM Aβ for 24 hours to establish the baseline for cell survival with short term Aβ-induced cytotoxicity with a significant decrease of 53.42% from the control ($p<0.001$). Treatment of combined linarin and luteolin at 0.5μM significantly increased the cell survival 32.83% from the cells treated with Aβ ($p<0.001$). This increase was not significantly greater than the increase caused by either linarin or luteolin individually at 0.5μM. Treatment of combined linarin and luteolin at 0.05μM significantly increased survival 40.60% from cells treated with Aβ ($p<0.001$). Combined treatment at 0.05μM did not significantly increase cell survival more than at 0.5μM (**Fig. 9**).

GT1-7 cells were treated with 5μM Aβ for 72 hours to establish the baseline for cell survival with long term Aβ-induced cytotoxicity with a significant decrease of 42.21% from the control ($p<0.001$). Treatment of combined linarin and luteolin at 0.05μM significantly increased the cell survival 15.94% from the cells treated with Aβ ($p<0.001$). This increase in cell survival was significantly less than the increase caused by linarin at 0.05μM ($p<0.001$) but not significantly more than the increase caused by luteolin at 0.05μM (**Fig. 10**).

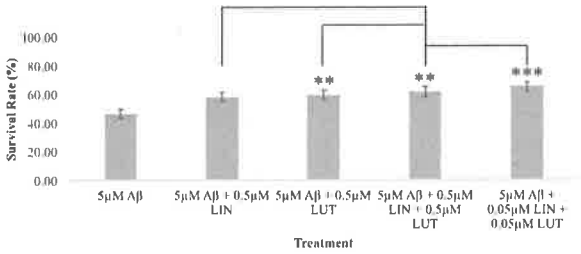

Figure 9. Survival rates of GT1-7 cells treated with Aβ after 24 hour treatments. GT1-7 cells treated with Aβ were treated with linarin, luteolin, combined linarin and luteolin at 0.5μM, and combined linarin and luteolin at 0.05μM for 24 hours to evaluate the potential neuroprotective effects of linarin, luteolin, and combined linarin and luteolin against Aβ-induced cytotoxicity on neuronal cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

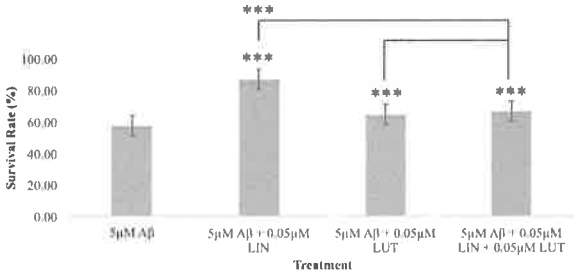

Figure 10. Survival rates of GT1-7 cells treated with Aβ after 72 hour treatments. GT1-7 cells treated with Aβ were treated with linarin, luteolin, combined linarin and luteolin at 0.05μM for 72 hours to evaluate the potential long term neuroprotective effects of linarin, luteolin, and combined linarin and luteolin against Aβ- induced cytotoxicity at 0.05μM concentration on neuronal cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

3.2 MTT Assay with RAW264.7 Mouse Macrophage Cells

3.2.1 The Effects of Individual Linarin and Luteolin on the Survival Rates of RAW264.7 Cells and RAW264.7 Cells Treated with Aβ

Treatment of 0.5μM linarin on RAW264.7 cells for 24 hours caused the cell survival to significantly decrease 20.46% from the 100.00% survival control baseline ($p<0.01$), while treatment on cells treated with Aβ caused the survival to insignificantly decrease 3.39% from the 61.33% survival baseline of RAW264.7 cells treated with 5μM Aβ (**Fig. 11**). Treatment of 0.5μM luteolin caused survival to significantly decrease 46.45% ($p<0.001$), while treatment on cells treated with Aβ caused survival to significantly decrease 35.82% ($p<0.01$) (**Fig. 12**).

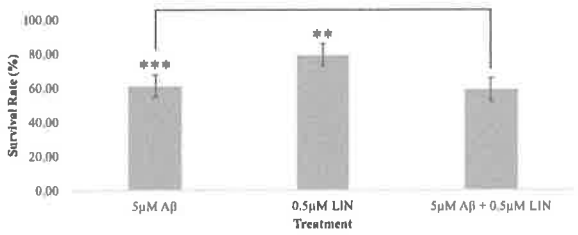

Figure 11. Survival rates of RAW264.7 cells after 24 hour treatments of linarin. RAW264.7 cells and RAW264.7 cells treated with Aβ were treated with linarin at 0.5μM for 24 hours to evaluate the potential cytotoxic and neuroprotective effects of linarin on immune cells individually ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

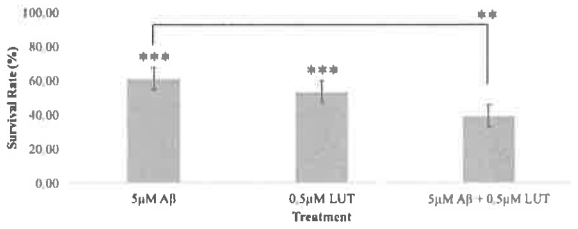

Figure 12. Survival rates of RAW264.7 cells after 24 hour treatments of luteolin. RAW264.7 cells and RAW264.7 cells treated with Aβ were treated with luteolin at 0.5μM for 24 hours to evaluate the potential cytotoxic and neuroprotective effects of luteolin on immune cells individually ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

Treatment of 0.5μM linarin on RAW264.7 cells at 72 hours caused the cell survival to significantly decrease 29.79% from the 100.00% survival control baseline ($p<0.001$), while treatment on RAW264.7 cells treated with Aβ caused the survival to significantly increase 98.69% from the 32.40% survival baseline of RAW264.7 cells treated with 5μM Aβ ($p<0.001$) (**Fig. 13**). Treatment of 0.5μM luteolin caused the cell survival to significantly decrease 64.35% ($p<0.001$), while treatment on cells treated with Aβ caused the survival to significantly increase 34.36% ($p<0.01$) (**Fig. 14**).

