Naringenin's Mitigation of Rotenone-Induced Cytotoxicity: a Potential New Candidate for Treating Parkinson's Disease

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***Abstract* — In the past five years, the prevalence of Parkinson's disease (PD) has increased by over 50% with an estimated 10 million people affected worldwide. Alpha-synuclein (αS) expression is a major hallmark of PD and has been linked to the downregulation of mitochondrial complex 1, neuronal death, and sporadic PD. Recent research has focused on polyphenols as potential targets for developing new treatments for PD because of their antioxidant properties. This study investigates the potential of the polyphenol naringenin to decrease αS expression, which would increase dopamine release in rotenone-induced PD. We tested naringenin against rotenone-induced cytotoxicity through caspase assays, colorimetric assays, and ELISA assays. We found that naringenin was able to mitigate both caspase activity and cytotoxicity in HTB-11 and U937 cells, representing neuronal and immune cells respectively. These findings suggest that naringenin has a protective effect on cells challenged by rotenone, potentially capable of slowing down the progression of PD.**

I. Introduction

Parkinson's Disease (PD) is a neurodegenerative disease caused by the selective degeneration of dopaminergic (DA) neurons in the substantia nigra of the midbrain [1]. The degeneration of DA neurons in PD is primarily due to apoptosis, with mitochondrial toxins exacerbating this process [4]. Important characteristics of PD include the appearance of alpha-synuclein (αS) and Lewy bodies in the neurons present in the substantia nigra pars compacta of the brain, which induces down-regulation of mitochondrial complex 1 and increased amount of reactivated oxygen species (ROS) [2].

Polyphenols are a diverse group of naturally occurring compounds found in various plant-based foods. Their antioxidant and anti-inflammatory effects make them potential therapeutic targets. These compounds are neutralizers of free radicals and protect cells from the oxidative damage implicated in numerous diseases, including cardiovascular conditions, neurodegenerative disorders, and cancer [5].

Naringenin, a polyphenol in the class of flavonoid, has emerged as a promising treatment option for PD. Scientists have demonstrated its ability to stimulate DA neurons and promote the production of glial cell line-derived neurotrophic factor (GDNF), supporting neuron growth and survival [8]. These studies also suggest that naringenin protects against dopaminergic neurodegeneration and oxidative damage by activating cellular defense mechanisms and inhibiting apoptotic pathways [8]. In addition, it has the ability to enhance the release of neurotrophic factors from astroglial cells, protect dopaminergic cells, improve motor skills, and reduce oxidative stress and neuroinflammation associated with PD [8]. However, these studies often investigate the effects of naringenin on neurons in general, not in the context of specific neurodegenerative diseases like PD.

The isoflavone rotenone is a naturally derived compound often used as pesticide and obtained from several plant species. Due to its lipophilic properties, it is able to inhibit mitochondrial complex 1 as well as the mitochondrial electron transport chain by crossing the blood brain barrier [11]. It has been found to kill DA neurons in a dose- and time-dependent manner by dramatically enhancing the release of lactate dehydrogenase (LDH) into the culture medium [6]. Additionally, rotenone can increase hallmarks of PD, such as αS expression, Lewy bodies, and dose-dependent ATP depletion. This means that rotenone plays a significant part in inducing PD through the creation of αS.

As mentioned above, flavonoids, as scavengers for highly reactive species such as free radicals, can increase oxidative damage and limit the expression of oxidative reactions. Naringenin in particular is able to mitigate mitochondrial oxidative stress damage, which plays an important role in neurodegeneration in PD [9]. On the other hand, rotenone was found to induce mitochondrial ROS production in isolated HL-60 cell mitochondria, primarily by blocking mitochondrial respiratory chain complex I [10]. If naringenin scavenged free radicals through mitochondrial complex 1, providing protection from rotenone, there is a possibility that it can lessen rotenone's impact in rotenone-induced PD.

