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Chemical Determinants of Pro-oxidant and Oral Cancer Inhibitory Activity of Dietary Polyphenols

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A thesis
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ABSTRACT

Oral cancer is a serious yet largely overlooked problem both nationally and globally. In the United States alone, a little over fifty thousand people are diagnosed with oral cancer each year, and approximately ten thousand people die from oral cancer per year. People with oral cancer experience many debilitating symptoms such as problems with speaking, swallowing, and eating. However, many of these severe symptoms do not appear until the disease has progressed to a much later stage, and the survival rate for oral cancer decreases significantly upon late detection. In order to discover and develop chemopreventive agents as well as therapeutics for such a severe disease, researchers have begun investigating natural compounds, such as dietary polyphenols, for potential use. Polyphenols are found in a wide variety of dietary sources, such as kale and other bitter greens, citrus fruits, red onions, red wine, tea, and coffee. These organic compounds have frequently been studied for their antioxidant, anti-inflammatory and anti-cancer properties. In normal healthy cells, polyphenols are recognized for their antioxidant behavior, but in cancerous cells, polyphenols are suspected to display prooxidant behavior as a way to inhibit cancer cell growth. This experiment focused on evaluating if prooxidant behavior was exhibited by polyphenols when the compounds were used to treat human oral cancer cells (SCC-25). My hypothesis predicted that dietary polyphenols relied on the induction of mitochondrial oxidative stress as the critical mechanism for their oral cancer inhibitory activity. First, I examined general cytotoxicity of apigenin, chrysin, eriodictyol, fisetin, naringenin, and quercetin. Then I assessed polyphenol cytotoxicity with an antioxidant present, and I measured intracellular reactive oxygen species (ROS) production after polyphenol treatment. All polyphenols examined in my experiments had a dose-dependent cytotoxicity in SCC-25 cells. Cell viability was restored in

most SCC-25 cells after co-treatment with a general antioxidant, but co-treatment with fisetin or eriodictyol and the antioxidant did not attenuate cytotoxicity. SCC-25 cells treated with quercetin displayed a significant increase in ROS production after 24 hours, but some SCC-25 cells had only a slight increase or no difference in ROS production after treatment with other polyphenols. There was no clear correlation between ROS production and cytotoxicity, as quercetin was the least cytotoxic, but SCC-25 cells treated with quercetin produced the most ROS after 24 hours. If further studies confirm these findings, polyphenols that are cytotoxic in oral cancer cells could be used to develop novel oral care treatments.

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Chapter 1

Introduction

Oral Cancer

In the United States alone, cancer is the second major cause of death, following cardiovascular disease.[1] Oral cancer is a disease that is most common in European and Asian populations, but cases of oral cavity and pharynx cancers are slowly on the rise in the United States.[2] Recent estimates from the American Cancer Society predict there will be about 54,000 new cases of the disease in the United States during 2022. In addition, they estimate that there will be approximately 11,230 deaths from oral cancer that same year.[3]

90% of all oral cancers are squamous cell carcinomas.[2] Common risk factors that have been linked to squamous cell carcinoma are heavy tobacco use, heavy alcohol consumption, human papillomavirus (HPV) infection or other viral infections, genetic factors, and poor oral hygiene.[4] Sores, lesions, and white plaques often develop inside the mouth as a result of the cancer, but often these symptoms are not painful or noticeable without proper screening until the cancer has progressed to a more severe stage. Once oral cancer has progressed significantly, the symptoms can be very extreme, and patients often struggle with speaking, swallowing, and eating as a result of their disease.[4]

When the disease is detected at late stages like this, serious and costly intervention methods like surgery and chemotherapy are typically required. Surgery can leave patients very disfigured, and this can put severe stress on a patient and worsen their mental health. Suicide risk is 2.7 times higher in women with oral cancer and 3 times higher in men with oral cancer compared to the general U.S. population.[5] In addition, even after receiving treatment, the five-year survival rate for late stage (stage III and IV) oral cancer is less than 25%.[6] Considering the five-year survival rate after early detection of oral cancer (stage I and II) is 80%,[6] this is an important disease that requires early attention and immediate action.

Polyphenols

Polyphenols are a diverse family of naturally occurring organic compounds that are found in many plant foods, and they are given the name since they have multiple phenolic structural units in their chemical structure. Many polyphenols exist as glycosides, meaning that their polyphenolic skeleton is attached to a sugar, and this improves their water solubility.[7] The polyphenolic family consists of over 8000 known phenolic compounds, and these are most commonly classified into the following subclasses: phenolics, stilbenes, flavonoids, tannins, and lignans.[8] The chemical structures for compounds in each subclass in the family vary greatly from each other (Figure 1), and this is because these groups have been classified by number of phenolic rings and the different structural elements that bind the rings together.

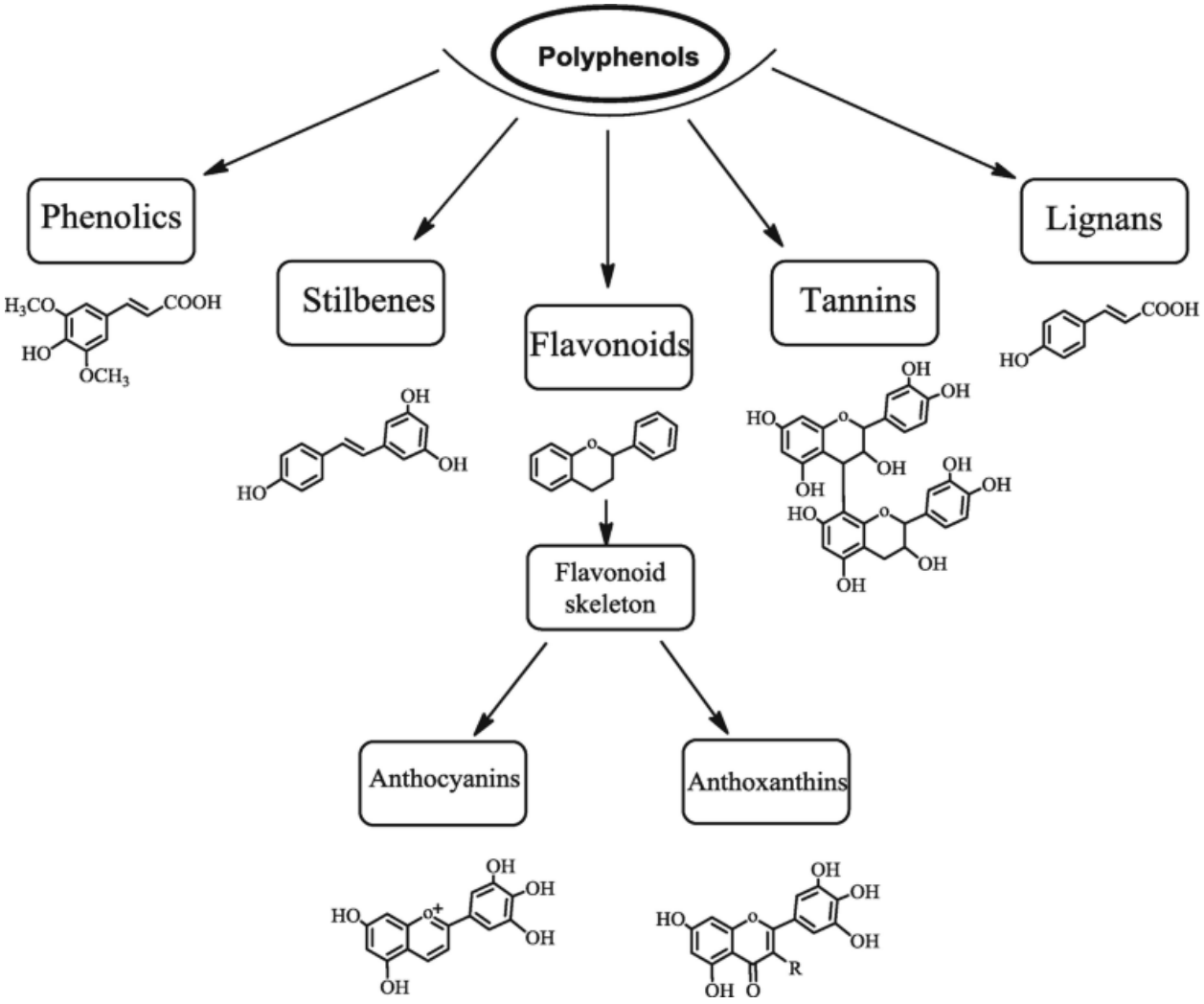


Figure 1. Classification of polyphenols by chemical structure

Polyphenols are secondary plant metabolites, and their differences in chemical structure are what account for the variation in color, bitterness, and astringency of their dietary source.[8] In addition, the differences in beneficial properties found in the dietary sources can also be attributed to each polyphenol's chemical structure. Polyphenols have been widely studied for their antioxidant behavior, anti-inflammatory properties, and their overall role in human health. A large amount of *in vitro, in vivo,* and epidemiological studies have found polyphenol-rich foods to be protective

against the development of chronic diseases like diabetes, osteoporosis, neurodegenerative diseases, and cancer.[8] Many of these diseases have been linked to oxidative stress from reactive oxygen and nitrogen species. Polyphenols are believed to act as antioxidants and free radical scavengers that enhance and complement the functions of other antioxidants and enzymes, and these compounds work together to form a cellular defense against oxidative stress.[9] However, there is also increasing evidence that some polyphenols might act as signaling molecules to modulate cell signaling pathways in ways similar to phosphoinositide 3-kinase (PI 3-kinase), Akt, and a variety of tyrosine and MAP kinases.[10] As a result, it is likely that polyphenols protect against disease through a variety of mechanisms, and their properties might be due to their unique chemical structures.

