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Comparative *in vitro* Bioavailability of Dietary Flavonoids in Oral Cancer Cells

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ABSTRACT

Oral cancer is in the top 10 most common cancer worldwide and is a leading cause of death. In our previous study, it was found that minor differences in flavonoid structure led to more significant differences in anticancer activity---the cytotoxic effects of flavonoids of interest against oral cancer were compared and ranked from the most to the least: Apigenin > Fisetin > Naringenin > Chrysin > Eriodictyol > Quercetin. Many studies demonstrated that the uptake of flavonoids into cells determines their biological actions, such as the mechanism underlying cytotoxicity. Therefore, this study aimed to quantify the cellular uptake of flavonoids by oral cancer cells in order to understand the relationship between the cellular uptake and their cytotoxicity. It is hypothesized that the cellular uptake of these flavonoids by oral cancer cells predicts their cytotoxicity. However, the hypothesis was rejected. Further studies are needed to determine and quantify the metabolites of flavonoids formed by the oral cancer cells, as several researchers have reported that flavonoid metabolites can retain anticancer activities.

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Chapter 1: Introduction

1. Oral Cancer

Oral cancer is the 8th most common cancer worldwide and is a leading cause of death.[1,2] Despite the recent improvements in oral cancer treatment, oral cancer is problematic due to the delayed clinical symptoms and no specific biomarker resulting in poor diagnosis.[1]

1.1 Definition

Oral cancer is a malignant condition that arises on the lip or oral cavity, including the gingiva, tongue, mouth floor, salivary glands, parotid, and throat.[1,2] The tumors primarily arise from epithelium cells. They initiate at the vermillion border of the lips then spread to the buccal mucosa throughout the mouth, tongue, floor, and palate.[3] More than 90% of oral cancer are squamous cell carcinomas (OSCC).[1,2]

1.2 Epidemiology

Oral cancer is more prevalent in males than females in most ethnic groups.[1] Hispanic and black men have higher oral cancer rates than white men.[4] According to the World Health Organization (WHO), in 2020, 330,000 death and 657,000 new cases of oral cancer were reported. These numbers were estimated to be doubled by 2035.[2] The recent study on global patterns and trends in cancers of the lip, tongue, and mouth in 2020 showed that the highest

incidence of oral cancer was found in South-Central Asia and parts of Oceania where the use of oral tobacco and betel quid is common.[3]

1.3 Risk factors

There are multiple risk factors of oral cancer. However, tobacco use and excessive alcohol drinking (more than 5 drinks on the same occasion) are the major risk factors, involved in approximately 90% of total oral cancer cases.[1]

Tobacco Compared to non-smokers, smoking increases the risk of developing oral cancer by three times.[1]

Alcohol Alcohol can cause many adverse effects that increase oral cancer risk. For example, it increases the permeability of oral mucosa and interferes with DNA synthesis and repair. Chronic alcohol consumption is also associated with an innate and acquired immunity impairment.[1]

Human papillomavirus (HPV) Infection with HPV16 is classified by the International Agency for Research on Cancer (IARC) as the cause of cancers of the oral cavity, pharyngeal tonsil, and HPV18 has been associated with developing in oral cancer.[1,4]

Age The risk of developing oral cancer increases with age. Oral cancer occurs typically in individuals over the age of 40.[4]

2. Flavonoids

Flavonoids are secondary metabolites of plants with variable phenolic structures.[5] They are widely found in vegetables, fruits, and certain beverages such as wine and tea.[5] Flavonoids

have attracted attention in recent years as they exhibit a broad spectrum of health-promoting effects.[5] They possess antioxidative, anti-inflammatory, antiallergic, antiviral, anticarcinogenic properties, and many more.[5,6]

2.1 Structure and classification

The basic structure of flavonoids comprises two aromatic rings (A and B) and an oxygenated heterocyclic ring (C).[7] Flavonoids can be divided into six subclasses: flavonols, flavones, flavanones, isoflavones, flavanols (also known as flavan-3-ols or catechins), and anthocyanins.[7,8]