In addition, we are interested in comparing the effects of naringenin to cannabidiol (CBD), as it is an established promising treatment for PD with anti-inflammatory and antioxidant effects [3]. CBD has been shown to improve motor symptoms related to PD and counter abnormal signaling in the PD-affected brain, playing a crucial role in the basal ganglia circuitry [3]. Studies have also demonstrated CBD's neuroprotective effects against rotenone -induced neuronal damage in primary cultures through its induction of cell stress and inhibition of the heme oxygenase (HO) reaction [7]. By inhibiting the HO reaction, CBD may enhance the cell stress response and confer full protection against oxidative stress in vivo [7].

We hypothesize that naringenin mitigates rotenone-induced immune and neuronal cell death through mitochondrial complex 1 at certain concentrations and can also reverse the effects of rotenone cytotoxicity. We wish to compare the effects of naringenin on neuronal and immune cells to that of CBD. To do so, we will conduct *In Silico* screening that determines the binding affinity of naringenin, rotenone and CBD to mitochondrial complex 1, and colorimetric assays that determine cell survival and cytotoxicity levels after treatment. We will also conduct the caspase assay which determines if naringenin and CBD are

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able to induce caspase mediated apoptosis, and the ELISA assay that determines the change in αS levels. A marker that will be used in these experiments are the caspases and endoproteases that are central to both intrinsic (mitochondria-mediated) and extrinsic (cell death receptor-mediated) apoptotic pathways, which have been shown to play a role in neuronal cell death. As apoptosis is dependent on caspases, new approaches to slowing the progression of neurodegenerative diseases have focused on caspase inhibition [4].

II. Materials and Methods

We used the virtual screening software Python Prescription (PyRx) to model the interactions between naringenin, CBD, and rotenone with mitochondrial complex 1 to determine if they intersect. CBD, rotenone and naringenin were all downloaded from PubChem in SDF 2D format. They were then converted into PDB format using the Online SMILES Translator and Structure File Generator. Then, the macromolecule (mitochondrial complex 1) was downloaded from the Protein Data Bank in PDB format. Both the ligands and the macromolecule were then input into the PyRx software, which processed the results. Finally, the Vina Wizard conducted a docking analysis within the PyRx workspace, and binding affinities (kcal/mol) and the upper and lower bond of the root-mean-square deviation (RMSD) were calculated.

The two cell lines used were u937 cells, which represented immune cells, and HTB-11 cells, which represented neuronal cells. Both cell lines were purchased from the American Type Culture Collection (ATCC), in Manassas. They were stored using a cell culture incubator, and incubated in an atmosphere of 5% CO2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assays were used to measure the cell proliferation rate, cytotoxicity, and quantification of cell growth and viability under treatment. Lactate Dehydrogenase (LDH) colorimetric assays additionally determined the percentage of damaged cells in a given sample. For both of the assays, the cell samples were placed in 96 well plates. The first row of the plate was set as the control, and the next three were treated with only naringenin at concentrations of 0.1uM, 1 uM and 10uM. The fifth row was treated with rotenone at concentrations of 1uM and 50uM. The last three rows were treated with a combination of both rotenone and concentrations of 0.1uM, 1 uM and 10 uM of naringenin. The cells were then incubated for 24 hours. This process was repeated with CBD for a MTT assay, where the first row of the plate was the control, the second was rotenone, the third was rotenone with CBD at 10uM, then rotenone combined with both CBD and naringenin at 10uM, then rotenone and naringenin at 10uM, and lastly, just CBD.

To calculate the percent change of the change in cell survival (viability percentage) for all the MTT assays, we used this formula:

$$-100 \times \frac{sample\ cell\ survival - control\ cell\ survival}{control\ cell\ survival}$$

In order to calculate the percent cytotoxicity compared to the original value of rotenone for the LDH assays, we used this formula:

$$100 \times \frac{sample\ released\ LDH - ROT\ released\ LDH}{sample\ total\ LDH - control\ released\ LDH}$$

To compare the means between the control group and various treatment samples, multiple T-Tests were performed. The p-values were then calculated using significance levels p=0.05, p=0.01, and p=0.001.The error bars on the graphs indicate standard deviations.