Out of the large number of polyphenolic compounds, my project focuses on a small group of flavonoids: apigenin, chrysin, eriodictyol, fisetin, naringenin, and quercetin; that have small changes in chemical structure (Figure 2). All of these flavonoids share the same skeletal structure of a 15 carbon skeleton with two aromatic rings connected by a three carbon linking chain (Figure 3).

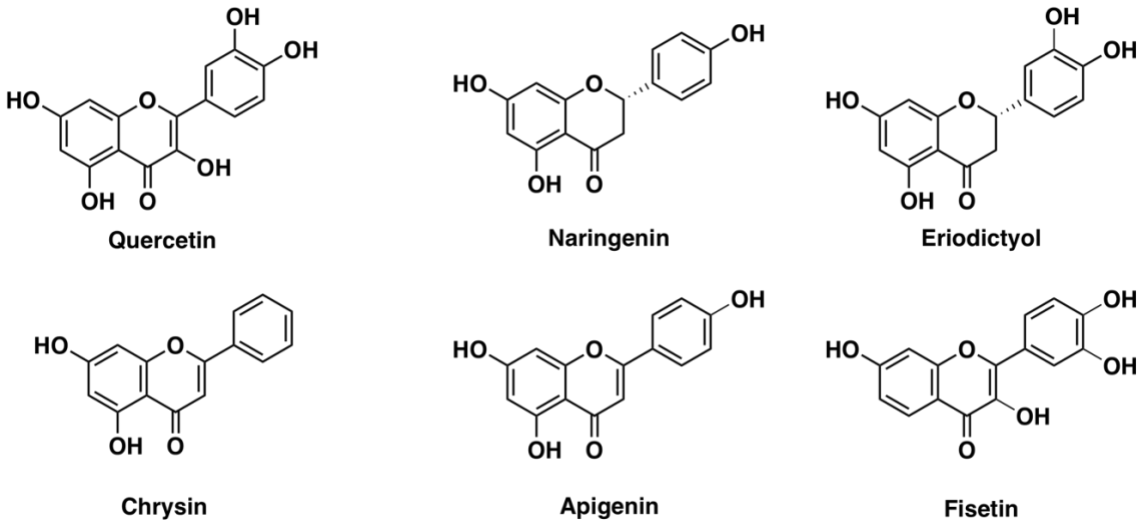


Figure 2. Chemical structures of polyphenols of interest. Structures were constructed using ChemDraw.

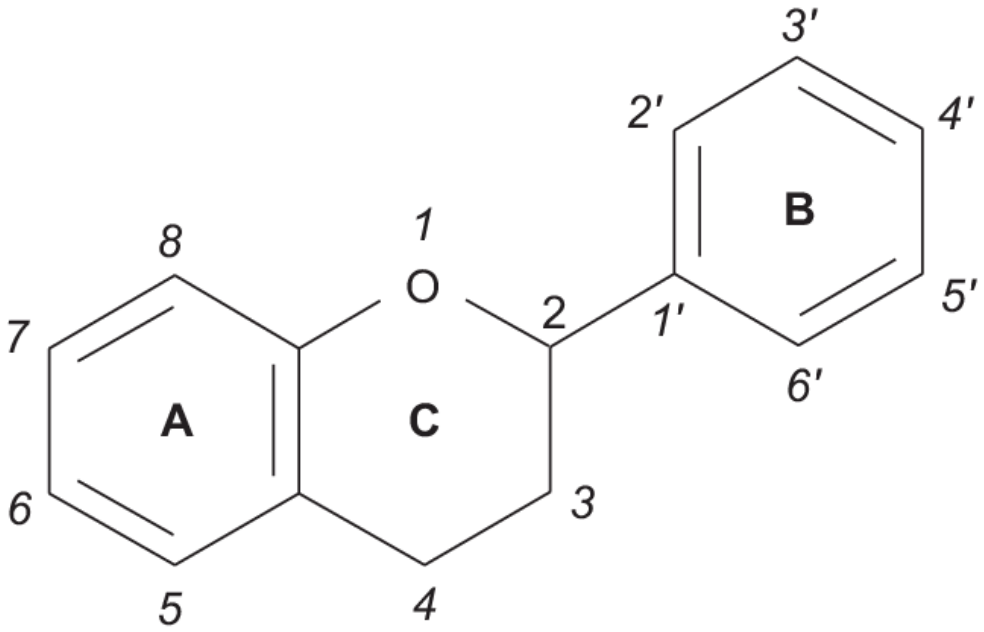


Figure 3. General structure of flavonoid compounds

Apigenin

Apigenin, which is chemically known as 4',5,7-trihydroxyflavone, is a member of the flavonoid classification. Like other flavonoids, apigenin is composed of two aromatic rings connected by a three carbon bridge, and this forms a diphenyl propane structure (C6-C3-C6). According to its chemical name, apigenin also has three hydroxy substituents, which can be removed or substituted to form a variety of flavone derivatives.[7] In its natural form, apigenin is usually glycosylated. It is found in this form in apples, parsley, celery, thyme, oregano, basil, chamomile tea, red wine, and many similar plant foods and plant-based beverages.[11]

Chrysin

Chrysin (5,7-dihydroxyflavone) is a flavone found in passion fruit, bee propolis, honey, mushrooms, and other plant sources.[12] Compared to apigenin, chrysin has no hydroxyl groups on its B ring of its flavone backbone. Chrysin has been widely studied for its anti-inflammatory properties and protective role in related diseases in the heart, brain, and kidney. Chrysin's anti-inflammatory behavior is due to its ability to inhibit nuclear factor kappa B (NFκB) activation,[13] downregulate tumor necrosis factor alpha (TNF- α) and interleukin 1β (IL-1β), suppress histamine release, and control many other pharmacological activities.[14]

Eriodictyol

Eriodictyol ((2*S*)-3′,4′,5,7-Tetrahydroxyflavan-4-one) is part of another flavonoid subclass: flavanones. Eriodictyol is a flavanone that has hydroxyl substituents at positions 5 and 7 on the

A ring and on positions 3’ and 4’ on the B ring (Figure 3). In human cells, eriodictyol has been found to activate Nrf2 and induce phase II enzymes heme-oxygenase (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO-1) to protect against oxidative stress.[15] In mice macrophages, eriodictyol has been found to suppress NFκB activation,[16] similar to chrysin. Eriodictyol is mostly found in lemons, grapefruits, and other citrus fruits, but it is also found in peppermint.[17]

Fisetin

Fisetin (3, 3’, 4’, 7-tetrahydroxyflavone) is a flavone commonly found in apples, persimmons, kiwis, cucumbers, nuts, and wine.[18] In both cell and animal models, fisetin has displayed anti-inflammatory, anti-diabetic, neuroprotective, and cardioprotective effects.[19] Fisetin is a strong antioxidant, and studies have found that the hydroxyl groups in the 3, 3’, and 4’ positions are much more effective at scavenging free radicals than the hydroxyl group at the 7 position on the A ring.[20] This compound has also been studied as an inhibitor to PI3K/Akt and mTOR pathways, and it is involved in downregulating these pathways and cell growth and proliferation in many cancer cell types.[20]

Naringenin

Naringenin (4',5,7-trihydroxyflavanone) is flavanone found in figs, tomatoes, bergamot, and other citrus fruits. Like many other flavonoids discussed, naringenin has anti-inflammatory and anti-cancer activity, but it also has been regarded as an antibacterial and antiviral agent in many cell and animal studies. Few clinical studies have been conducted with naringenin, but one study

in patients with high cholesterol found that administering a 400 mg capsule of naringenin each day significantly reduced LDL cholesterol levels and increased activity of antioxidant enzymes superoxide dismutase and catalase after 8 weeks.[21]

Quercetin

Quercetin, chemically named 3, 3', 4', 5, 7-pentahydroxyflavanone, is a flavanol that accounts for approximately 75% of all flavonoid intake in U.S. adults.[22] Quercetin is found as a glycoside in many dietary sources, which include kale, broccoli, capers, onions, apples, tea, and many other plant foods.[23] Clinical studies have found supplementation with quercetin regulates glutathione synthesis to help improve antioxidant capacity in patients with necrotizing enterocolitis, an inflammatory gastrointestinal disease.[24] In gastrointestinal epithelial cells, quercetin has been found to prevent oxidative damage through unknown mechanisms.[25] In human oral squamous carcinoma (SCC-9) cells, treatment with quercetin inhibited cell growth,[26] which indicates its importance in cell cycle regulation and tumor growth.