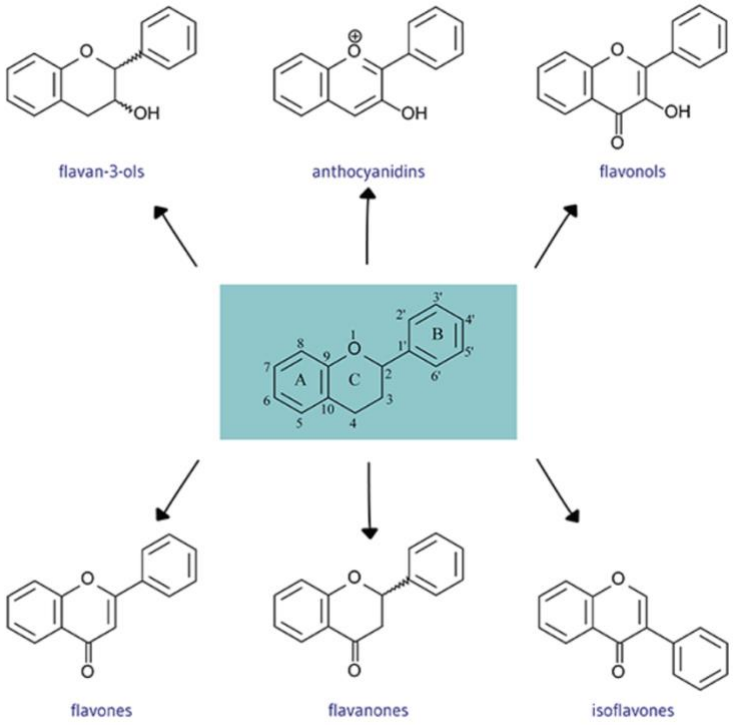

Figure 1: Basic structure and subclasses of flavonoid[9]

2.2 Flavonoids used in our experiment

In the current study, we focused our attention on six different flavonoids with small differences in chemical structure (Figure 2). These flavonoids include naringenin, quercetin, eriodictyol, apigenin, fisetin, and chrysin.

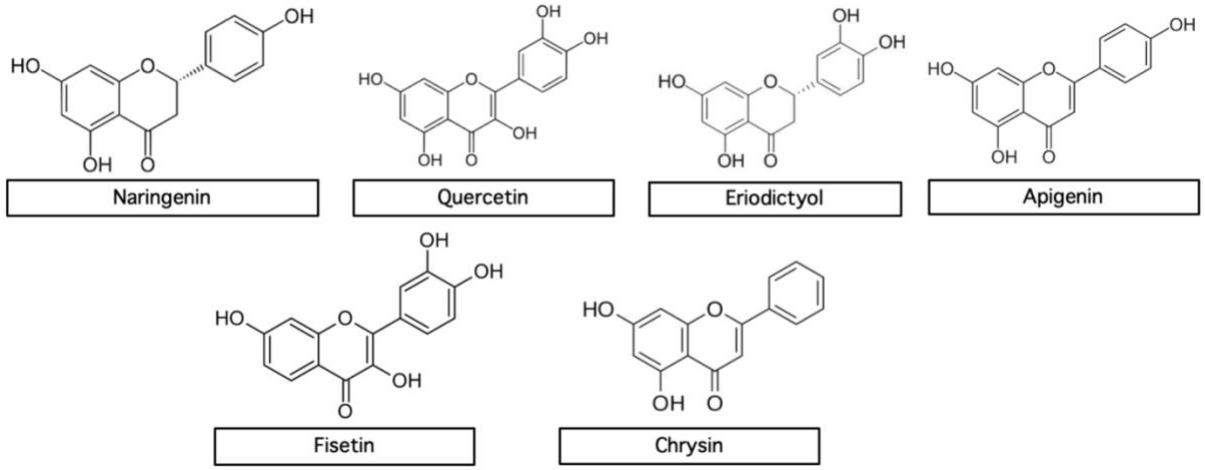

Figure 2: The chemical structure of flavonoids of interest

These flavonoids are found extensively in several foods and plant sources. Table 1 summarizes their subclasses and dietary sources.