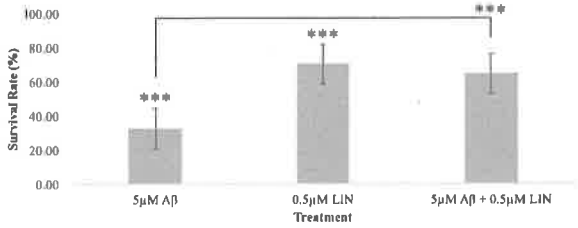

Figure 13. Survival rates of RAW264.7 cells after 72 hour treatments of linarin. RAW264.7 cells and RAW264.7 cells treated with Aβ were treated with linarin at 0.5μM for 72 hours to evaluate the potential cytotoxic and neuroprotective long term effects of linarin on immune cells individually ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

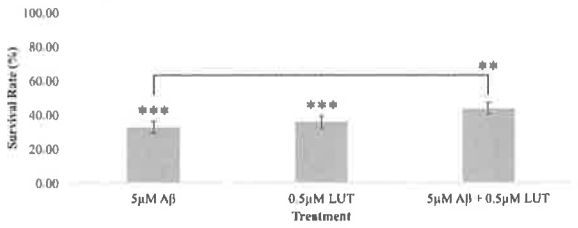

Figure 14. Survival rates of RAW264.7 cells after 72 hour treatments of luteolin. RAW264.7 cells and RAW264.7 cells treated with Aβ were treated with luteolin at 0.5μM for 72 hours to evaluate the potential cytotoxic and neuroprotective long term effects of luteolin on immune cells individually ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

While both linarin and luteolin caused significant decreases in cell survival in RAW264.7 cells for both short term and long term treatments, luteolin was significantly decreased cell survival more than linarin ($p<0.001$). Although both substances decreased survival on RAW264.7 cells with Aβ-induced cytotoxicity at 24 hours, only luteolin has significant effects. While both substances significantly increased cell survival against Aβ-induced cytotoxicity at 72 hours, linarin significantly increased survival more than luteolin ($p<0.001$).

3.2.2 The Effects of Concentration on Cytotoxicity Induced by Linarin, Luteolin, and Combined Linarin and Luteolin on the Survival Rates of RAW264.7 Cells

Treatment of combined linarin and luteolin at 0.5μM significantly decreased cell survival 62.39% from the 100.00% survival control baseline ($p<0.001$). Combined linarin and luteolin significantly decreased cell survival more than both linarin ($p<0.001$) and luteolin ($p<0.001$) individually at 0.5μM

(**Fig. 15**). Treatment of combined linarin and luteolin at 0.05μM significantly decreased cell survival 47.58% from the control ($p<0.001$). Combined linarin and luteolin significantly decreased cell survival more than both linarin ($p<0.001$) and luteolin ($p<0.05$) individually at 0.05μM (**Fig. 16**).

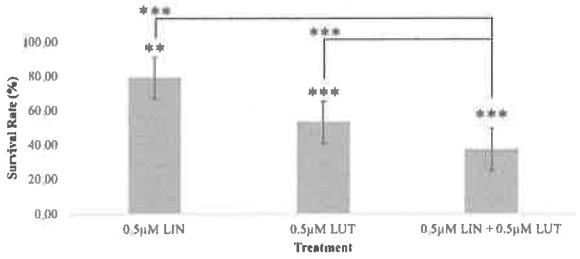

Figure 15. Survival rates of RAW264.7 cells after 24 hour treatments of 0.5μM. RAW264.7 cells were treated with linarin, luteolin, and combined linarin and luteolin at 0.5μM for 24 hours to evaluate the potential cytotoxic effects of linarin, luteolin, and combined linarin and luteolin at 0.5μM concentration on immune cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

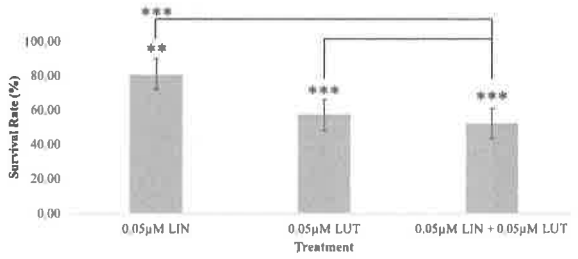

Figure 16. Survival rates of RAW264.7 cells after 24 hour treatments of 0.05μM. RAW264.7 cells were treated with linarin, luteolin, and combined linarin and luteolin at 0.05μM for 24 hours to evaluate the potential cytotoxic effects of linarin, luteolin, and combined linarin and luteolin at 0.05μM concentration on immune cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

Treatment of combined linarin and luteolin at 0.5μM significantly decreased cell survival 59.23% from the 100.00% survival control baseline ($p<0.001$). Combined linarin and luteolin at 0.5μM significantly decreased cell survival more than linarin individually ($p<0.001$) but did not significantly decrease survival more than luteolin individually (**Fig. 17**). Treatment of combined linarin and luteolin at 0.05μM significantly decreased cell survival 30.67% from the control ($p<0.001$). Combined linarin and luteolin at 0.05μM significantly decreased cell survival more than linarin ($p<0.05$) and significantly decreased cell survival less than luteolin ($p<0.001$) individually (**Fig. 18**).

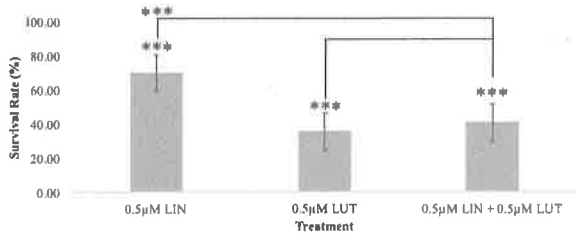

Figure 17. Survival rates of RAW264.7 cells after 72 hour treatments of 0.5μM. RAW264.7 cells were treated with linarin, luteolin, and combined linarin and luteolin at 0.5μM for 72 hours to evaluate the potential long term cytotoxic effects of linarin, luteolin, and combined linarin and luteolin at 0.5μM concentration on immune cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

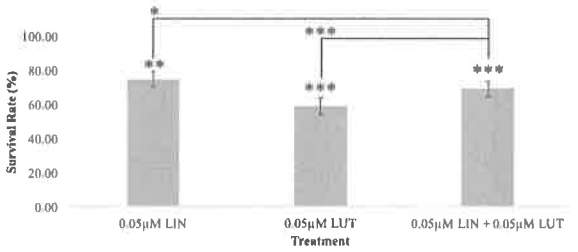

Figure 18. Survival rates of RAW264.7 cells after 72 hour treatments of 0.05μM. RAW264.7 cells were treated with linarin, luteolin, and combined linarin and luteolin at 0.05μM for 72 hours to evaluate the potential long term cytotoxic effects of linarin, luteolin, and combined linarin and luteolin at 0.05μM concentration on immune cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

While linarin and luteolin both decreased cell survival more at 0.5μM for both 24 and 72 hours, only luteolin significantly decreased cell survival more at 0.5μM than 0.05μM at 72 hours ($p<0.001$). Combined linarin and luteolin significantly decreased cell survival at both concentrations for both 24 and 72 hours, however, combined linarin and luteolin significantly decreased cell survival more at 0.5μM concentration at both 24 hours ($p<0.01$) and 72 hours ($p<0.001$).