Oxidative Stress

Oxidative stress occurs when cells are exposed to high levels of ROS. Reactive oxygen species include singlet oxygen, superoxide, peroxides, nitric oxide, hydroxyl radicals, and more. ROS is produced in the mitochondria during aerobic respiration by electrons released from the electron transport chain.[27] NAPDH oxidases found in the cytoplasm are also one of the largest contributors to the production of ROS, and these are enzymes that catalyze superoxide

production from O_2 and NADPH. Mitochondrial electron leakage and ROS production increases with age, and it is believed that this oxidative damage contributes to the development of many serious diseases like diabetes and cancer.[28]

There are higher levels of ROS in cancer cells than those found in normal cells, and it is believed that high levels of ROS are required for tumorigenesis. In addition, cells that are experiencing oxidative stress and damage tend to produce more radicals, accumulate more mutations, and activate more oncogenes to induce tumor growth in a "feed-in loop." Some results of oxidative stress are changes to mitochondrial membrane permeability, changes in mitochondrial function, and eventually cell death by apoptosis. One mechanism cancer cells use to avoid apoptosis is to activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway.[28]

Oral Cancer, Oxidative Stress, and Polyphenols

Polyphenols are known for antioxidant activity in normal cells, since they possess OH groups that can donate a hydrogen atom to free radicals, or they can inhibit prooxidant enzymes like NADPH oxidases.[9] Beyond these mechanisms, polyphenols are also known to potentially upregulate Nrf2, which is a transcription factor that drives additional antioxidant signaling.[29] However, in cancerous cells, the behavior of polyphenols can be largely dependent on their concentration in the cell. In low concentrations, certain polyphenols might support the cancer cells antioxidant defense, but in higher concentrations these same compounds might inhibit the defense and instead favor ROS production, oxidative damage, and cell death.[28]

In the Lambert Lab, it has already been determined that epigallocatechin-3-gallate (EGCG), a polyphenol found in green tea, shows inhibitory activity against oral cancer cells by inducing mitochondrial ROS and mitochondrial dysfunction.[30] EGCG shares antioxidant abilities with many other polyphenols, but now this finding provides more evidence highlighting the prooxidant abilities of polyphenols.

In my project, I aimed to extend the hypothesis tested in this existing data to see if other polyphenols exhibit anticancer activity in oral cancer cells, and I aimed to determine if production of intracellular ROS is the mechanism of their cytotoxicity. I investigated these aims by measuring the cytotoxicity of a group of polyphenolic compounds, the levels of reactive oxygen species present after cell treatment with a polyphenol, and the effects of adding an antioxidant and a polyphenol together.

Chapter 2

Materials and Methods

Materials

Apigenin, chrysin, fisetin, and naringenin were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Eriodictyol was purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). Quercetin and N-acetyl-L-cysteine were both obtained from Sigma-Aldrich (St. Louis, MO, USA). Thiazolyl Blue tetrazolium bromide (MTT) dye was ordered from Alfa Aesar (Haverhill, MA, USA). Dihydrofluorescein diacetate (DCFH-DA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nunc Lab-Tek II Chambered Coverglass system was ordered from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents used in these experiments were of the highest commercial grade available.

Methods

Cell Culture

Human oral squamous cell carcinoma cells (SCC-25) were purchased from ATCC (Manassas, VA, USA). SCC-25 cells were cultured in DMEM:F-12 (1:1) medium that was supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA, USA), 1% penicillin streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were grown at 37 °C under a 5% CO_2 atmosphere, and cells were passaged when confluent.

Cell Viability Assay

Polyphenol Cytotoxicity

The impact of each polyphenol on cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In the assay, SCC-25 cells were treated with polyphenol solution (0–200 µM) in serum-complete medium. Following 48-hour treatment, cells were washed with fresh medium once. MTT (1 mg/mL)-containing medium was added to the cells, and the cells were incubated for 30 min. Development of the formazan dye, which correlates with viability, was measured by Multiskan GO microplate spectrophotometer at 550 nm. The cell viability of polyphenol-treated cells was normalized to controls treated with media.

Co-treatment with N-acetylcysteine

SCC-25 cells were treated with 50-100 µM polyphenol solution in the presence or absence of 2 mM of a general antioxidant, N-acetyl-cysteine (NAC), for 48hr. Following 48-hour treatment, cells were washed with fresh medium once. 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (1 mg/mL)-containing medium was added to the cells, and the cells were incubated for 30 min. Development of the formazan dye was measured by Multiskan GO microplate spectrophotometer at 550 nm. The cell viability of co-treated cells was normalized to controls treated with media.

Determination of Intracellular ROS

Visualization of total intracellular ROS was accomplished by staining with 6-carboxy-2 ,7 -dichlorodihydrofluorescein diacetate, di(acetoxymethylester) (H2DCFDA) in combination with fluorescent microscopy. Cells were grown on a chambered, glass, microscope slide overnight and treated with 50-100 µM polyphenol solution for 24 hours. Following treatment, cells were incubated with 10 µM

H2DCFDA for 30 min at 37 ºC, rinsed twice with PBS, and examined by microscope (λex = 480 nm; λem = 520 nm). Fluorescence was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The corrected total cell fluorescence (CTCF) was calculated by the formula below: CTCF = Integrated density − (Area × Mean fluorescence of background). Mean CTCF was found for treatment with each polyphenol and normalized to the mean CTCF of controls treated with media.

Analysis

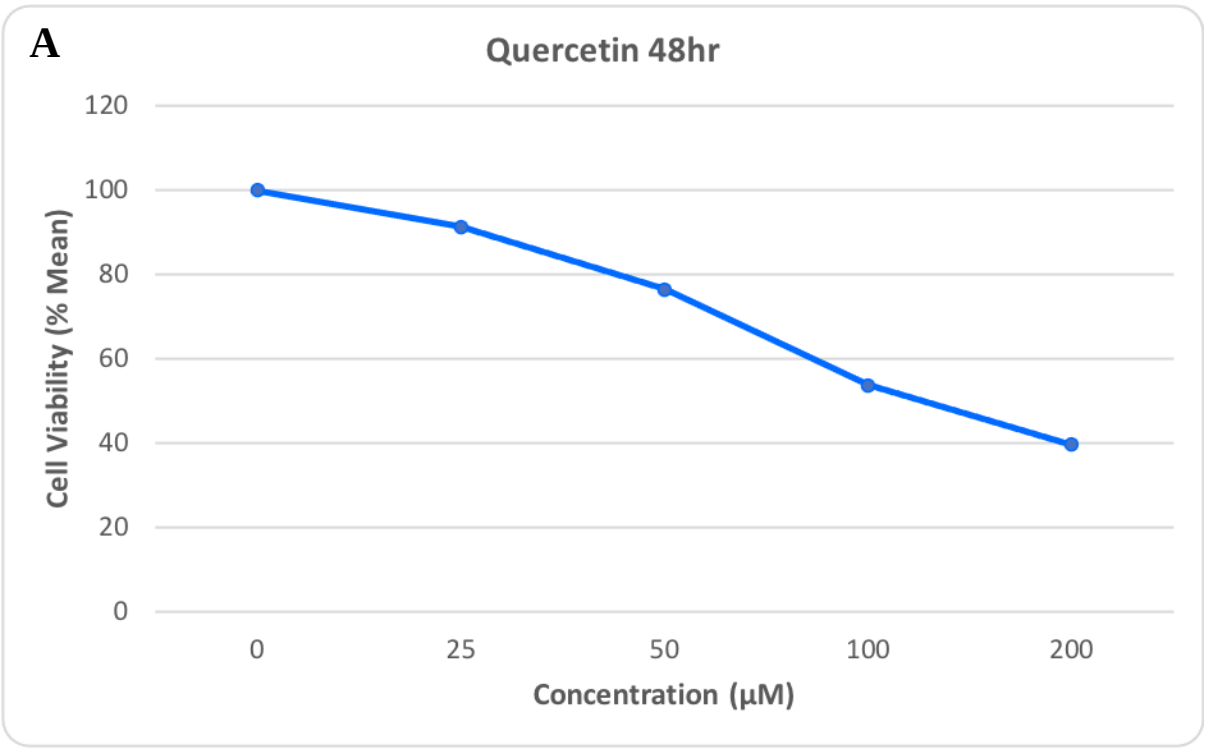
Statistical analysis was performed using Microsoft Excel. All results were normalized against a vehicle control and performed in triplicate measurements for reproducibility. Values are the means ± standard error of the mean (SEM). Student's unpaired t-test was performed with $p < 0.05$ considered significant. ***, $p < 0.001$; **, $p < 0.01$; * $p < 0.05$.