Table 1: Flavonoids, their classes, and dietary sources

Flavonoid	Subclass	Dietary sources	Reference
Naringenin	Flavanones	Citrus fruits, grape, cherry, grapefruit, cocoa	[5,10]
Eriodictyol	Flavanones	Lemon, rosehip	[5]
Apigenin	Flavones	Parsley, celery, chocolate, milk	[5,11]
Chrysin	Flavones	Propolis, honey, herbal extracts obtained from species of genus *Passiflora* or *Alpinia oxyphylla* Miquel	[12]
Quercetin	Flavanols	Apple, onion, tea, berries, tomato, grape, brassica vegetables, medical botanicals, including *Ginkgo biloba* and *Hypericum perforatum*	[13,14]
Fisetin	Flavanols	Strawberry, apple, grape, onion, cucumber, persimmon	[15]

2.2.1 Cancer preventive effects

There are at least three distinct mechanisms in which flavonoids may act as chemopreventive agents: (1) By inducing tumor cell death (apoptosis), (2) Inhibition of carcinogen metabolic activation and (3) Inhibition of tumor cell proliferation by inactivation or down-regulation of pro-oxidant enzymes or signal transduction enzymes.[16] Below is the results of research demonstrating the anticancer properties of each of flavonoids of interest, mainly against oral and gastrointestinal tract cancers.

2.2.1.1 Naringenin

The anticancer activity of naringenin against oral cancer has been demonstrated in a few studies. The study by Maggioni et al. showed that naringenin can inhibit the proliferation of oral squamous cell carcinoma (OSCC) cell line, SCC-25 cell.[17] It was found that the cell treatment with naringenin (concentrations range from 150 μM to 300 μM) for 48 hours significantly decreased SCC-25 cell viability, compared to control ($P < 0.01$). The 24-h treatment of SCC-25 cells with 250 μM of naringenin also negatively impacted cell cycle distribution by increasing and decreasing the percentage of cells in G0/G1 phase and the S phase, respectively. At the same concentration and treatment time, western blot analysis showed that naringenin reduced Cyclin D1 expression, which was consistent with the G0/G1 phase arrest observed. Moreover, naringenin can inhibit SCC-25 cell migration as evaluated by the Boyden Chamber migration assay, indicating inhibition of metastasis.[17] Another study by Sulfikkarali et al. demonstrated that oral administration of free naringenin and naringenin-loaded nanoparticles (NARNPs) shows oral cancer-preventive effects against 7,12-dimethyl benz(a)anthracene (DMBA)-induced oral

carcinogenesis in male golden Syrian hamsters.[18] By giving free naringenin (50 mg NAR/kg body weight/day) to DMBA-induced hamsters, only 3 of 10 animals developed OSCC. And, giving NARNPs (50 mg NARNPs/kg body weight/day) to DMBA-induced hamsters completely prevented the tumor incidence and the formation of oral squamous cell carcinoma.[18]

In addition, naringenin exhibits the ability to inhibit other several cancer types, such as breast, skin, colorectal, brain, lung, liver, prostate, bladder, mammary carcinosarcoma cancers. Potential mechanisms for the anticancer properties of naringenin includes GSK3β inactivation, JAK2/STAT3 downregulation, activation of p38/MAPK and caspase-3, and the suppression of the gene and protein activation of NF-kB and COX-2.[19]

2.2.1.2 Quercetin

Quercetin has been shown to have inhibitory effects on colorectal, lung, gastric, breast, ovarian, and hepatic cancers. Cancer preventive effects of quercetin were reported due to several mechanisms such as cell apoptosis, inhibition of angiogenesis, inhibition of P-gp channel, and reduction of oncogene expression.[20] Quercetin was found to inhibit the proliferation of liver cells by causing apoptosis and/or cell cycle arrest. In 13 hepatocellular carcinoma cell lines *in vitro* experiment, after 72 h of treatment with 100 μM of quercetin, the viable cell number decreased to around 20 to 70%.[21] 12 out of 13 cell lines were observed cell cycle arrest with the variation of arrest phase from cell line to cell line (G0/G1 phase, G2/M phase, or S phase cell cycle arrest).[21] Yoshizumi et al. suggested that quercetin-mediated apoptosis may be due to the disruption of microtubules and mitochondria, release of cytochrome *c*, induction of stress proteins, and activation of caspases.[22,23] In the DMBA-induced carcinogenesis hamster model, quercetin also

showed chemopreventive properties against oral squamous cell carcinoma (OSCC) through modulation of NF-κB signaling and its target genes Bcl-2 and Bax.[24]