The Caspase-3 Colorimetric Assay was conducted to measure Caspase-3's activities to assess whether rotenone induces apoptosis. A chromophore p-nitroaniline (pNA) solution was added, which causes a deeper color change if there is more caspase and a lighter color change if there is less caspase. Following the cell culture procedures, cells were transferred to a six-well plate, and treated with their respective treatments (add 5 uL of NAR 100 uM, and NAR 10 uM to the wells). Then, we incubated the cells for 24 hours, after which the cells were collected in 1.5 ml tubes with 50ul of lysis buffer in each tube. Following the protocol of the assay kit provider (Caspase Colorimetric Apoptosis Assay, 2019), 50ul of assay buffer, 45 ul of lysis buffer, and 5ul of cell lysate were added to each well of a 96-well plate. Then, we added 5 uL of the pNA substrate solution, mixed the contents in each well, and took readings of the amount of absorbency at 0 minutes, 15 minutes, 45 minutes, and 1 hour with a microplate reader (iMark, USA) at 415 nm. Finally, we calculated the percent change of the caspase activity over the hour by this formula:

$$100 \times \frac{sample\ average\ absorbency - control\ average\ absorbency}{control\ average\ absorbency}$$

We measured αS and phosphorylated alpha-synuclein (p-Ser129) concentrations through an enzyme-linked immunosorbent assay (ELISA) to determine if either naringenin or CBD has an effect on the neuronal apoptosis caused by rotenone. p-Ser129 is often regarded as a helpful biomarker and also causes cognitive and motor dysfunction. A graph was generated to compare both the αS and p-Ser129 levels between the control and treatment groups.

III. Results

Molecular dockings of rotenone (ROT), naringenin (NAR), and cannabidiol (CBD) with the bovine mitochondrial cytochrome bc1 complex (1L0L) macromolecule found that all three ligands possess significant binding potential to mitochondrial complex 1

(Table 1), suggesting a strong bond between the ligands and the macromolecules in each pair.

Ligand	Macromolecule	Binding Affinity
NAR	1L0L	-9.1
ROT	1L0L	-8.9
CBD	1L0L	-8.8

Table 1. Binding Affinities of NAR, ROT and CBD molecularly docked to 1L0L assessed using PyRx showing strong bonds between the ligands and the macromolecules.

We also found that both naringenin and rotenone interact with mitochondrial complex 1 at approximately the same place (Figure 1), suggesting that naringenin can compete with rotenone at the binding site. Since rotenone causes apoptosis when bound to mitochondrial complex 1, we conclude that naringenin mitigates rotenone-induced apoptosis through interactions with mitochondrial complex 1.

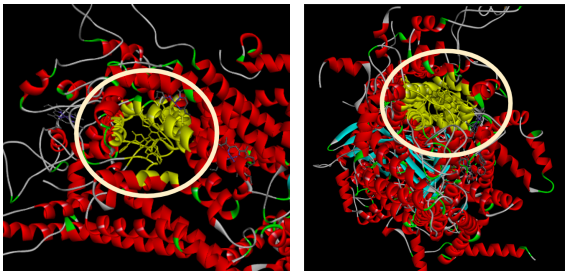

Figure 1. Interactions between mitochondrial complex 1, naringenin and rotenone. Left Panel: The area where mitochondrial complex 1 and rotenone intersect. Right Panel: The area where mitochondrial complex 1 and naringenin intersect

Next, we performed a MTT assay that examined u937 cells, which represent immune cells, and the amount of rotenone-induced cytotoxicity that different concentrations of naringenin were able to mitigate. We found that cells that were treated with 0.1uM naringenin significantly ($p<0.05$) reduced the increase of cytotoxicity caused by rotenone (Figure 2). Compared to the control, cells at 0.1uM naringenin + rotenone displayed a 7.6% increase in cytotoxicity, while rotenone combined with 1uM and 10uM of naringenin displayed greater increases of cytotoxicity (compared to the control) of 29.1% and 33.7%, similar to the 33.1% increase exhibited by cells with rotenone alone added (Figure 2). The results suggest that the concentration of 0.1uM of naringenin offers the best protective effects against the cytotoxicity induced by rotenone on u937 cells.