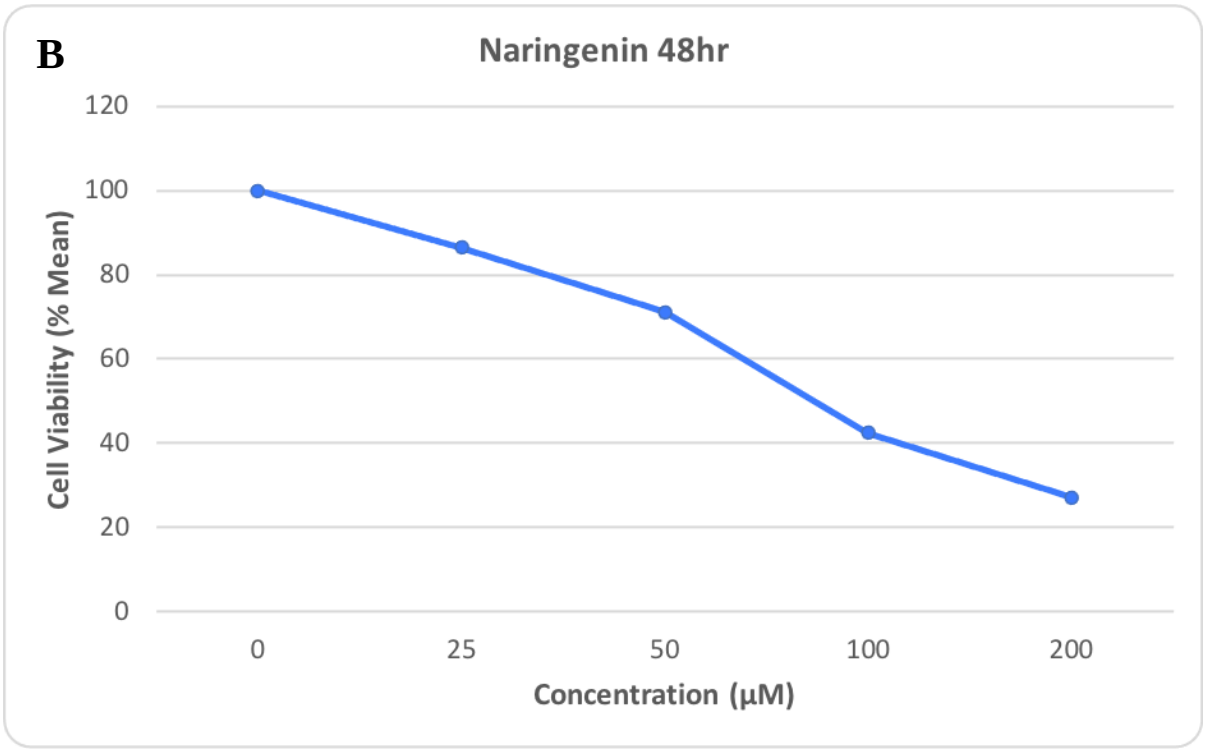
Chapter 3

Results

Effect of Polyphenol Treatment on SCC-25 Cell Viability

This set of experiments evaluated the cytotoxicity of polyphenols apigenin, chrysin, eriodictyol, fisetin, naringenin, and quercetin in SCC-25 cells (Figure 4). All polyphenols tested exhibited a dose-dependent cytotoxicity when evaluating SCC-25 cells treated with concentrations of 0-200 µM polyphenol for 48 hours. Based on the data collected, quercetin (Figure 4A) was the least cytotoxic out of all compounds tested. Fisetin was the most cytotoxic out of all compounds tested (Figure 4F). In order of increasing cytotoxicity, the polyphenols are ranked as follows: quercetin, naringenin, apigenin, eriodictyol, chrysin, fisetin.




B
Naringenin 48hr
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Cell Viability (% Mean)
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100
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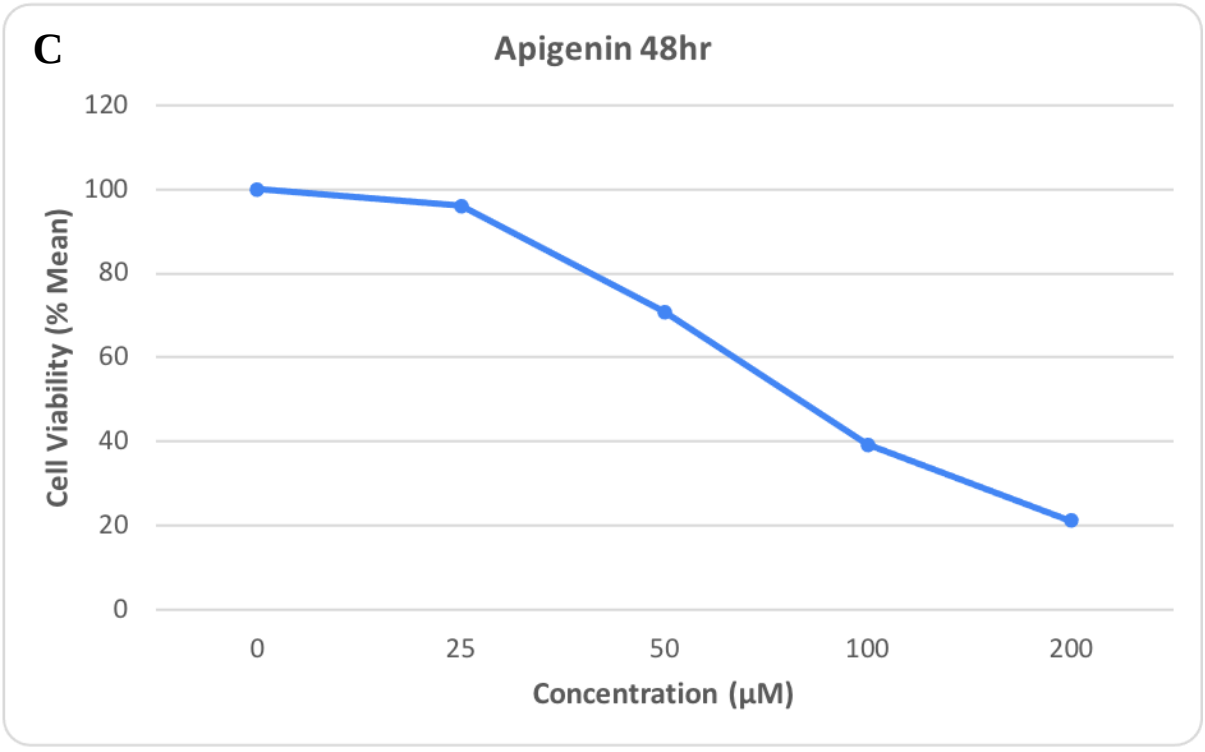
Concentration (µM)


C
Apigenin 48hr
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Cell Viability (% Mean)
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25
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100
200
Concentration (µM)

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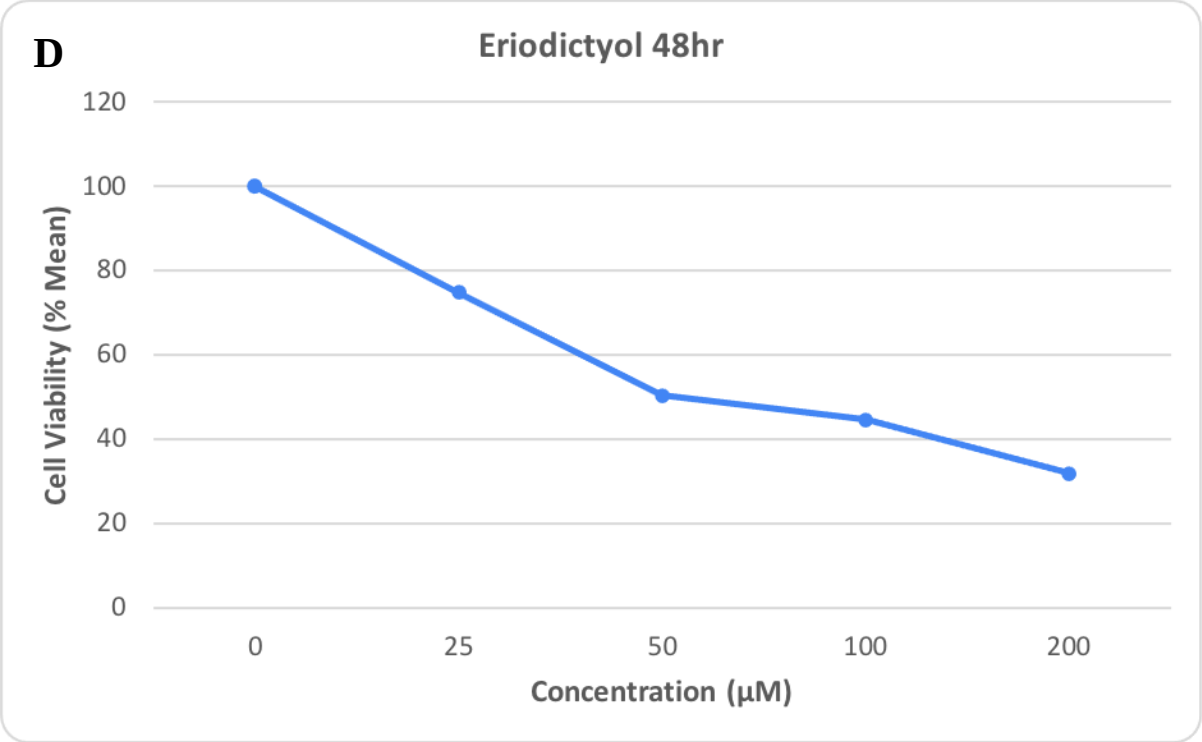
Eriodictyol 48hr
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80
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Cell Viability (% Mean)
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100
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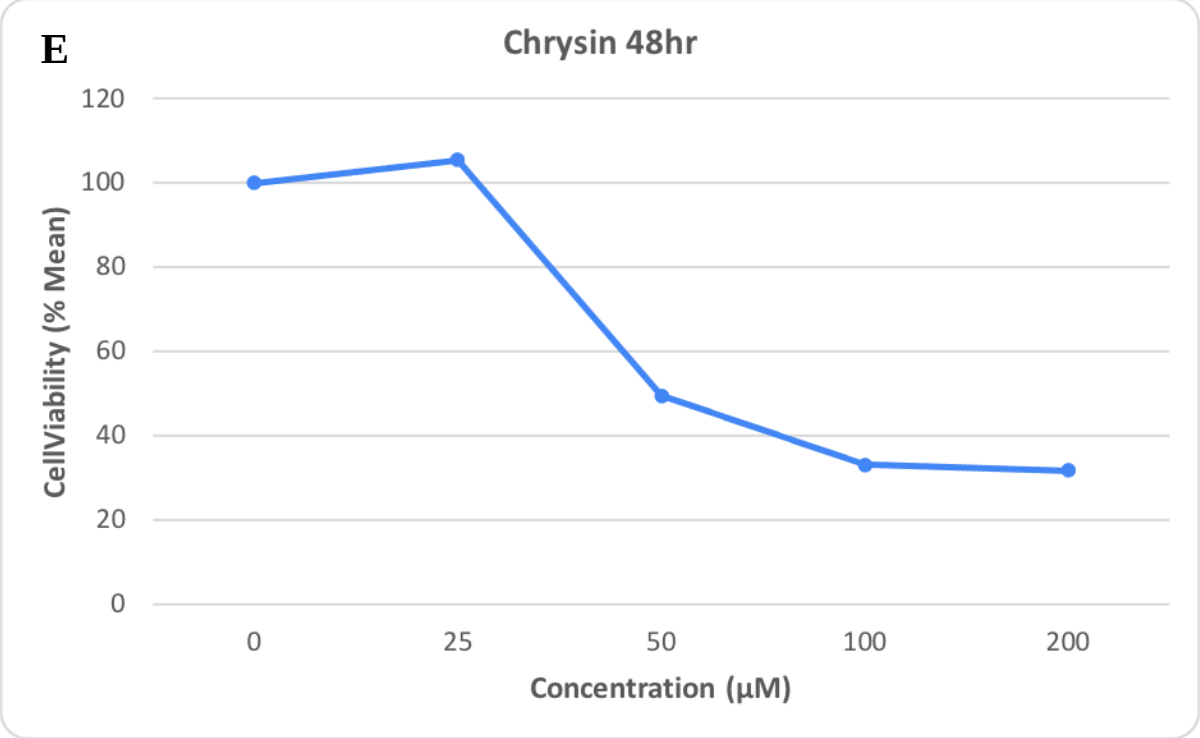
Concentration (µM)