3.2.3 The Effects of Linarin, Luteolin, and Combined Linarin and Luteolin on the Survival Rates of RAW264.7 Cells Treated with Aβ

RAW264.7 cells were treated with 5μM Aβ for 24 hours to establish the baseline for cell survival with short term Aβ-induced cytotoxicity with a significant decrease of 38.68% from the control ($p<0.001$). Treatment of combined linarin and luteolin at 0.5μM significantly decreased cell survival 19.46% from the cells treated with Aβ ($p<0.01$). Combined linarin and luteolin at 0.5μM did not significantly decrease cell survival more than linarin individually but significantly decreased cell survival less than luteolin individually ($p<0.05$). Treatment of combined linarin and luteolin at 0.05μM significantly decreased survival 43.17% from cells treated with Aβ ($p<0.001$). Combined treatment at 0.05μM significantly decreased cell survival more than combined treatment at 0.5μM ($p<0.01$) (**Fig. 19**).

RAW264.7 cells were treated with 5μM Aβ for 72 hours to establish the baseline for cell survival with long term Aβ-induced cytotoxicity with a significant decrease of 67.60% from the control ($p<0.001$). Treatment of combined linarin and luteolin at 0.5μM significantly increased the cell survival 41.32% from the cells treated with Aβ ($p<0.001$). Combined linarin and luteolin at 0.5μM significantly increased cell survival less than linarin individually ($p<0.01$) but did not significantly increase cell survival more than luteolin individually. Treatment of combined linarin and luteolin at 0.05μM significantly increased survival 35.11% from cells treated with Aβ ($p<0.001$). Combined treatment at 0.05μM did not significantly increase cell survival less than combined treatment at 0.5μM (**Fig. 20**).

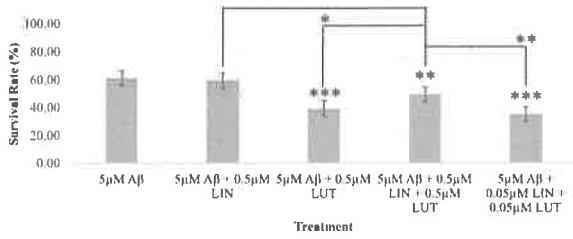

Figure 19. Survival rates of RAW264.7 cells treated with Aβ after 24 hour treatments. RAW264.7 cells treated with Aβ were treated with linarin, luteolin, combined linarin and luteolin at 0.5μM, and combined linarin and luteolin at 0.05μM for 24 hours to evaluate the potential neuroprotective effects of linarin, luteolin, and combined linarin and luteolin against Aβ-induced cytotoxicity on immune cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

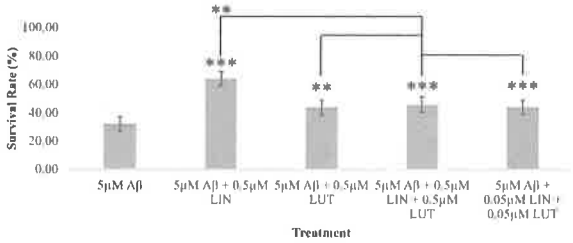

Figure 20. Survival rates of RAW264.7 cells treated with Aβ after 72 hour treatments. RAW264.7 cells treated with Aβ were treated with linarin, luteolin, combined linarin and luteolin at 0.5μM, and combined linarin and luteolin at 0.05μM for 72 hours to evaluate the potential long term neuroprotective effects of linarin, luteolin, and combined linarin and luteolin against Aβ-induced cytotoxicity on immune cells ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

3.3 Image Analysis with SK-N-SH Human Neuroblastoma Cells

3.3.1 The Effects of Linarin and Luteolin on the Cell Counts of SK-N-SH Cells and SK-N-SH Cells Treated with Aβ

The untreated SK-N-SH cells had a cell count average of 482.5 cells per 786,432.0000 square pixels, as shown in **Image A** (**Fig. 21**), which established a baseline for healthy SK-N-SH cells. Treatment of 0.1μM linarin on SK-N-SH cells for 24 hours caused the cell count to significantly decrease 12.54%, as shown in **Image B** (**Fig. 21**), from the control baseline ($p<0.05$). Treatment of 0.1μM luteolin

on SK-N-SH cells caused the cell count to significantly decrease 16.74%, as shown in **Image C** (**Fig. 21**), from the control baseline ($p<0.05$). Although luteolin caused a greater decrease in cell count than linarin, the difference was insignificant (**Fig. 22**).

The SK-N-SH cells treated with 0.5μM Aβ had a cell count average of 286.75 cells per 786,432.0000 square pixels, as shown in **Image D** (**Fig. 21**), which established a baseline for Aβ-treated SK-N-SH cells. The treatment of 0.5μM Aβ cause the cell count to significantly decrease 40.57% from the cells treated with Aβ ($p<0.001$). Treatment of 0.1μM linarin on SK-N-SH cells treated with Aβ caused the cell count to significantly increase 41.76%, as shown in **Image E** (**Fig. 21**), from the cells treated with Aβ ($p<0.05$). Treatment of 0.1μM luteolin on SK-N-SH cells treated with Aβ caused the cell count to significantly increase 37.49%, as shown in **Image F** (**Fig. 21**), from the cells treated with Aβ ($p<0.01$). Although linarin caused a greater increase in cell count than luteolin on SK-N-SH cells treated with Aβ, the difference was insignificant (**Fig. 22**).