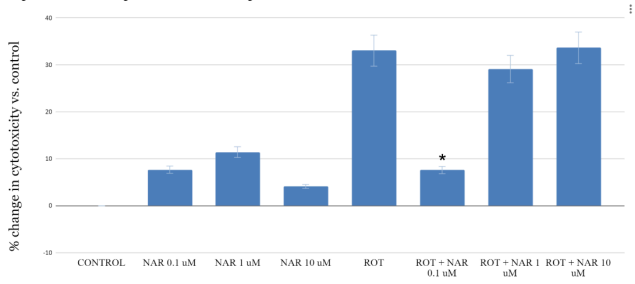

Figure 2. Naringenin significantly mitigated rotenone caused reduction of cell survival by 25.5% ($p<0.05$) at a concentration of 0.1 uM in u937 cells compared to original rotenone levels. *=$p<0.05$, NAR=naringenin, ROT = rotenone

A MTT assay was then conducted for the HTB-11 cells which represent neurons. We found that in cells incubated with rotenone, the treatment of 10uM naringenin significantly ($p<0.05$) mitigated rotenone-induced reduction of cell survival (Figure 3). Compared to the control, cells that were treated with 10uM naringenin + rotenone had a viability percentage (cell survival rate) of 90.6%, while rotenone combined with 0.1uM or 1uM of naringenin displayed viability percentage increases of 55.7% and 58.2%, even less than that exhibited by cells treated only with rotenone. The results suggest that a concentration of 10uM of naringenin + rotenone gives HTB-11 cells the best protective effects against rotenone on cell death.

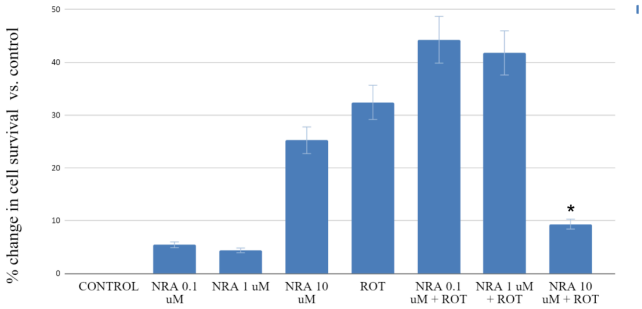

Figure 3. Naringenin significantly mitigated rotenone caused reduction of cell survival by 23.0% ($p<0.05$) in HTB-11 cells at 10uM compared to original ROT levels. *=$p<0.05$, NRA=naringenin, ROT = rotenone

After that, HTB-11 cells were treated with naringenin and rotenone at different concentrations in a LDH assay. We found that cells at 10uM naringenin + rotenone had a 42.2% increase in cytotoxicity compared to the original value of rotenone (Figure 4), which corresponds with the results obtained from our MTT assay with HTB-11 cells (Figure 3). Among the p-values collected from this data, only the results for naringenin and rotenone at 10uM were significant ($p<0.001$). The other p-values were all greater than 0.5, showing that naringenin didn't cause a significant amount of LDH to be released from the cells at NRA 0.1uM and 1uM.

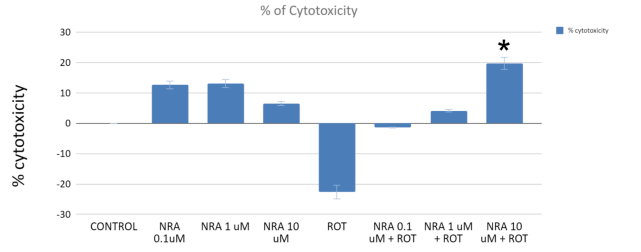

Figure 4. Naringenin at a concentration of 10uM significantly mitigated rotenone caused cytotoxicity by 42.2% ($p<0.001$) in HTB-11 cells. *=$p<0.001$ compared to ROT