There are limited numbers of epidemiological studies on the relationship between the consumption of quercetin-rich foods and cancer risk. A case-control study in Swedish populations by Ekström et al. indicated a strong inverse relationship between quercetin intake and the risk of non-cardia gastric adenocarcinoma.[25] The odds ratio (OR) for the highest quantile (≥11.9 mg) of daily quercetin intake was 0.57 (95% CI [0.40–0.83]).[25]

2.2.1.3 Eriodictyol

So far, there has been no report on the anticancer properties of eriodictyol against oral cancer cells. However, eriodictyol was found to be protective against colorectal cancer. *In vitro* study reported some potent eriodictyol on exhibiting antitumor activity [IC = 54.2 μM] in the HCT116 carcinoma cell line.[26] Another study by Huang et al. indicated that eriodictyol suppresses the malignant progression of colorectal cancer by downregulating TSTA3 expression and thus decreasing fucosylation.[27] Cancers are known to exhibit abnormal glycosylation. Glycosylation plays a crucial role in the folding, polymerization, maturation, and transport of proteins, affecting cellular functions.[28] Fucosylation is one of the most common glycosylation modifications that contribute to many malignant transformation events of cancer cells, such as clone formation, proliferation, migration, and invasion.[27,28]

2.2.1.4 Apigenin

Apigenin can inhibit tumor initiation, progression, and metastasis in various cancer types, such as oral, pancreatic, and colorectal cancers.[29] It has been well recognized for promoting cell cycle arrest and apoptosis via the p53-related pathway.[29] A study conducted by Maggioni et al. investigated antiproliferative mechanisms of apigenin against SCC-25 cells and HaCaT human keratinocytes.[30] They observed that apigenin treatment resulted in cell cycle arrest at both G0/G1 and G2/M phases and found that the most effective dose-time combination of apigenin treatment was 100 μM for 24 and 48 h. Western blot analysis showed that apigenin decreased cyclin D1 and E expressions and inactivated CDK1, which is functionally related to cyclin B1.[30]

2.2.1.5 Fisetin

One study demonstrates the anti-proliferative activities of fisetin in human OSCC.[31] It has been suggested that fisetin inhibited cell proliferation and promoted apoptosis by repressing PAK4 expression. PAK4 is involved with cell proliferation and invasion. In several cancer cell lines and tumor tissues, PAK4 is found to be overexpressed and abnormally amplified.[31]

Another study shows the anti-proliferative activities of fisetin against digestive cancers through the activation of caspases in tumors.[32] Human hepatocellular carcinoma (HepG-2), colorectal carcinoma (CaCo-2), and pancreatic cancer (Suit-2) cells were used in the study. In this study, fisetin was also found to inhibit cytochrome P450 3A4, as well as glutathione-S-transferase activity, and the effect was dose-dependent. It was shown in other reports that fisetin induces cell cycle arrest, apoptosis and inhibits the growth of colon cancer cells through multiple signaling pathways such as the activation of CDKN1A, SEMA3E, GADD45B, and GADD45A

and down-regulation of TOP2A, KIF20A, CCNB2, and CCNB1 genes.[32] Furthermore, several studies have shown that fisetin protects against other types of cancer, such as prostate, breast, cervical, bladder, and lung cancers.[32]

2.2.1.6 Chrysin

Xie et al. demonstrate that chrysin induces apoptosis of oral squamous carcinoma KB cell line.[33] Cells were treated with different concentrations of chrysin (1 – 32 μM) for 24 h. Chrysin inhibited proliferation in a dose-dependent manner, and this was accompanied by an increase in cell apoptosis, activating caspase-3/7, decreasing mitochondrial membrane potential, and suppressing AKT and PI3K phosphorylation.[33]

Another study by Wang et al. reported that chrysin also protects against colorectal cancer by blocking cell-cycle progression.[34] As a result of chrysin-treated SW480 colon cancer cells, the G2/M phase arrest was observed in a dose-dependent manner. The percentage of cells in G2/M significantly decreased at doses greater than 40 μM.[34]

2.2.2 Pharmacokinetics

The pharmacokinetic parameters of flavonoids of interest have been studied extensively in mice, rats, and humans. The doses, dosage forms, and plasma pharmacokinetic parameters are varied between studies. A summary table for polyphenol pharmacokinetic and biotransformation studies were included in the Appendix.

