Michelle Li

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

Research Plan/Project Summary

a. RATIONALE:

Alzheimer's Disease (AD) is a progressive neurodegenerative disease associated with dementia, which is the loss of cognitive functions and behavioral abilities, such as memory loss and impaired reasoning. In the United States, AD ranks as the sixth leading cause of death (National Institute on Aging, 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 (Alzheimer's Association). Hallmarks of AD are acetylcholine (ACh) deficiency, inflammation, amyloid-β (Aβ) aggregation, tau (τ) protein hyperphosphorylation, senile plaques, and neurofibrillary tangles (NFT) (Pan et al., 2019). The Aβ peptides that help characterize AD are derived from an amyloid precursor protein (APP) via γ-secretase and β-secretase cleavage. 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). 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 (Huang et al., 2016).

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). Its anti inflammatory, acetylcholinesterase (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 is 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). Luteolin is an important flavone with antioxidative, anti cancerous, 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, as well as traditional medicinal plants (Nabavi et al., 2015). The antioxidative and neuroprotective effects of luteolin are associated with the NRF2 pathway and the upregulation of the NRF2 protein levels (Ashaari

et al., 2018). It exhibits 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). In Tg2576 mice, it has been able to reduce cerebral Aβ accumulation, 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. The effects of linarin on neuronal and immune cells have yet to be investigated directly. Therefore, this study will investigate the effects of linarin and luteolin on the survival rates of GT1-7 mouse hypothalamus cells and RAW264.7 mouse macrophage 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 interleukin-1-beta (IL-1β) concentration in RAW264.7 cells. The similar effects of these substances through varying pathways indicate 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 was 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.

b. RESEARCH QUESTION(S), HYPOTHESIS(ES), ENGINEERING GOAL(S), EXPECTED OUTCOMES:

Research Questions:

Do linarin and luteolin have neuroprotective effects against Aβ-induced cytotoxicity on neuronal and immune cells?

Do linarin and luteolin have adverse effects on neuronal and immune cells?

What are the mechanisms behind the effects of linarin and luteolin on neuronal and immune cells?

Hypotheses:

Linarin and luteolin will have neuroprotective effects on both neuronal and immune cells by ameliorating Aβ plaque toxicity and Aβ-induced inflammation.

Linarin and luteolin utilize different pathways for similar effects and will thus have synergistic neuroprotective effects when combined.

c.

Procedures:

Cell Cultures

- Culture GT1-7 mouse hypothalamic cells, RAW264.7 mouse macrophage cells, and SK-N-SH human neuroblastoma cells *in vitro* in flasks with 10mL minimum essential media (MEM) in an incubator at 37.0°C and 5.0% CO_2 concentration.
    - Purchase GT1-7 from Sigma-Aldrich.
        - GT1-7 Mouse Hypothalamic GnRH Neuronal Cell Line
        - SKU: SCC116
        - BSL-1
    - Purchase RAW264.7 from American Tissue Culture Collection (ATCC).
        - Organism: mouse
        - Cell type: macrophage; Abelson murine leukemia virus transformed
        - Tissue: Abelson murine leukemia virus-induced tumor; ascites
        - Disease: Abelson murine leukemia virus-induced tumor
        - SKU: TIB-71™
        - BSL-1
    - Purchase SK-N-SH from ATCC.
        - Organism: human
        - Tissue: brain; derived from metastatic site: bone marrow
        - Disease: neuroblastoma
        - SKU: HTB-11™
        - BSL-1

Well Plate Preparations

- Perform all well plate preparations under the fume hood and with gloves.
- Culture the cells in the flasks in the incubator. The cells will attach to the bottom of the flask.
- Discard the media in the flask but allow the cells to stay attached to the flask.

- Add 4mL trypsin protease to the flasks to detach the cells from the flask.
    - Wait for the solution to become cloudy and the flask wall to become clear.
- Add 5mL MEM to the flask, using the media to rinse the flask bottom.
- Move the cell solution into a tube.
- Centrifuge the tube at 3400 rpm for 4 minutes to create a cell pellet.
    - Discard the supernatant.
- Add 8mL of media into a new tube and add 2mL of the cell solution into the tube.
    - Mix well.
- Dispense cell solution into well plate.
    - 100μL for each well of a 96-well plate.
    - 2000μL for each well of a 6-well plate.
- Incubate the new well plates for at least 1 day to allow the cells attach to the bottom of the well.