An ELISA assay was used to monitor the amount of αS in cultured cells. We found that after treating the cells at

higher concentrations of naringenin, such as 10uM, there is a downregulation of αS expression compared to the control (Figure 5). Moreover, the percent change in αS for rotenone and naringenin at 100uM was 1.73% ($p < 0.001$) and 0.64% with rotenone and CBD at 100uM ($p < 0.05$). These are all significant given that the p-values compared to the original amount of rotenone are all less than 0.05. Similarly, the change in phosphorylated alpha-synuclein (p-Ser129) for rotenone and naringenin at 100uM was 0.49% ($p < 0.001$) and 0.14% with rotenone and CBD at 1000uM ($p < 0.01$). Lastly, the percent change in p-Ser129 for rotenone and CBD at 100uM is 0.13% ($p < 0.001$). These are also all significant, since the p-values compared to the control are all less than 0.05 (Figure 5). We observed that the percent change in both αS and p-Ser129 decreases as the concentration of naringenin increases in the presence of rotenone, suggesting that high concentration of naringenin is beneficial for decreased apoptosis of DA neurons and less expression of both αS and p-Ser129. Finally, the percent change in p-Ser129 decreases as the concentration of rotenone and CBD decreases, while the percent change in αS increases as the same concentration decreases. This suggests that naringenin is a more efficient treatment at mitigating αS expression in rotenone-induced PD.

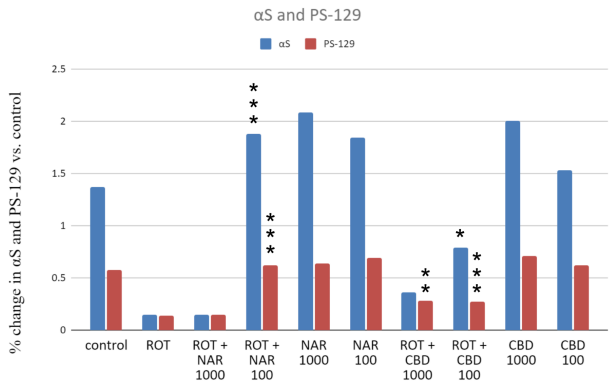

Figure 5. At a concentration of 100uM, naringenin also increased the amount of αS by 1.73% ($p<0.001$) and p-Ser129 by 0.49% ($p<0.001$). At a concentration of 1000uM of cannabidiol, the amount of p-Ser129 increased by 0.14% ($p<0.01$), and at a concentration of 100uM, the amount of αS increased by 0.64% ($p<0.05$) and p-Ser129 increased by 0.13% ($p<0.001$). *=$p<0.05$ compared to original amount of rotenone; **=$p<0.01$ compared to original amount of rotenone; ***=$p<0.001$ compared to original amount of rotenone. CBD = cannabidiol

From the Caspase-3 Colorimetric Assay, we found that 10uM of naringenin and rotenone increased caspase activity by 60% (Figure 6), which is a 22.6% decrease from caspase assay when treated with only rotenone. Additionally, naringenin and rotenone together at 100uM increased caspase activity by about 51% (Figure 6), which is a 30.6% decrease from only rotenone. The percent change of caspase activity increases as the concentration of rotenone and naringenin decreases, suggesting an inverse relationship between caspase activity and concentration of naringenin. As caspase activity levels are directly correlated to apoptosis rates, this means that the more naringenin was present, the less apoptosis occurred.

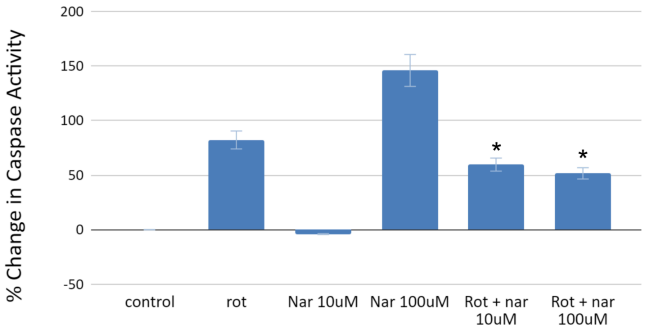

Figure 6. At a concentration of 100uM, naringenin mitigated rotenone-induced caspase activity by 30.6% ($p<0.05$), and at a concentration of 10uM, caspase activity was mitigated by 22.6% ($p<0.05$). *=$p<0.05$ compared to rotenone.