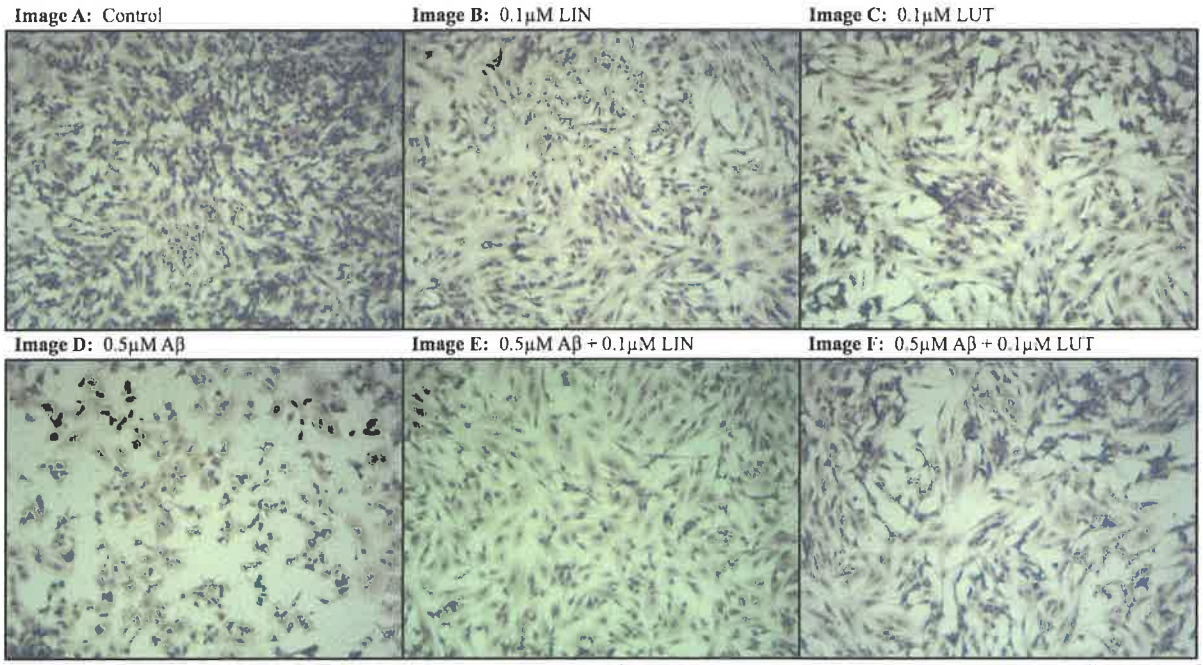

Figure 21. Images of SK-N-SH cells in a 786,432.0000 square pixel area after 24 hour treatments. SK-N-SH human neuroblastoma cells were treated with linarin and luteolin at 0.1μM concentration to test the potential effects of these substances on SK-N-SH cells and SK-N-SH cells treated with Aβ for 24 hours. Images of the treated SK-N-SH cells were taken using an electron light microscope at 100x magnification. The images were used to analyze the differentiating cell counts between the various treatments of SK-N-SH cells and SK-N-SH cells treated with Aβ for 24 hours. The control cells in **Image A** were used as a baseline to analyze the effects on normal cells of 0.1μM concentration treatment of linarin and luteolin. Treatment of 0.1μM of linarin significantly decreased the cell count of healthy cells by 12.54% ($p<0.05$), as shown in **Image B**. Treatment of 0.1μM of luteolin significantly decreased the cell count of healthy cells by 16.74% ($p<0.05$), as shown in **Image C**. Treatment of 0.5μM of Aβ significantly decreased the cell count of healthy cells by 40.57% ($p<0.001$), as shown in **Image D**. The cells treated with 0.5μM Aβ in **Image D** were used as a baseline to analyze the effects on Aβ-treated cells of 0.1μM concentration treatment of linarin and luteolin. Treatment of 0.1μM of linarin significantly increased the cell count of Aβ-treated cells by 41.76% ($p<0.05$), as shown in **Image E**. Treatment of 0.1μM of luteolin significantly increased the cell count of Aβ-treated cells by 37.49% ($p<0.01$), as shown in **Image F** (created by student researcher).

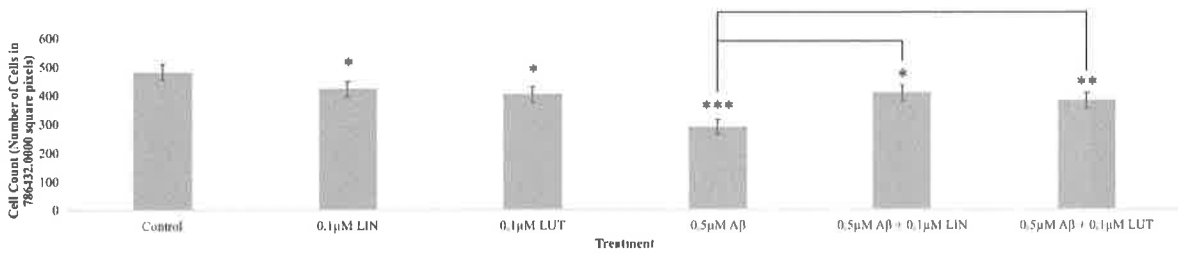

Figure 22. Cell counts of the number of SK-N-SH cells in a 786,432.0000 square pixel area after 24 hour treatments. SK-N-SH human neuroblastoma cells were treated with linarin and luteolin at 0.1μM concentration to test the potential cytotoxic effects of these substances on SK-N-SH cells and protective effects on SK-N-SH cells treated with Aβ for 24 hours ($p<0.05$*, $p<0.01$**, $p<0.001$***) (created by student researcher).

3.4 APP ELISA with SK-N-SH Human Neuroblastoma Cells

3.4.1 The Effects of Linarin and Luteolin on the Concentrations of APP in SK-N-SH cells and SK-N-SH cells Treated with Aβ

The untreated SK-N-SH cells had an APP concentration average of 5,054.83pg/mL, which established a baseline for healthy SK-N-SH cells. Treatment of 0.1μM linarin on SK-N-SH cells for 24 hours caused the APP concentration to increase 5.71% from the control baseline. Treatment of 0.1μM luteolin on SK-N-SH cells caused the APP concentration to increase 9.06% from the control baseline (**Fig. 23**).