Reagents

- Purchase linarin from Cayman Chemical Company.
- Dissolve 5mg of linarin with 5mL dimethyl sulfoxide (DMSO) to create a stock solution at 1mL concentration. Prepare dilutions of linarin at 100μM, 10μM, and 1μM concentrations using phosphate-buffered saline (PBS) by adding 50μL of linarin solution to 450μL of PBS for each subsequently lower concentration.
    - During treatments, label linarin as LIN.
    - Keep the stock solution in the freezer at -20°C and the dilutions in the refrigerator.
- Purchase luteolin from Sigma-Aldrich.
- Dissolve 5mg luteolin with 5mL DMSO to create a stock solution at 1mL concentration. Prepare dilutions of luteolin at 100μM, 10μM, and 1μM concentration using 50% DMSO and 50% distilled water by adding 100μL luteolin solution to 450μL of DMSO and 450μL of water for each subsequently lower concentration.
    - During treatments, label luteolin as LUT.
    - Keep the stock solutions and dilutions in the refrigerator.

MTT Assay

- Perform all treatments under the fume hood with gloves.
- Treat 96-well plates of cells with linarin, luteolin, and combined linarin and luteolin.
    - Test various concentrations of linarin and luteolin: 0.5μM and 0.05μM.
        - Calculate concentrations based on concentration of treatment, amount of treatment added, and amount of cell solution in well.

- Test GT1-7 and RAW264.7 to study neuronal and immune cells
- Test effects on normal cells and cells treated with Aβ.
- Test short term with 24 hours and long term with 72 hours.

- Follow tables regarding treatments on 96-well plates:

Treatment for GT1-7 Cells for 24 Hours		
Row	**Treatments Added**	**Final Treatment Concentration**
1	None	Control
2	5uL LIN x1000	0.05μM LIN
3	5uL LIN x100	0.5μM LIN
4	5uL LUT x1000	0.05μM LUT
5	5uL LUT x100	0.5μM LUT
6	5uL AB x10	5μM Aβ
7	5uL AB x10 + 5uL LIN x100	5μM Aβ + 0.5μM LIN
8	5uL AB x10 + 5uL LUT x100	5μM Aβ + 0.5μM LUT
9	5uL AB x10 + 5uL LIN x100 + 5uL LUT x100	5μM Aβ + 0.5μM LIN + 0.5μM LUT
10	5uL AB x10 + 5uL LIN x1000 + 5uL LUT x1000	5μM Aβ + 0.05μM LIN + 0.05μM LUT
11	5uL LIN x100 + 5uL LUT x100	0.5μM LIN + 0.5μM LUT
12	5uL LIN x1000 + 5uL LUT x1000	0.05μM LIN + 0.05μM LUT

Treatment for GT1-7 Cells for 24 Hours		
Row	**Treatments Added**	**Final Treatment Concentration**
1	None	Control
2	5uL AB x10	5μM Aβ
3	5uL LIN x1000	0.05μM LIN
4	5uL AB x10 + 5uL LIN x1000	5μM Aβ + 0.05μM LIN
5	5uL LUT x1000	0.05μM LUT
6	5uL AB x10 + 5uL LUT x1000	5μM Aβ + 0.05μM LUT
7	5uL AB x10 + 5uL LIN x1000 + 5uL LUT x1000	5μM Aβ + 0.05μM LIN + 0.05μM LUT

Treatment for RAW264.7 Cells for 24 Hours		
Row	**Treatments Added**	**Final Treatment Concentration**
1	None	Control
2	5uL LIN x1000	0.05μM LIN

3	5uL LIN x100	0.5μM LIN
4	5uL LUT x1000	0.05μM LUT
5	5uL LUT x100	0.5μM LUT
6	5uL AB x10	5μM Aβ
7	5uL AB x10 + 5uL LIN x100	5μM Aβ + 0.5μM LIN
8	5uL AB x10 + 5uL LUT x100	5μM Aβ + 0.5μM LUT
9	5uL AB x10 + 5uL LIN x100 + 5uL LUT x100	5μM Aβ + 0.5μM LIN + 0.5μM LUT
10	5uL AB x10 + 5uL LIN x1000 + 5uL LUT x1000	5μM Aβ + 0.05μM LIN + 0.05μM LUT
11	5uL LIN x100 + 5uL LUT x100	0.5μM LIN + 0.5μM LUT
12	5uL LIN x1000 + 5uL LUT x1000	0.05μM LIN + 0.05μM LUT

Treatment for RAW264.7 Cells for 74 Hours		
Row	**Treatments Added**	**Final Treatment Concentration**
1	None	Control
2	5uL LIN x1000	0.05μM LIN
3	5uL LIN x100	0.5μM LIN
4	5uL LUT x1000	0.05μM LUT
5	5uL LUT x100	0.5μM LUT
6	5uL AB x10	5μM Aβ
7	5uL AB x10 + 5uL LIN x100	5μM Aβ + 0.5μM LIN
8	5uL AB x10 + 5uL LUT x100	5μM Aβ + 0.5μM LUT
9	5uL AB x10 + 5uL LIN x100 + 5uL LUT x100	5μM Aβ + 0.5μM LIN + 0.5μM LUT
10	5uL AB x10 + 5uL LIN x1000 + 5uL LUT x1000	5μM Aβ + 0.05μM LIN + 0.05μM LUT
11	5uL LIN x100 + 5uL LUT x100	0.5μM LIN + 0.5μM LUT
12	5uL LIN x1000 + 5uL LUT x1000	0.05μM LIN + 0.05μM LUT

- Incubate cell plates for designated treatment times at 37.0°C and 5.0% CO_2 concentration.
- Add 10μL yellow MTT dye per well.
    - Incubate for 2 hours to allow MTT to undergo reaction from yellow to purple.
- Add 70μL DMSO per well.