Finally, we compare the effects of CBD and naringenin in the presence of rotenone. The addition of rotenone at 10uM increased cytotoxicity in the cells by 54.58% (Figure 7). 10uM of CBD decreased % change in cytotoxicity to 34.40%, while 10uM of naringenin decreased cytotoxicity levels to 45.65% (Figure 7). Most noticeably, CBD and naringenin together decreased cytotoxicity to only 6.48%, much lower than any of the two used alone, suggesting a potential beneficial effect of combined treatment (Figure 7).

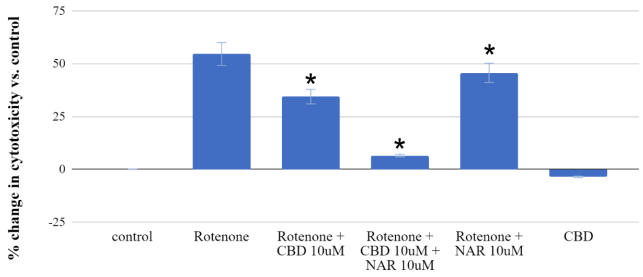

Figure 7. At a combined concentration of 10uM of cannabidiol and 10uM of naringenin in HTB-11 cells, rotenone-induced caspase activity was mitigated by 28.0% ($p<0.05$). *=$p<0.05$ compared to rotenone

IV. Conclusions

This research highlights the positive effects of naringenin towards decreasing rotenone-induced cytotoxicity.

In silico screening revealed strong binding potential of rotenone, naringenin, and cannabidiol to mitochondrial complex 1, indicating naringenin's ability to compete with rotenone at the binding site and mitigate rotenone-induced apoptosis. We proposed a model where naringenin and rotenone are observed to be able to interact at mitochondrial complex 1, where naringenin is able to scavenge the free radicals that rotenone produces.

In examining u937 immune cells through the MTT assay, we found that 0.1uM naringenin significantly reduced rotenone-induced cytotoxicity, indicating that this concentration of naringenin provides the most effective

protection against rotenone-induced cytotoxicity in u937 cells.

In HTB-11 cells which represent neurons, the MTT assay demonstrated that treatment with 10uM naringenin significantly mitigated rotenone-induced reduction of cell survival, providing the most effective protection against cell death compared to lower concentrations of naringenin.

Treatment of HTB-11 cells for an LDH assay showed that cells treated with 10uM naringenin + rotenone showed a significant increase in released cytotoxicity, indicating that lower concentrations of naringenin did not significantly increase LDH release from the cells.

The ELISA assay showed that higher concentrations of naringenin, particularly 10uM, reduced αS expression compared to the control, leading to a reduction in neuronal apoptosis. αS expression is also significantly decreased in both αS and phosphorylated alpha-synuclein (p-Ser129) levels compared to rotenone at 100uM and CBD at 100uM, showing an inverse relationship between rotenone/naringenin concentration and their effects on αS and p-Ser129 expression.

A similar inverse relationship was found between caspase activity and the concentration of naringenin through the caspase assay, which also suggests that higher concentrations of naringenin led to reduced caspase activity and thus, reduced apoptosis rates.

Finally, we observed that naringenin at 10uM and CBD at 10uM combined offer the best protection against rotenone-induced cytotoxicity, reducing cytotoxicity from 54.58% to 6.48%. The combined positive protective effects are much more pronounced than CBD or naringenin used alone

Our results have significant implications for rotenone-induced PD treatment, as they demonstrate that naringenin offers neuroprotective effects against rotenone-induced cytotoxicity, and this effect can be maximized when combined with CBD. Although the specific molecular pathways involved remain unclear, future mechanistic studies could investigate the downstream signaling cascades, oxidative stress markers, and apoptotic pathways, and how they contribute to the neuroprotective effects of naringenin. The current study primarily focused on in vitro cell culture models. Future research could involve in vivo studies using animal models of Parkinson's disease to continue to evaluate the neuroprotective effects of naringenin, which would provide a more comprehensive understanding of the potential therapeutic benefits of naringenin in a physiological context.

V. References

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