The SK-N-SH cells treated with 0.5μM Aβ had an APP concentration average of 9,172.15pg/mL, which established a baseline for Aβ-treated SK-N-SH cells. The treatment of 0.5μM Aβ cause the APP concentration to increase 81.45% from the cells treated with Aβ. Treatment of 0.1μM linarin on SK-N-SH cells treated with Aβ caused the APP concentration to decrease 22.47% from the cells treated with Aβ. Treatment of 0.1μM luteolin on SK-N-SH cells treated with Aβ caused the APP concentration to decrease 29.49% from the cells treated with Aβ (**Fig. 23**).

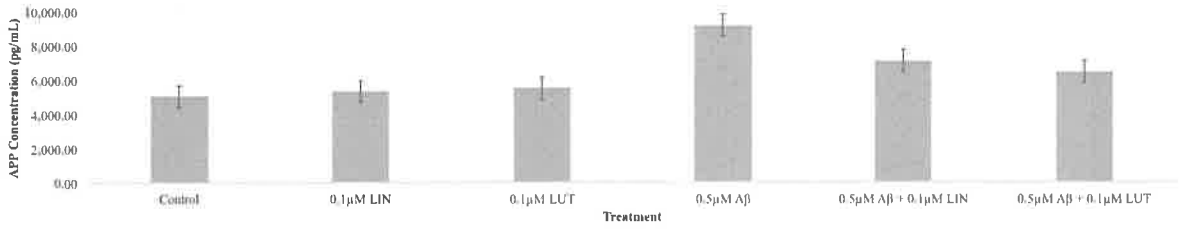

Figure 23. Concentrations of APP in pg/mL present in SK-N-SH cells after 24 hour treatments. SK-N-SH human neuroblastoma cells were treated with linarin and luteolin at 0.1μM concentration to test the potential effects of these substances on the concentrations of APP present in SK-N-SH cells and in SK-N-SH cells treated with Aβ for 24 hours (created by student researcher).

3.5 IL-1β ELISA with RAW264.7 Mouse Immune Cells

3.5.1 The Effects of Linarin and Luteolin on the Concentrations of IL-1β in RAW264.7 Cells and RAW264.7 Cells Treated with Aβ

The untreated RAW264.7 cells had an IL-1β concentration average of 5.07pg/mL, which established a baseline for healthy RAW264.7 cells. Treatment of 0.1μM linarin on RAW264.7 cells for 24

hours caused the IL-1β concentration to decrease 12.77% from the control baseline. Treatment of 0.1μM luteolin on RAW264.7 cells caused the IL-1β concentration to decrease 12.35% from the control baseline (**Fig. 24**).

The RAW264.7 cells treated with 0.5μM Aβ had an IL-1β concentration average of 7.88pg/mL, which established a baseline for Aβ-treated RAW264.7 cells. The treatment of 0.5μM Aβ cause the IL-1β concentration to increase 35.64% from the cells treated with Aβ. Treatment of 0.1μM linarin on RAW264.7 cells treated with Aβ caused the IL-1β concentration to decrease 26.91% from the cells treated with Aβ. Treatment of 0.1μM luteolin on RAW264.7 cells treated with Aβ caused the IL-1β concentration to decrease 36.61% from the cells treated with Aβ (**Fig. 24**).

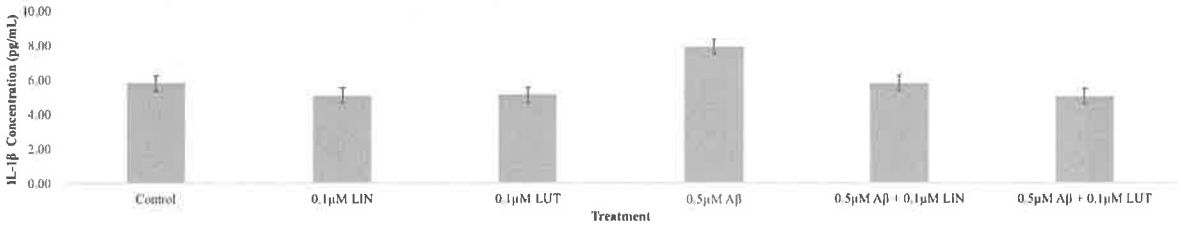

Figure 24. Concentrations of IL-1β in pg/mL present in RAW264.7 cells after 24 hour treatments. RAW264.7 mouse immune cells were treated with linarin and luteolin at 0.1μM concentration to test the potential effects of these substances on the concentrations of IL-1β present in RAW264.7 cells and in RAW264.7 cells treated with Aβ for 24 hours (created by student researcher).

4. Discussion

4.1 The Cytotoxic and Neuroprotective Against Aβ Effects of Linarin and Luteolin on GT1-7 Mouse Hypothalamic Cells and RAW264.7 Mouse Macrophage Cells

This study aimed to investigate the effects of linarin and luteolin, individually, on neuronal and immune cells, both healthy and with Aβ-induced cytotoxicity, by utilizing MTT assays. Individually, treatments of linarin and luteolin at 0.5μM were significantly decreased cell survival of GT1-7 cells and RAW264.7 cells after 24 hours and 72 hours ($p<0.01$), indicating cytotoxic effects of the substances on healthy neuronal and immune cells, although neither substance was significantly more cytotoxic than the other at either time on either cell type (**Fig. 3, 4, 5, 6, 11, 12, 13, and 14**). Both linarin and luteolin decreased cell survival more on immune cells after 72 hours, indicating the substances tend to have more cytotoxic effects on immune cells than neuronal cells (**Fig. 13 and 14**). For both cell types, only luteolin had significant effects after 24 hours ($p<0.001$) (**Fig. 4 and 12**), while linarin was more neuroprotective after 72 hours (**Fig. 5 and 13**). Treatment on neuronal cells treated with Aβ had short term and long term neuroprotective properties, treatment on immune cells treated with Aβ has short term toxic effects and long term neuroprotective properties. These results are consistent with the findings of studies that determined the ability of linarin to block Aβ-induced cytotoxicity in PC12 rat cells, thus reducing cell death associated with Aβ toxicity in a dose dependent manner (Lou et al., 2011). They are also consistent

with the findings that indicate that luteolin is able to ameliorate neurotoxicity caused by Aβ fragments through its antioxidative properties (Choi et al., 2014).