E


Chrysin 48hr
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CellViability (% Mean)
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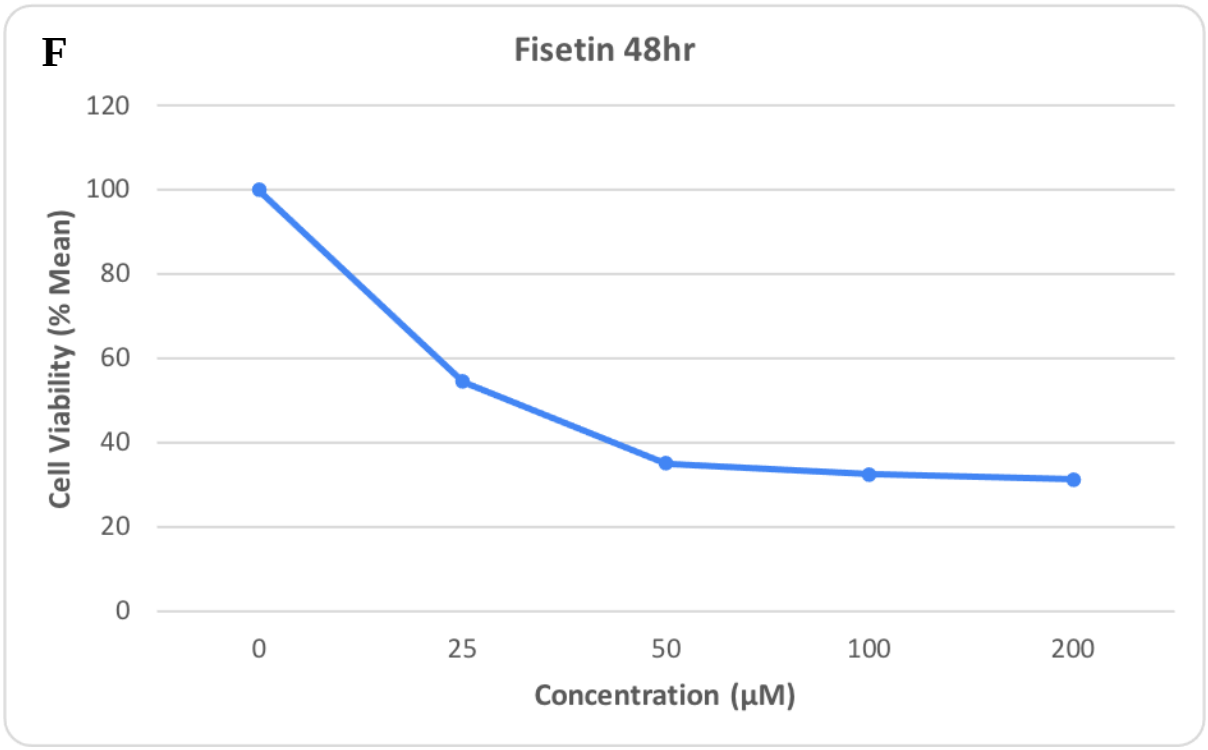

Figure 4. (A-F) Effect of polyphenol solutions (0-200 µM) on viability of human oral cancer cells (SCC-25) after 48 hours treatment. Cell viability was determined by MTT assay. Data represents the means ± SEM (n = 6).

Effect of N-acetylcysteine on SCC-25 Cell Viability

After conducting the cytotoxicity assay on each of the polyphenols, an approximate IC50 value was taken from the results of each experiment and used for this next set of cytotoxicity experiments. In this set of experiments, a general antioxidant, N-acetylcysteine (NAC) was added alone or in co-treatment with a polyphenol to SCC-25 cells, and cell viability was once again measured after 48 hours via MTT assay. Since the polyphenols are suspected to be cytotoxic through prooxidant behavior, co-treatment with NAC was used to see if adding in an antioxidant would reduce oxidative stress and restore cell viability.

Treatment with NAC alone had no effect on cell viability. Co-treatment of NAC with polyphenols fisetin and eriodictyol showed no significant change in cell viability levels compared to those of cells treated with fisetin (Figure 5B) or eriodictyol (Figure 4E) alone. However, cells co-treated with NAC and one of the polyphenols apigenin, chrysin, naringenin, or quercetin (Figure 5) showed significant restoration in cell viability compared to the cell viability levels of cells treated with these polyphenols alone.

SCC-25 cells that received a quercetin co-treatment had a significant 1.4-fold increase in cell viability when compared to quercetin treatment alone. Apigenin co-treatment resulted in a cell viability of 88%, which is a significant 1.8-fold increase in cell viability compared to cells treated with apigenin. Most notably, the greatest protective effects of NAC were seen in the co-treatments with either chrysin (Figure 5C) or naringenin (Figure 5D). Chrysin co-treatment resulted in a cell viability of 43% compared to 19% of chrysin treatment alone, which is a

significant 2.3-fold increase. Naringenin co-treatment with NAC resulted in a significant 2.4-fold increase in cell viability compared to cells treated with naringenin alone.