- Allow to sit for 10 minutes to allow DMSO to break up cell membrane and dissolve purple MTT formazan.

- Analyze cell plate with iMark™ Microplate Absorbance Reader set to a wavelength of 595nm and collect data with the Microplate Manager 6 Software.
  - Transfer data to an Excel spreadsheet.

Image Analysis

- Perform all treatments under the fume hood with gloves.
- Treat 6-well plate with linarin and luteolin
  - Test SK-N-SH to study human neuronal cells
  - Test effects on normal cells and cells treated with Aβ
- Follow table regarding treatments on 6-well plates:

Treatment for SK-N-SH Cells for 24 Hours		
Well	**Treatments Added**	**Final Treatment Concentration**
1	None	Control
2	10uL AB x10	0.5μM Aβ
3	20uL LIN x100	0.1μM LIN
4	10uL AB x10 + 20uL LIN x100	0.5μM Aβ + 0.1μM LIN
5	20uL LUT x100	0.1μM LUT
6	10uL AB x10 + 20uL LUT x100	0.5μM Aβ + 0.1μM LUT

- Incubate cell plate for 24 hours at 37.0°C and 5.0% CO_2 concentration.
- Remove cell media from well plate.
  - Be careful not to disturb the cell culture on the bottom of the wells.
- Add 1mL of hema 3 fixation, a light blue solution, to each well.
  - Allow to sit for 1 minute.
  - Remove hema 3 fixation.
- Add 1mL of hematoxylin stain, a dark purple solution, to each well.
  - Allow to sit for 2 minutes.
  - Remove hematoxylin.
- Use distilled water to gently rinse the excess stains from the wells.
- Plate well plate under an electron light microscope set to 100x magnification.

- Link microscope to computer with ScopeImage 9.0 application to take pictures of cell cultures.
- Take 4 pictures of different areas of the well for each well.

- Analyze images of cells using ImageJ application.
  - Use Image-based Tool for Counting Nuclei (ITCN) plug-in set to width of 40 pixels and minimum distance of 20.0 pixels.
  - Count cells in pictures and record on Excel sheet.

ELISA

- Perform all treatments under the fume hood with gloves.
- Treat 6-well plates
  - Test SK-N-SH and RAW264.7 to study neuronal and immune cells
  - Test effects on normal cells and cells treated with Aβ
- Follow tables regarding treatments on 96-well plates:

Treatment for SK-N-SH Cells for 24 Hours		
Well	**Treatments Added**	**Final Treatment Concentration**
1	None	Control
2	10uL AB x10	0.5μM Aβ
3	20uL LIN x100	0.1μM LIN
4	10uL AB x10 + 20.uL LIN x100	0.5μM Aβ + 0.1μM LIN
5	20uL LUT x100	0.1μM LUT
6	10uL AB x10 + 20.uL LUT x100	0.5μM Aβ + 0.1μM LUT

Treatment for RAW264.7 Cells for 24 Hours		
Well	**Treatments Added**	**Final Treatment Concentration**
1	None	Control
2	10uL AB x10	0.5μM Aβ
3	20uL LIN x100	0.1μM LIN
4	10uL AB x10 + 20uL LIN x100	0.5μM Aβ + 0.1μM LIN
5	20uL LUT x100	0.1μM LUT
6	10uL AB x10 + 20uL LUT x100	0.5μM Aβ + 0.1μM LUT

- Incubate cell plate for 24 hours at 37.0°C and 5.0% CO_2 concentration.
- Remove cell media from the plates.
- Add 1mL of distilled water to each well.
    - Put cell plates through 4 freeze-thaw cycles to break up the cell membranes.
- Scrape well bottoms and transfer all the cell solutions into a separate tube for each well.
- Prepare ELISA test according to the kit instructions.
    - Human APP ELISA and Human IL-1β ELISA kits purchased from Boster Biological Technology Co., Ltd..
- Centrifuge cell solutions from the cell plate and add the supernatants to the 96 well plates from the ELISA kits.
    - Use SK-N-SH cells for APP ELISA and RAW264.7 cells for IL-1β ELISA.
- Continue to follow ELISA kit instructions for several washes to remove unbound material from the plate.
- Analyze the well plates with iMark™ Microplate Absorbance Reader and collect data with the Microplate Manager 6 Software.
    - Transfer data to an Excel spreadsheet.
- Analyze the standard curve of optical density vs. concentration. Compare the collected data to the standard curve to calculate quantitative measurements of antigens in the samples.