4.2 The Cytotoxic and Neuroprotective Against Aβ Effects of Combined Linarin and Luteolin on GT1-7 Mouse Hypothalamic Cells and RAW264.7 Mouse Macrophage Cells

This study aimed to investigate the effects of combined linarin and luteolin on neuronal and immune cells, both healthy and with Aβ-induced cytotoxicity, by utilizing MTT assays. Combined linarin and luteolin caused more decreases in cell survival for GT1-7 cells after 24 hours than individual treatments at 0.5μM and less than individual treatments at 0.05μM (**Fig. 7 and 8**). While both concentrations were significantly cytotoxic ($p<0.001$), neither was significantly different than individual treatments, indicating that combined linarin and luteolin does not have synergistic cytotoxic effects. Combined treatment caused significant decreases in cell survival for RAW264.7 cells after 24 hours and 72 hours ($p<0.001$), significantly more so than both individual treatments after 24 hours ($p<0.001$) but not after 72 hours (**Fig. 15, 16, 17, and 18**). This indicated that combined treatment has short term and long term cytotoxic properties on both neuronal and immune cells, although combined treatment does not have synergistic effects in comparison to individual treatments (**Fig. 7, 8, 15, 16, 17, and 18**).

Combined linarin and luteolin caused significant increases in cell survival for GT1-7 cells treated with Aβ after 24 hours and 72 hours ($p<0.001$), not significantly more so than both individual treatments at 0.5μM after 24 hours but significantly less so than linarin ($p<0.001$) and insignificantly more so than luteolin after 72 hours (**Fig. 9 and 10**). This indicates that combined linarin and luteolin does not have synergistic neuroprotective effects on neuronal cells. Combined treatment caused significant decreases in cell survival after 24 hours ($p<0.001$) and significant increases after 72 hours ($p<0.001$) for RAW264.7 cells treated with Aβ (**Fig. 19 and 20**). Combined treatment did not have synergistic neuroprotective effects on immune cells. While combined short term treatment is neuroprotective against Aβ on neuronal cells and is toxic on immune cells, combined long term treatment is neuroprotective on both types of cells against Aβ (**Fig. 9, 10, 19 and 20**). This was the first study to investigate the potential synergistic effects of linarin and luteolin as potential AD treatments by testing these substances in tandem on neuronal and immune cells. Thus, the findings of the lack of synergistic effects between linarin and luteolin of both cytotoxicity on healthy cells and neuroprotective ability against Aβ-induced cell death are a novel finding.

4.3 The Cytotoxic and Neuroprotective Against Aβ Effects of Linarin and Luteolin on SK-N-SH Human Neuroblastoma Cells

This study investigated the effects of linarin and luteolin on the cell counts of human neuronal cells by utilizing image analysis. Both linarin and luteolin at 0.1μM were significantly cytotoxic against controlled SK-N-SH cells ($p<0.05$). While luteolin caused greater decreases in cell count than linarin, the differences were not significant. Linarin and luteolin were also both significantly neuroprotective against

Aβ-induced cell death ($p<0.05$). While linarin caused greater increases in cell count than luteolin from the Aβ-treated cell count baseline, the differences were not significant. Overall, both linarin and luteolin have significant cytotoxic effects ($p<0.05$) as well as significant neuroprotective effects ($p<0.05$) against Aβ-induced cytotoxicity on human neuronal cells. In addition, neither substance had significantly greater effects than the other, cytotoxic or neuroprotective (**Fig. 22**). This indicates that linarin and luteolin make promising AD treatments against human cells, albeit they should be tested with caution in lieu of their cytotoxic effects against healthy cells. Previous studies of linarin have indicated that it is able to increase cell viability against Aβ toxicity in PC12 rat adrenal cells and previous studies of luteolin have indicated that it is able to ameliorate Aβ toxicity in mouse mixed cortical cell cultures, as well as reduce Aβ accumulation in Tg2576 mice (Lou et al., 2011; Choi et al., 2014; Kwon, 2017). The results of this study were consistent with the findings of previous studies that linarin and luteolin are able to ameliorate Aβ-induced cytotoxicity, however, this study was the first to study and compare the effects of linarin and luteolin against Aβ-induced cell death on human neuronal cells.

4.4 The Effects of Linarin and Luteolin on APP Concentration in SK-N-SH Human Neuroblastoma Cells and SK-N-SH Cells Treated with Aβ

This study investigated the effects of linarin and luteolin on the APP concentrations in human neuronal cells by utilizing a human APP ELISA. Both linarin and luteolin at 0.1μM were found to increase the concentration of APP in control SK-N-SH cells, although luteolin caused greater increases than linarin (**Fig. 23**). The increase of APP, which is a normal part of neuronal growth and repair, indicates that linarin and luteolin may also cause cell damage, which elicits a greater need for neuronal repair. Both linarin and luteolin caused decreases in APP concentration in SK-N-SH cells with Aβ-induced APP concentration increases, although luteolin caused greater decreases than linarin (**Fig. 23**). This decrease in APP from the Aβ-induced increase indicates that linarin and luteolin may ameliorate Aβ-induced cytotoxicity by decreasing the amount of the molecule that generates Aβ plaques. Luteolin may cause greater effects on the APP concentrations in human neuroblastoma cells than linarin (**Fig. 23**). Overall, this demonstrates that a possible mechanism behind the neuroprotective effects against Aβ of linarin and luteolin may be due to the effects of linarin and luteolin on the molecule that generates the Aβ precursor. These findings are consistent with the findings of studies that determined that pretreatment of linarin is able to ameliorate Aβ-induced cell death by decreasing the apoptotic rate of the cells in a neuroprotective manner against Aβ-induced increases in apoptotic death (Lou et al., 2011). This is also consistent with the findings that indicate that luteolin is able to reduce amyloidogenesis induced by APP mutations and reduce cerebral Aβ accumulation, potentially due to its antioxidative ability by dispersing Aβ plaques (Kwon, 2017; Ashaari et al., 2018).