2.2.2.1 Naringenin

Some studies have investigated the pharmacokinetics of naringenin in healthy humans. Rebello et al. evaluated pharmacokinetic profiles of healthy adults over 24 hours after an oral dose of 150 mg and 600 mg of naringenin in an extract from whole oranges (NAR) administered in capsule form.[35] The C_{max} of naringenin was 307.5% higher in the NAR600 dose compared to the NAR150 dose (C_{max} = 15.76 μM and 48.45 μM for NAR150 and NAR600, respectively; P = 0.01). In another pharmacokinetic study of the naringenin (1.95 mg/kg) after single oral administration in the form of solid dispersion capsules in six healthy volunteers, naringenin was rapidly absorbed, and its concentrations in plasma were observed 20 min after dosing and reached C_{max} of 7.39 μM in 3.5 hours.[36] In a comparison of these studies, administration of NAR150 and NAR160 produced C_{max} that were two-fold and three-fold higher in less time (T_{max} = 3.17 h and 2.14 for NAR150 and NAR600, respectively).[35,36]

2.2.2.2 Quercetin

There have been extensive quercetin pharmacokinetic studies in healthy humans. The doses and forms used in these studies are varied, including the quercetin aglycone capsule (0.123 mg/kg, 0.307 mg/kg, and 0.768 mg/kg), Quercetin-500 Plus Capsules containing 500 mg quercetin, dietary treatments (apples, onions, and mixture of two; each deliver ~100 mg of quercetin aglycone equivalents), quercetin fortified oral food products (RealFX™ Q-Plus™ Chews (8.22 mg/kg quercetin), Quercetin fortified Tang® suspension (7.61 mg/kg quercetin), and First Strike™ Bar (6.97 mg/kg quercetin)).[37-40] Interestingly, although with the higher dose of quercetin in Quercetin-500 Plus Capsules than dietary treatments of apples, onion, or the mixture

of two, and quercetin fortified oral food products, AUC, C_{max}, and $t_{½}$ of capsule administration was lower than the rest treatments.[38-40]

A pharmacokinetic study in male Sprague-Dawley rats demonstrated that after oral administration of quercetin (100 mg/kg), Quercetin-3-O- β-glucuronide (Q3G) was detected as a major active component in plasma and tissue with $AUC_{(0-t)}$ of 24625.1 mg ·h/L or $AUC_{(0-\infty)}$ of 31328.4 mg ·h/L.[41] These values are 18-fold higher than free quercetin with $AUC_{(0-t)}$ of 1394.6 mg ·h/L or $AUC_{(0-\infty)}$ of 1400.7 mg ·h/L.[41]

2.2.2.3 Eriodictyol

The pharmacokinetics studies of eriodictyol in Sprague-Dawley rats were done using either racemic eriodictyol (20 mg/kg) or *Drynariae rhizoma* extract.[42,43] A study by Yáñez et al. was interested in the stereospecific pharmacokinetics of eriodictyol.[42] They found that both enantiomers reported similar half-lives in serum, namely 4.163 h for R(+)-eriodictyol and 3.678 h for S(-)-eriodictyol. They also found that glucuronide metabolites are due to stereospecific differences as they observed higher concentrations than the S(-)-enantiomer glucuronide than R(+)-eriodictyol glucuronides.[42]

Xu et al. investigated the pharmacokinetics of eight main flavonoids, including eriodictyol, in rat plasma after oral administration of the *Drynariae rhizoma* extract using the selective ultraperformance liquid chromatography-mass spectrometry (UPLC-MS/MS) method.[43] For eriodictyol, the administration of 4 g/kg dried root of *D. rhizoma* extract containing 2.64 mg/kg eriodictyol) resulted in a peak plasma concentration of 894.09 ng·h/mL. The time to reach peak plasma concentration was 6 hours, with a respective half-life of 2.49 hours.[43]

2.2.2.4 Apigenin

A study of the pharmacokinetics of apigenin in humans following a single oral dose of parsley bolus containing 0.74 mg apigenin/kg found that C_{max} was 192.22 nmol/L and AUC was 61.98 min·μmol/L.[44] Other pharmacokinetic studies of apigenin were mostly conducted in rats either via oral or intravenous administration. The doses in rat studies ranged from 5.4 mg/kg to 100 mg/kg.[45-48] The forms of doses affected the pharmacokinetic profiles in a rat model, as shown in the study conducted by Alshehri et al.[48] With the same doses (10 mg/kg), oral administration of pure apigenin resulted in higher C_{max} and AUC_{0-24}, but lower Tmax and $t_{½}$, compared to oral administration of a commercial capsule.[48]