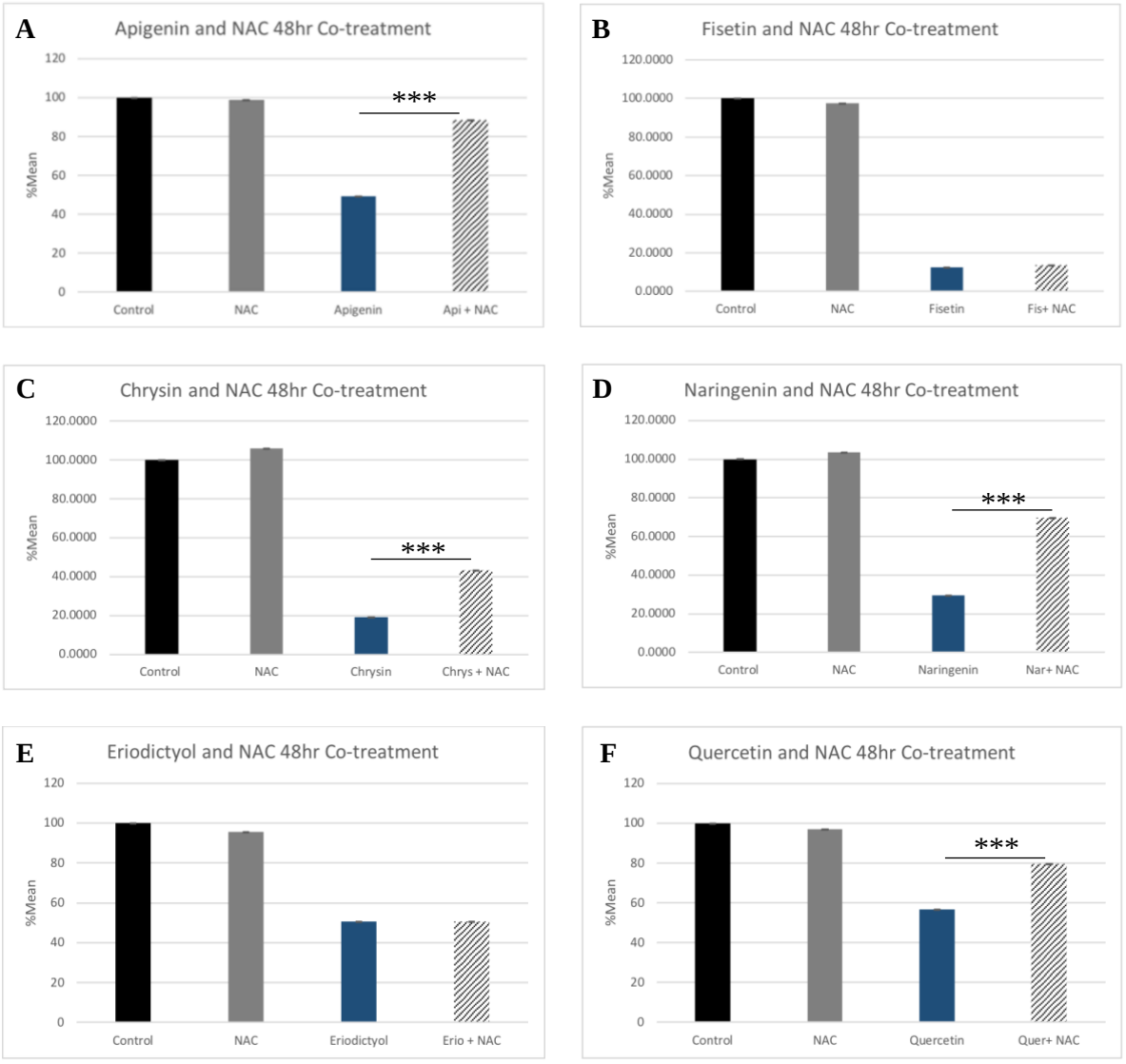

Figure 5. (A-F) Effect of co-treatment of polyphenol solutions (50-100 µM) and N-acetylcysteine (2mM) on viability of human oral cancer cells (SCC-25) after 48 hours treatment. IC50 values were selected based on data from cytotoxicity assay (Fig 4.) Cell viability was determined by MTT assay. Data represents the means ± SEM (n = 6). Student's unpaired t-test was performed with p< 0.05 considered significant. ***, p < 0.001; **, p < 0.01; *, p< 0.05

Induction of Intracellular Oxidative Stress

In this set of experiments, the estimated IC50 of the polyphenols of interest were used to treat SCC-25 cells for a period of 24 hours. Since fisetin was much more cytotoxic even at similar IC50 concentrations to the other compounds, I was unable to obtain images that contained a sufficient amount of viable cells for analysis, so fisetin had to be excluded from this analysis. Compared to the control group, treatment of SCC-25 cells with chrysin (Figure 11B) or naringenin (Figure 11D) resulted in no change in ROS production. Treatment of SCC-25 cells with apigenin resulted in a significant decrease in intracellular ROS after normalization to the control group (Figure 11A). Treatment of SCC-25 cells with eriodictyol resulted in a slight increase in intracellular ROS when normalized to the control group (Figure 11C). Treatment of SCC-25 cells with quercetin resulted in a 3.5-fold increase in ROS when normalized to the control (Figure 11E), and this was found to be statistically significant after performing an unpaired t test ($p<0.05$).

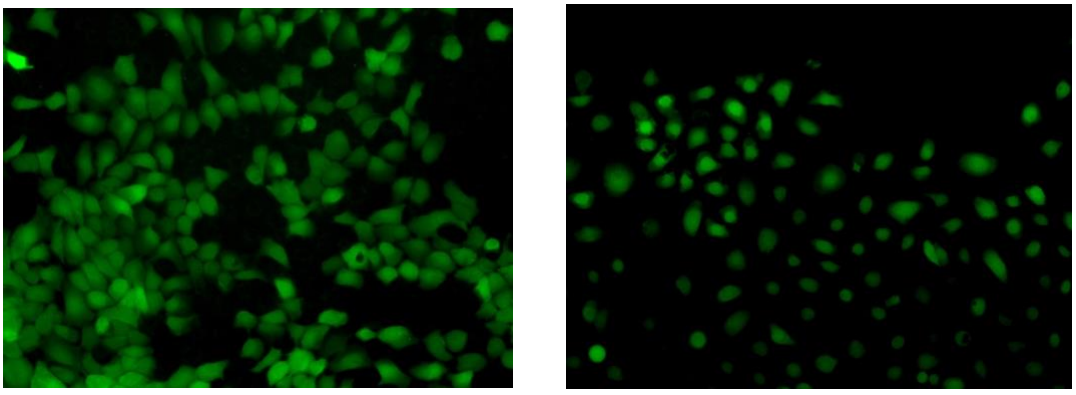

Figure 6. SCC-25 cells treated with control (left) and 100 µM apigenin (right) for 24 hours.

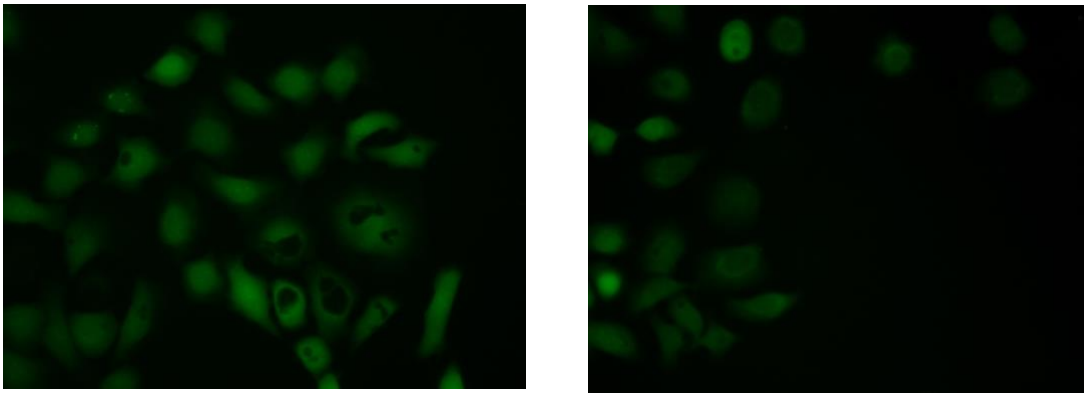

Figure 7. SCC-25 cells treated with control (left) and 100 µM chrysin (right) for 24 hours.

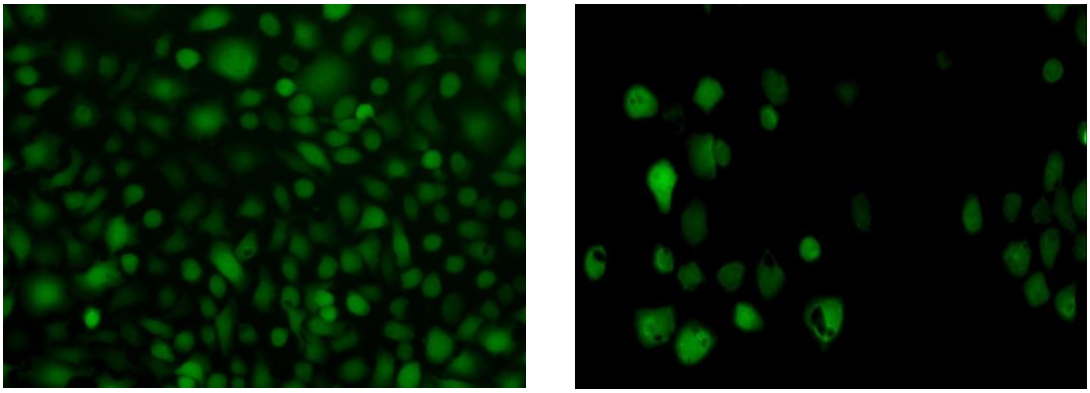

Figure 8. SCC-25 cells treated with control (left) and 100 µM eriodictyol (right) for 24 hours.

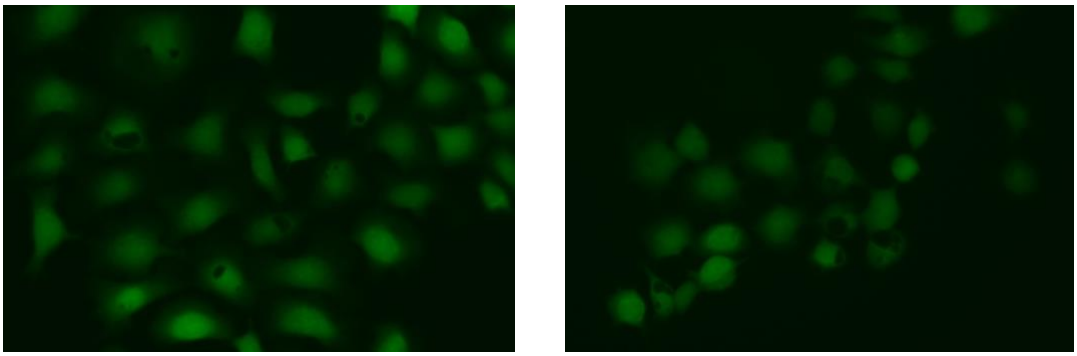

Figure 9. SCC-25 cells treated with control (left) and 50 µM naringenin (right) for 24 hours.