4.5 The Effects of Linarin and Luteolin on IL-1β Concentration in RAW264.7 Mouse Macrophage Cells and RAW264.7 Cells Treated with Aβ

This study also investigated the effects of linarin and luteolin on the IL-1β concentrations in mouse immune cells. Both linarin and luteolin at 0.1μM were found to decrease the concentration of IL-1β in control RAW264.7 cells, although linarin causes greater decreases than luteolin (**Fig. 24**). The decrease of IL-1β, which is a necessary part of the inflammatory response, may indicate a decrease in the cells that produce this molecule, demonstrating the cytotoxic effects of linarin and luteolin on healthy cells. Both linarin and luteolin caused decreases in IL-1β concentration in RAW264.7 cells with Aβ-induced IL-1β concentration increases, although luteolin caused greater decreases than linarin (**Fig. 24**). This decrease in IL-1β from the Aβ-induced increase indicates that linarin and luteolin may ameliorate Aβ-induced cytotoxicity by decreasing the amount of proinflammatory cytokines produced by the inflammatory responses of the immune cells. Luteolin may cause greater effects against Aβ-induced inflammation in mouse immune cells than linarin (**Fig. 24**). Overall, this demonstrates that a possible mechanism behind the neuroprotective effects against Aβ of linarin and luteolin may be due to the effects of linarin and luteolin against the proinflammatory cytokines produced in response to the Aβ plaques. These findings are consistent with the findings of other studies that have determined that linarin has anti-inflammatory properties and is able to inhibit inflammation via the inactivation of the NF-κB pathway, MAPKs, TNF, and the PI3K/Akt pathway, as well as the downregulation of LPS-induced oxidative stress and proinflammatory cytokine release (Pan et al., 2019; Han et al., 2018). They are also consistent with another study that indicated that luteolin has an anti-inflammatory agent with the ability to suppress inflammation in mice by inhibiting cytokine expressions, NF-κB signalling, and TLR4 signaling in immune cells, as well as the ability to inhibit TNF alpha-induced and LPS-induced inflammation by inhibiting NF-κB signaling (Nabavi et al., 2015; Kwon, 2017; Ashaari et al., 2018).

5. Conclusions and Future Work

This project investigated the effects of linarin and luteolin, individually and combined, on both healthy cells and cells with Aβ-induced cytotoxicity. The effects of these substances were studied on GT1-7 mouse hypothalamic cells, RAW264.7 mouse macrophage cells, and SK-N-SH human neuroblastoma cells to investigate their effects on both neuronal cells and immune cells, as linarin and luteolin have potential as treatments for AD, a neurodegenerative disease that involves both types of cells. While luteolin has been studied on both immune and neuronal cells, linarin has been less thoroughly studied and this current project is the first to study them as combined treatments. This study thus investigated the effects of linarin and luteolin on healthy and Aβ-treated neuronal and immune cells. The impacts of these substances on the APP concentration in neuronal cells and on the IL-1β concentration in

immune cells were also investigated to gain a deeper insight into the mechanisms of the protective properties of linarin and luteolin.

Both short term and long term treatments of linarin and luteolin at both high and low concentrations caused significant cytotoxic effects on healthy neuronal and immune cells ($p<0.01$). While individual linarin and luteolin were neuroprotective against Aβ in both the short term and long term for neuronal cells, they were only neuroprotective in the long term for immune cells. Combined linarin and luteolin caused significant cytotoxic effects on both neuronal and immune cells ($p<0.001$), however, they were generally not significantly more cytotoxic than individual treatments. Combined linarin and luteolin also caused significant neuroprotective against Aβ effects on neuronal cells in both the short term and the long term ($p<0.001$), as well as on immune cells only in the long term ($p<0.001$), although the combined treatments were not significantly more neuroprotective than individual treatments. These results indicated that linarin and luteolin have demonstrated cytotoxic effects on healthy cells, which prompts the use of caution when further investigating these substances as AD treatments. While linarin and luteolin have been shown to have neuroprotective properties against Aβ that make them individual potential novel AD treatments, they have not demonstrated the synergistic effects that would have made combined treatment potentially more effective than individual treatments.

Both linarin and luteolin were significantly cytotoxic on healthy human neuronal cells ($p<0.05$) and significantly neuroprotective against Aβ-induced cytotoxicity ($p<0.05$). While both substances had significant effects, neither had significantly greater effects than the other, cytotoxic or neuroprotective. This indicated that both substances individually make promising AD treatments against Aβ for humans, albeit with caution of their potential cytotoxic effects, and neither substance currently stands to be significantly more effective than the other as treatment.

Both linarin and luteolin caused increases in APP concentration in healthy neuronal cells, indicating that they may cause damage to the cells and increase the need for neuronal repair. However, they also caused decreases in APP concentration in neuronal cells with Aβ-induced APP concentration increases, indicating that the substances may ameliorate the need for neuronal repair as well as decrease the precursor molecule to toxic Aβ plaques. In addition, both linarin and luteolin caused decreases in IL-1β concentration in healthy immune cells, indicating that they may decrease the number of cells producing cytokines and compromise the inflammatory response of the cells. However, they also caused decreases in IL-1β concentration in immune cells with Aβ-induced IL-1β concentration increases, indicating that the substances may ameliorate the inflammation caused by Aβ-toxicity by decreasing the number of proinflammatory cytokines released by Aβ presence. Overall, linarin and luteolin have been shown to cause decreases in Aβ-induced cytotoxicity and inflammation, making these substances

promising novel AD treatments against Aβ. This also indicated that linarin and luteolin cause similar effects and may potentially affect the same pathways.

Overall, the neuroprotective effects of linarin and luteolin on neuronal and immune cells with Aβ-induced cytotoxicity demonstrated these substances to be promising novel AD treatments. However, the cytotoxic effects of these substances on healthy cells also prompt caution in further investigations, as these ramifications may cause adverse side effects. In addition, linarin and luteolin have not been shown to have synergistic effects, indicating that combined treatment will not be a more effective AD treatment than individual treatments. Neither substance has been shown to be significantly more effective than the other in ameliorating Aβ-induced cytotoxicity, indicating that neither substance has been demonstrated to be a potentially more effective AD treatment. Linarin and luteolin have been shown to have neuroprotective effects against Aβ through the decrease of Aβ precursor molecule and proinflammatory cytokines, as shown by the decrease of APP and IL-1β concentrations.