2.2.2.5 Fisetin

Only a few studies have been performed to assess its bioavailability in body tissues. I obtained two fisetin pharmacokinetic studies in male Sprague-Dawley rats.[49,50] Shia et al. found that after an intravenous bolus of fisetin (10 mg/kg), there was a rapid decrease of fisetin parent form ($t_{1/2}$ = 2.7 min) with the appearance of sulfate and glucuronide conjugates of fisetin.[49] They found that the AUC_{0-720} of fisetin sulfates/glucuronides was 4.6-fold that of fisetin glucuronides and 14.0-fold that of the fisetin parent form. However, after the oral administration of fisetin (50 mg/kg), the fisetin parent form could be quantitated only in a few specimens within 90 min. They are then hydrolyzed by sulfatase and glucuronidase, resulting in a dramatic increase of fisetin sulfates/glucuronides in the plasma.[49]

In another study by Huang et al., fisetin (30 mg/kg dissolved in PEG 400) was given intravenously.[50] The results reveal that free fisetin is rapidly transformed into its major

conjugated form through phase II metabolism in the liver, in agreement with the findings of Shia et al. The concentration of fisetin in both plasma and bile decreased rapidly within 30 min. Within 15 minutes of dosing, fisetin glucuronides and sulfates were detected, but levels declined relatively slowly ($t_{½}$ = 134.4 min and 208.8 min for glucuronides and sulfates, respectively).[50]

2.2.2.6 Chrysin

The pharmacokinetic studies of chrysin in rats have been conducted using herbal extracts, such as *Alpiniae Oxyphyllae* Fructus (Yuzhi) and *Radix scutellariae*, or a pure compound.[51-54] The doses of herbal extract in rat studies ranged from 0.25 mg/kg to 58.8 mg/kg mg/kg. The results showed that with oral administration of a pure compound (20 mg/kg), T_{max} was 4.0 h, while the T_{max} was < 1.0 h when chrysin was administered orally in herbal extracts.[51-54]

One study conducted a pharmacokinetic study on human volunteers.[55] Seven healthy participants were given oral administration of 400 mg of chrysin capsule. The results showed that chrysin-sulfate was the major form detected in the plasma with a mean AUC of 1490 ng/mL·h. Free chrysin was detected in a less significant amount in the plasma with a mean AUC of 64 ng/mL·h.[55]

2.2.3 Cellular uptake

To the best of our knowledge, no information is available on the cellular uptake of flavonoids of interest by oral cancer cells. However, the cellular uptake of some flavonoids has been investigated in Caco-2 human colon adenocarcinoma cells.[56,57] One study by Fang et al. investigated the cellular uptake of apigenin, chrysin, quercetin, and naringenin in Caco-2 cells.[56]

The Caco-2 cells were treated with 40 μM flavonoid for 1 h. The intracellular concentrations of the flavonoids were quantified by HPLC with a standard curve of each compound and normalized to the protein concentration within the same well. This resulted in cellular uptake in the 10^{-3} mol/g proteins unit. The cellular uptakes of flavonoids that accumulated in the cells after incubation for 1 h in the Caco-2 cell were reported to be 9.33 ± 1.65, 10.99 ± 0.91, 11.77 ± 0.62, and 5.91 ± 1.22 (× 10^{-3} mol/g proteins) for apigenin, chrysin, quercetin, and naringenin, respectively.[56]

Another study by Boyer et al. also used Caco-2 cells to examine the uptake of quercetin aglycone and quercetin 3-glucoside as purified compounds and from shallot and apple peel extracts.[57] Caco-2 cells were incubated with 1 mL of 50 μM quercetin or 1 mL of 100 μM quercetin 3-glucoside in serum-free Hanks' balanced salt solution (HBSS) for 1, 2, 4, 8, 12, or 24 h. The results were that, in quercetin-treated cells, quercetin levels peaked after only 20 min (8.6 ± 0.8 nmol), stabilized after 40 min, and decreased after 60 min ($P < 0.05$). In quercetin 3-glucoside-treated cells, peak accumulation of quercetin 3-glucoside occurred at 40 min (0.95 ± 0.12 nmol), but declined after 60 min ($P < 0.05$). Another experiment in this study was that Caco-2 cells were incubated for 40 min with 0 – 150 mg/mL apple peel or shallot extract in HBSS. It was shown that quercetin and quercetin 3-glucoside from the shallot and apple were taken up by Caco-2 monolayers in a dose-dependent manner. Interestingly, quercetin uptake from the shallot extract was greater than the uptake of pure quercetin ($P < 0.05$).[57]