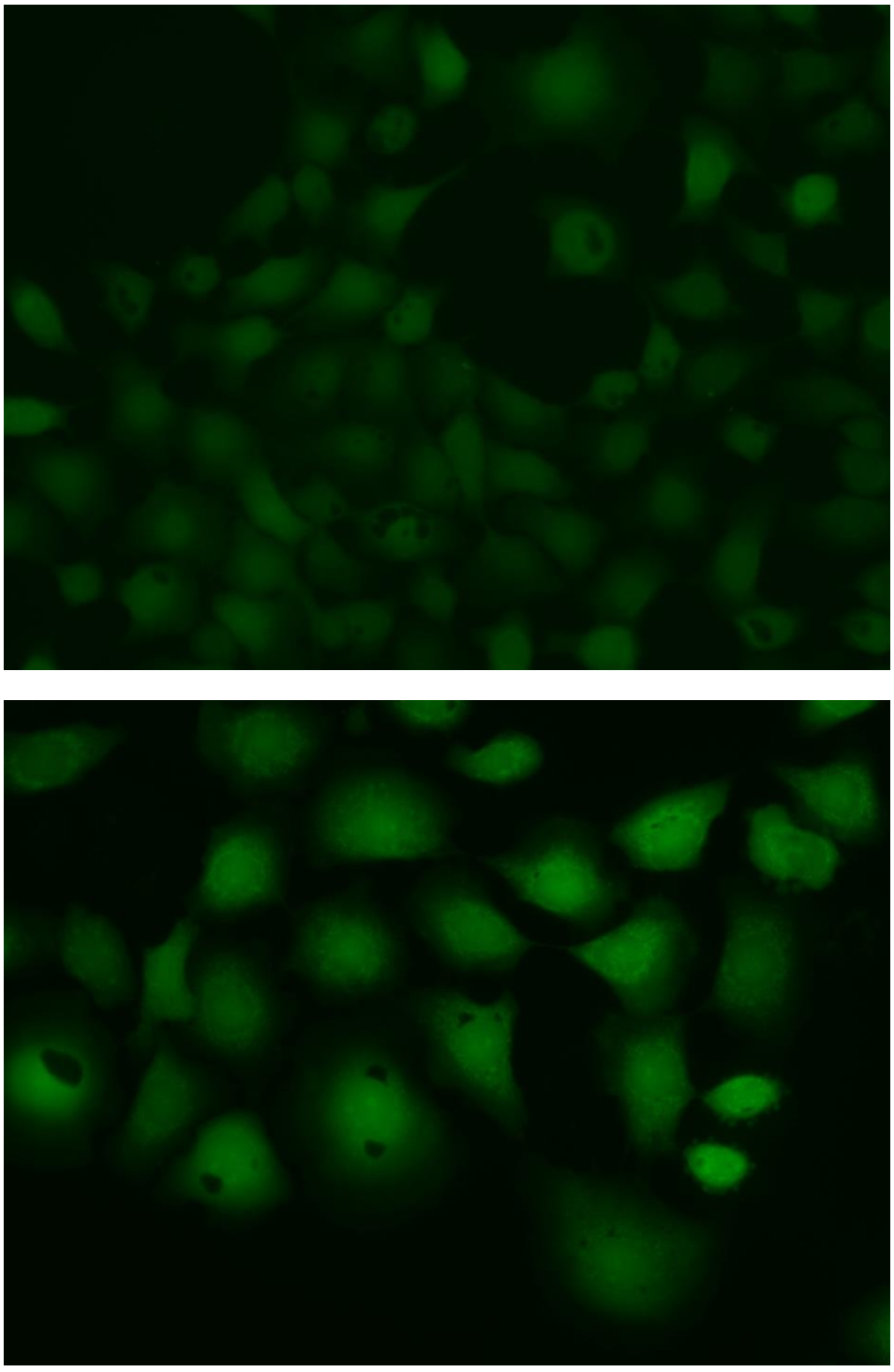

Figure 10. SCC-25 cells treated with control (top) and 50 µM quercetin (bottom) for 24 hours.

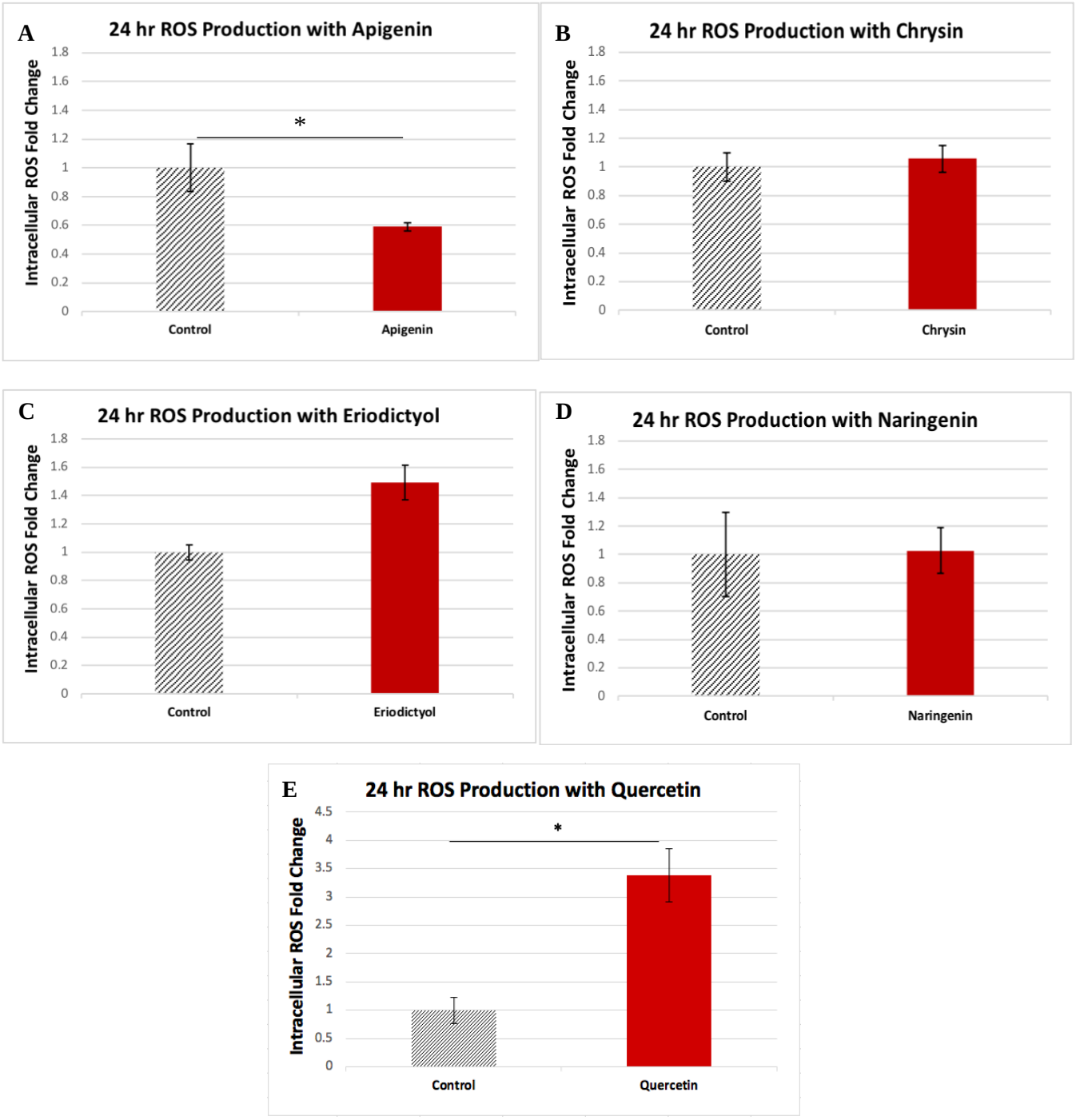

Figure 11. (A-E) Effect of polyphenol solutions on oxidative stress in human oral cancer cells. SCC-25 cells were treated with 50-100 µM polyphenol solution or vehicle for 24 hours. ROS was visualized by fluorescence microscopy and quantified with ImageJ. ROS was normalized to controls treated with complete media. Data represents means ± SEM (n=3-4). Student's unpaired t-test was performed with $p < 0.05$ considered significant. *, $p < 0.05$.

Chapter 4

Discussion

Even though polyphenols are most commonly studied for their antioxidant behavior, they also have the ability to act as prooxidants and produce reactive oxygen species. Although there are thousands of compounds that are classified as polyphenols, I focused on a small group of flavonoids that are closely related in terms of structure. The goal of my project was to evaluate the anticancer activity and prooxidant behavior of these compounds, and I wanted to be able to relate any functional differences to slight differences in each chemical structure. In order to help establish a relationship between polyphenol structure and anti-cancer activity in oral cancer cells, I evaluated overall cytotoxicity and measured the amount of intracellular reactive oxygen species present after SCC-25 cells were treated with each polyphenol. In order to support these findings, I also investigated the effects of adding an antioxidant, N-acetylcysteine (NAC) to the polyphenol treatment.

In my cytotoxicity experiments, I was able to compare the strengths of each compound in reducing oral cancer cell viability. Fisetin was determined to be the most cytotoxic out of all compounds tested. Chrysin and eriodictyol were also close in terms of cytotoxicity, and these structures share a OH on the 7 position of the A ring with fisetin. Eriodictyol also shares a 4’ OH on its B ring with fisetin and an OH on the 5 position of the A ring with chrysin. Quercetin was found to be the least cytotoxic, and its structure has hydroxyl groups at the 5 and 7 positions of the A ring like naringenin and apigenin, which similar in cytotoxicity. Quercetin’s structure also has a 4’ OH seen in all of these discussed compounds.