In the future, this research can be expanded by testing linarin and luteolin *in vivo* with a transgenic AD *C. elegans* model to validate the neuroprotective effects of these substances within a simple living organism and can continue to be expanded by testing *in vivo* with transgenic AD mice to investigate the neuroprotective effects of these substances within a more complex living organism. By continuing to test these substances on increasingly complex organisms and validating their neuroprotective abilities against Aβ-induced toxicity, linarin and luteolin can continue to be developed towards becoming clinical drug trials, thus progressing the research of developing natural drugs to treat AD. Linarin and luteolin can also be examined further by studying their potential neuroprotective properties against additional hallmarks of AD, such as their ability to ameliorate τ protein hyperphosphorylation and inhibit AChE. This would investigate the potentials of linarin and luteolin as AD treatments against additional factors that may play a role in AD pathogenesis, which could prompt scientists to study different methods of therapy for AD to develop the most effective treatment. This research can also be expanded by further investigating other plant-based chemicals that have exhibited neuroprotective properties, in order to consider them as potential AD treatments as well. There is a wide variety of plant-based chemicals that have anti-oxidative and anti-inflammatory properties that could be studied, as individual treatments and combined treatments against AD-related factors. While linarin and luteolin have not been shown to have synergistic effects against Aβ-induced toxicity, there may be other chemicals that do produce synergistic treatment effects against AD, which could be investigated in order to develop a more effective natural treatment.

References

Alzheimer's Disease Fact Sheet. (n.d.). Retrieved August 31, 2019, from http://www.nia.nih.gov/health/alzheimers-disease-fact-sheet.

Ashaari, Z., Hadjzadeh, M.-A.-R., Hassanzadeh, G., Alizamir, T., Yousefi, B., Keshavarzi, Z., & Mokhtari, T. (2018). *The Flavone Luteolin Improves Central Nervous System Disorders by Different Mechanisms: A Review. Journal of Molecular Neuroscience, 65(4), 491–506.*

Bhullar, K. S., & Rupasinghe, H. P. V. (2013). *Polyphenols: Multipotent Therapeutic Agents in Neurodegenerative Diseases. Oxidative Medicine and Cellular Longevity, 2013, 1–18.*

Calvo-Rodríguez, M., de la Fuente, C., García-Durillo, M., García-Rodríguez, C., Villalobos, C., & Núñez, L. (2017). *Aging and amyloid β oligomers enhance TLR4 expression, LPS-induced Ca2+ responses, and neuron cell death in cultured rat hippocampal neurons. Journal of Neuroinflammation, 14(1).*

Choi, S. M., Kim, B. C., Cho, Y. H., Choi, K. H., Chang, J., Park, M. S., Kim, M. K., Cho, K. H., Kim, J. K. 2014. Effects of Flavonoid Compounds on beta-amyloid-peptide-induced Neuronal Death in Cultured Mouse Cortical Neurons. Chonnam Medical Journal, 50(2), 45-51.

Feng, X., Wang, X., Liu, Y., & Di, X. (2015). Linarin Inhibits the Acetylcholinesterase Activity In-vitro and Ex-vivo. *Iranian journal of pharmaceutical research: IJPR, 14*(3), 949–954.

Gu, S. M., Lee, H. P., Ham, Y. W., Son, D. J., Kim, H. Y., Oh, K. W., … Hong, J. T. (2018). *Piperlongumine Improves Lipopolysaccharide-Induced Amyloidogenesis by Suppressing NF-KappaB Pathway. NeuroMolecular Medicine, 20(3), 312–327.*

Heras-Sandoval, D., Pérez-Rojas, J. M., Hernández-Damián, J., & Pedraza-Chaverri, J. (2014). *The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. Cellular Signalling, 26(12), 2694–2701.*

Hooper, C., Killick, R., & Lovestone, S. (2007). *The GSK3 hypothesis of Alzheimer's disease. Journal of Neurochemistry, 104(6), 1433–1439.*

How Is Alzheimer's Disease Treated. (n.d.). Retrieved August 31, 2019, from https://www.nia.nih.gov/health/how-alzheimers-disease-treated.

Huang, W.-J., Zhang, X., & Chen, W.-W. (2016). *Role of oxidative stress in Alzheimer's disease. Biomedical Reports, 4(5), 519–522.*

Kim, S., Chin, Y.-W., & Cho, J. (2017). *Protection of Cultured Cortical Neurons by Luteolin against Oxidative Damage through Inhibition of Apoptosis and Induction of Heme Oxygenase-1. Biological and Pharmaceutical Bulletin, 40(3), 256–265.*

Kwon, Y. (2017). *Luteolin as a potential preventive and therapeutic candidate for Alzheimer's disease. Experimental Gerontology, 95, 39–43.*

Lou, H., Fan, P., Perez, R. G., & Lou, H. (2011). Neuroprotective effects of linarin through activation of the PI3K/Akt pathway in amyloid-β-induced neuronal cell death. Bioorganic & Medicinal Chemistry, 19(13), 4021–4027.

Medications for Memory. (n.d.) Retrieved August 31, 2019 from https://www.alz.org/alzheimers-dementia/treatments/medications-for-memory.

Nabavi, S. F., Braidy, N., Gortzi, O., Sobarzo-Sanchez, E., Daglia, M., Skalicka-Woźniak, K., & Nabavi, S. M. (2015). *Luteolin as an anti-inflammatory and neuroprotective agent: A brief review. Brain Research Bulletin, 119, 1–11.*

Rojo, A. I., Pajares, M., Rada, P., Nuñez, A., Nevado-Holgado, A. J., Killik, R., … Cuadrado, A. (2017). *NRF2 deficiency replicates transcriptomic changes in Alzheimer's patients and worsens APP and TAU pathology. Redox Biology, 13, 444–451.*

Silveyra, M.-X., García-Ayllón, M.-S., de Barreda, E. G., Small, D. H., Martínez, S., Avila, J., & Sáez-Valero, J. (2012). *Altered expression of brain acetylcholinesterase in FTDP-17 human tau transgenic mice. Neurobiology of Aging, 33(3), 624.e23–624.e34.*

Pan, H., Zhang, J., Wang, Y., Cui, K., Cao, Y., Wang, L., & Wu, Y. (2019). *Linarin improves the dyskinesia recovery in Alzheimer's disease zebrafish by inhibiting the acetylcholinesterase activity. Life Sciences.*

What Is Alzheimer's Disease. (n.d.). Retrieved August 31, 2019, from https://www.alz.org/alzheimers-dementia/what-is-alzheimers.