Risk and Safety:

- All cells are Biosafety Level 1.
    - All treatments involving cells must be performed under a fume hood with gloves and with supervision by mentor.
    - The fume hood surfaces must be cleaned with ethanol or bleach before and after experimentation.
    - Cell culture materials must be disposed of through the biohazard container.

Data Analysis:

- All data will be presented as mean ± standard error (SEM). The error bars of the charts will represent standard error.

- Two-tailed student's t-tests with $p<0.05$ indicating the threshold for significant will be performed. Significance will be denoted on figures using asterisks ($p<0.05$*, $p<0.01$**, $p<0.001$***), when applicable.
- The percentage of change in comparison to the control, as well as the percentage of change in comparison to the Aβ-treated baselines, will be calculated. These will be used to compare the effects of the treatments, when applicable.

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1. Human participants research:

- N/A

2. Vertebrate animal research:

- N/A

3. **Potentially hazardous biological agents research:**

- All cells are established cells lines and biosafety level 1.
    - GT1-7 was purchased from Sigma-Aldrich.
        - GT1-7 Mouse Hypothalamic GnRH Neuronal Cell Line
        - SKU: SCC116
    - RAW264.7 was purchased from American Tissue Culture Collection (ATCC).
        - Organism: mouse
        - Cell type: macrophage; Abelson murine leukemia virus transformed
        - Tissue: Abelson murine leukemia virus-induced tumor; ascites
        - Disease: Abelson murine leukemia virus-induced tumor
        - SKU: TIB-71™
    - SK-N-SH was purchased from ATCC.
        - Organism: human
        - Tissue: brain; derived from metastatic site: bone marrow
        - Disease: neuroblastoma
        - SKU: HTB-11™
- All treatments involving cells must be performed under a fume hood with gloves and with supervision by mentor.
- The fume hood surfaces must be cleaned with ethanol or bleach before and after experimentation.
- Cell culture materials must be disposed of through the biohazard container.

4. **Hazardous chemicals, activities, devices:**

- All treatments involving chemicals must be performed under a fume hood with gloves and with supervision by mentor.
- All chemicals will be involved in cell culture treatment. All cell culture materials will be disposed through a biohazardous container.
- Linarin:
    - From Cayman Chemical Company.
    - Adverse human health effects and symptoms:
        - May be irritating to the mucous membranes and upper respiratory tract.
        - May be harmful by inhalation, ingestion, or skin absorption.
        - May cause eye, skin, or respiratory system irritation.
        - Toxicological properties have not been thoroughly investigated.

    - Precautions to be taken in handling:
        - Avoid breathing dust/fume/gas/mist/vapour/spray.
        - Avoid prolonged or repeated exposure.
- Luteolin:
    - From Sigma-Aldrich.
    - Not a hazardous substance or mixture.
    - First aid measures:
        - Move out of dangerous area.
        - If inhaled, move person into fresh air. If not breathing, give artificial respiration.
        - In case of skin contact, wash off with soap and water.
        - In case of eye contact, flush eyes with water.
        - If swallowed, rinse mouth with water.
- Dimethyl sulfoxide (DMSO):
    - From Sigma-Aldrich
    - Flammable liquid (Category 4).
        - Combustible liquid.
        - Keep away from heat/sparks/open flames/hot surfaces. No smoking.
        - In case of fire, use dry sand, dry chemical, or alcohol-resistant foam for extinction.
    - Rapidly absorbed through skin.
    - Indication of any immediate medical attention and special treatment needed:
        - No data available.
    - Handling:
        - Avoid contact with skin and eyes.
        - Avoid inhalation of vapour or mist.
        - Keep away from sources of ignition. No smoking.
- Phosphate buffered saline (PBS):
    - Not a hazardous substance or mixture.
- Tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide:
    - GHS Classification:
        - Skin irritation (Category 2).
        - Eye irritation (Category 2A).
        - Germ cell mutagenicity (Category 2).

        - Specific target organ toxicity - single exposure (Category 3), Respiratory system
    - Warning:
        - Causes skin irritation.
        - Causes serious eye irritation.
        - May cause respiratory irritation.
        - Suspected of causing genetic defects.
- Hema 3 Fixation:
    - From Sigma-Aldrich.
    - Flammable liquid (Category 2).
    - Acute oral toxicity (Category 3).
    - Acute dermal toxicity (Category 3).
    - Acute inhalation toxicity - vapors (Category 3).
    - Specific target organ toxicity - single exposure (Category 1).
- Hematoxylin:
    - From Sigma-Aldrich.
    - Not a hazardous substance or mixture.