When further exploring if each polyphenol was cytotoxic by causing oxidative stress, I found that NAC did not restore cell viability when combined with fisetin or eriodictyol. This was an unexpected finding for eriodictyol because treatment of SCC-25 cells with eriodictyol resulted in a modest increase in ROS. NAC showed protective effects in SCC-25 cells that were treated with apigenin, chrysin, naringenin, and quercetin. Since NAC was effective for restoring cell viability in oral cancer cells treated with apigenin, chrysin, naringenin, and quercetin, it is possible that these polyphenols have some prooxidant behavior.

Despite the NAC experiment results indicating protective effects against oxidative stress for chrysin and naringenin, the fluorescence analysis did not indicate any changes in ROS after 24 hours. Considering that these experiments were run using different timelines (24-hour treatment for ROS measurement, 48-hour treatment for cytotoxicity assay), it is possible that these compounds still caused cells to produce reactive oxygen species but the effects are not potent enough until beyond 24 hours.

Treatment with 100 µM apigenin resulted in a significant decrease in intracellular ROS, which could be attributed to apigenin acting as an antioxidant during the 24-hour treatment period. Since 48-hour treatment with apigenin decreased SCC-25 cell viability, which was able to be restored by antioxidant cotreatment, it is likely that ROS production caused by 100 µM apigenin occurs after 24 hours. Treatment of SCC-25 cells with eriodictyol resulted in a slight increase in ROS, but treatment with quercetin resulted in a significant increase in ROS despite quercetin being the least cytotoxic compound. Both quercetin and eriodictyol share a 3’ hydroxyl on the B

ring, but quercetin also has a 4' hydroxyl group on its B ring and a hydroxyl group on the 3 position of the C ring (only shared with fisetin).

Quercetin's additional OH groups make it a strong antioxidant, but within the unique environment of a cancer cell, this could explain why quercetin behaves as a strong prooxidant. Cancer alters the redox state of the cell, and when flavonoids form stable complexes with any metal ions present in the cell, such as Cu^{2+}, it has been shown that these complexes cause DNA damage and induce oxidative stress.[31,32] Specifically, studies have found that flavonoids with higher numbers of hydroxyl groups cause the most DNA damage through these complexes.[31]

In order to improve the reliability and reproducibility of this study, I made sure to have a sound experimental design. All experiments were run taking at least triplicate measurements. In addition, all experiments were performed using the proper controls. In my cytotoxicity experiments, I treated cells with media alone, and I used that absorbance value to normalize for all other experimental groups. In addition, when I was imaging cells to measure ROS, I imaged cells treated with media alone to establish a base level of fluorescence for normalization. During imaging, I took multiple images across each well in the chamber slide, and I evaluated the CTCF for at least 50 cells per control/experimental group to reduce bias and variability in the data. I also imaged cells treated with quercetin as a positive control alongside each experimental group to make sure the assay was running correctly. When using NAC for the co-treatment experiments, I included groups of cells treated with media alone and just NAC alone to make sure the reagent was performing as expected.

Even after using good experimental design, there were still a few limitations to my study. One limitation was that only one time point was taken for ROS determination (24 hours) and cytotoxicity measurement (48 hours). If I had been able to measure cytotoxicity and ROS levels across multiple time points, I would have been able to better establish if and when a switch occurs from antioxidant behavior to prooxidant behavior. Future work needs to be conducted over a wide range of time points to see if any of the flavonoids I tested produce ROS before or after 24 hours.

Another limitation was in determining IC50 values of each polyphenol. To simplify finding an IC50 value for each polyphenol, I chose a value from the concentrations tested (25, 50, 100, 200 μM) that resulted in approximately 50% cell viability. Since the IC50 values were not specifically measured, this could contribute to some variability in the findings in the antioxidant co-treatment and intracellular ROS experiments. Due to this estimation, I was also unable to find a fisetin concentration suitable enough to ensure there were enough cells present to analyze using the fluorescence microscopy technique. Given this issue, I could only make conclusions about fisetin's prooxidant abilities based on any changes observed in the antioxidant co-treatment experiment.

Overall, the results of these experiments supported part of my hypothesis that structural differences are important to each polyphenol's anticancer activity. Despite all of these polyphenols exhibiting a dose-dependent cytotoxicity in oral cancer cells, not all of the polyphenols exhibited strong prooxidant activity. This indicates that many of the polyphenols have different levels in cytotoxicity due to varying mechanisms that have yet to be determined.

However, quercetin did exhibit cytotoxicity in oral cancer cells, and this is likely explained by its ability to induce oxidative stress through excessive intracellular ROS production.

Out of all flavonoids tested, quercetin behaved as a prooxidant in SCC-25 cells, which was expected by my hypothesis. It would be useful to confirm the findings of this study by measuring the effects of quercetin treatment on the gene and protein expression of Nrf2 signaling pathway molecules in oral cancer cells. The Nrf2 signaling pathway has been linked to antioxidants like quercetin in previous studies, but it would be relevant to study this relationship in oral cancer cell lines specifically.

Since chrysin, eriodictyol, and fisetin were the most active out of the flavonoids I tested, it would be valuable to investigate if they are able to produce ROS at different time points than those measured, or if their cytotoxicity results from a different cellular interaction. Since eriodictyol produced a modest increase in ROS after 24 hours, this flavonoid might behave similar to quercetin but in a different timeline, especially since after 48 hours NAC co-treatment did not have an effect. Similarly, chrysin and fisetin treatment should be further assessed at different time points in SCC-25 cells since both compounds have multiple hydroxyl groups in their structure and high levels of cytotoxicity. Similar to quercetin, it would also be useful to look at these other flavonoids' potential involvement in cancer cell signaling by measuring modulations of the JAK/STAT pathway, ERK signaling pathway, or other factors involved in cell cycle arrest and cell survival.

If any future studies support that these flavonoids are strong candidates to target oral cancer, they should be evaluated to find concentrations that would be clinically relevant for people with oral cancer. Because these polyphenolic compounds are available in a wide variety of plant sources, the use of these compounds as cancer therapeutics has the potential to greatly reduce production costs. With the proper dosage, flavonoids could be considered as oral supplements or as additives in chewing gum, mouthwash, or toothpaste that could prevent and protect against oral cancer.

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EXPERIENCE

Undergraduate Researcher **University Park, PA**
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- Investigating pro-oxidant behavior of dietary polyphenols and the role of oxidative stress in inhibiting oral cancer
- Performing MTT cytotoxicity assay for polyphenol-treated SCC-25 oral cancer cells
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Investigative Lab Sciences Intern **West Point, PA**
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- Contributed to a target liability study by establishing an NMR metabolomics profile of a knockout mouse model
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Summer Research Intern – NIH STEP-UP Program **Washington, D.C.**
The George Washington University, Dr. Pedro Jose *05/2019 - 08/2019*

- Investigated pro-SAAS peptides as ligands involved in hypertension pathways
- Grew and maintained culture of human renal proximal tubule cells (hRPTCs)
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Research Fellow – RISE/SURF **New Brunswick, NJ**
Rutgers University, Dr. Debra Laskin *06/2020 - 11/2020*

- Conducted literature review investigating PGC1β as a critical mediator in the resolution of ozone-induced lung inflammation during 6-week RISE/SURF summer program
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RELEVANT TRAINING

HealthWorks Peer Educator - *Penn State* *08/2019 - 05/2020*

- Trained as a peer educator in a 3-credit Biobehavioral Health course focused on public health

- Led interactive workshops and designed social media content promoting health and wellness within the Penn State community for 40 hours a semester

Summer Pharmacology Fellow - *University of Michigan* *06/2020 - 08/2020*

- Participated in weekly "Conversations on Pharmacology" seminars hosted by University of Michigan faculty

LEADERSHIP EXPERIENCE

Organic Chemistry Lab Learning Assistant - *Penn State* *08/2020 – 12/2021*

- Teaching a group of 30+ students organic chemistry concepts during a 3hr/week workshop activity
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Recruitment Chair *05/2020 - 12/2020*

Phi Sigma Rho, Penn State Lambda Chapter

- Organized virtual recruitment open houses, bid day, and general events for Phi Sigma Rho
- Coordinated event invitations to over 60+ members and potential new members

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- Encouraged academic achievement for sorority sisters by tracking member study goals/GPAs

Chemistry Grader and Proctor - *Penn State* *08/2019- present*

- Reviewed and graded honors freshman chemistry homework and quizzes weekly
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PRESENTATIONS

1. **Capece G**, Smith C, Laskin D. *Investigating PGC1β as a Critical Mediator in the Resolution of Ozone-Induced Lung Inflammation*. Rutgers Summer Undergraduate Research Fellowship Symposium, Virtual. July 30, 2020.
2. **Capece G**, Kumar M, Armando I, Jose PA. *Pro-SAAS Peptides and Salt Resistance.* STEP-UP Research Symposium, Bethesda, MD. July 29-30, 2019.
3. **Capece G**, Lambert JD. *Chemical determinants of pro-oxidant and oral cancer inhibitory activity of dietary polyphenols.* Penn State Farm to Fork Symposium, University Park, PA. April 26, 2019.

GRANTS AND SCHOLARSHIPS

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MEMBERSHIPS

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SKILLS

Mammalian Cell Culture || Fluorescence Microscopy || ImageJ Software || NMR || Immunoblotting || Mouse Studies || Biotechnology || Electronic Lab Notebook || Science Writing