3. Purpose of study and hypothesis

The purpose of this study is to quantify the cellular uptake of flavonoids of interest by SCC-25 oral cancer cells so that we can understand the relationship between the cellular uptake of flavonoids and their cytotoxicity. It is hypothesized that cellular uptake predicts cytotoxicity. The flavonoid that accesses the cell to the greatest extent will cause the most cytotoxic effects on oral cancer cells.

Chapter 2: Materials and Methods

Chemicals and reagents

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 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) and Coomassie Plus (Bradford) Assay Reagent were ordered from Thermo Fisher Scientific (Waltham, MA, USA). For experimental purposes, a 100 mM stock solution of each compound was prepared in dimethylsulfoxide and stored at -80 ℃. All other reagents used in these experiments were of the highest commercial grade available.

Cell culture and growth condition

SCC-25 human oral squamous cell carcinoma cells were purchased from ATCC (Manassas, VA, USA). Cells were maintained in the DMEM: F-12 (1:1) medium supplemented with 10 % fetal bovine serum (Atlanta Biologicals, Atlanta, GA, USA) and 1% penicillin streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37 ℃ in 95% humidity and 5% CO_2.

Cellular uptake studies

Cells were seeded in six-well plates at a seeding density of 3.1×10^5 cells per well in a 2 mL growth medium and incubated for 48 h to allow attachment. Cells well were then treated

with 50 μM flavonoids in 2 mL of complete media per well and incubated for 1 h. After that, the old medium was removed, and the cells were washed three times with ice-cold PBS. The cells were harvested by adding 250 μL of a mixture of 0.2 % ascorbic acid and 0.01% EDTA・4Na, followed by washing the wells with an additional 100 μL of the mixture. Then, the harvested cells were sonicated using an ultrasonic processor (5 times for 5 s at setting 4). The lysed cells were centrifuged at 14,000 rpm for 10 min in a cold room. The supernatants were collected, and 50 μL of them was used for protein determination via the Bradford assay (using the standard microplate protocol from Thermo Fisher Scientific).[58] 150 μL of supernatants were mixed with an equal volume of ice-cold methanol. The mixture was centrifuged at 14,000 rpm for 10 min to precipitate the proteins. The supernatant (200 μL) was analyzed by HPLC.

Protein determination

The protein standards were prepared by diluting BSA (2.0 mg/mL) with water, using the dilution scheme of Bradford assay protocol from Thermo Fisher Scientific. [58] 10 μL of each standard or unknown sample and 200 μL of Coomassie Plus (Bradford) Assay Reagent were added to the microplate wells. The plate was mixed with a plate shaker for 30 s and incubated for 10 min at room temperature. The absorbance at 595 nm was then evaluated using the plate reader. The average Blank-corrected 595 nm measurement for each protein standard vs. its concentration in μg/mL were plotted to generate the standard curve. This standard curve was used to determine the protein concentration of each unknown sample.

HPLC analysis

Equipment and chromatographic conditions

Apigenin, Chrysin, Eriodictyol, and Naringenin

The analysis was performed using the Shimadzu HPLC system with two LC-20AD pumps, a SIL-20AC refrigerated autosampler, and an SPD-20AV UV-Vis detection system (Shimadzu Scientific, Kyoto, Japan). The analytic column used was a Supelcosil LC-18 column (150 mm × 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA). The mobile phases consisted of A (0.1% formic acid in water) and B (0.1% formic acid in methanol) and the gradient program was set as follows: solution B, 5-50% (0-15 min), 50-95% (15-35 min), 95% (35-40 min), and 5% (40.1-45 min). Eluant was monitored at 280 nm. The flow rate was 1 mL/min, and the injection volume was 100 μL.

Fisetin and Quercetin

The analysis was performed using the same HPLC system and analytic column as above. But, a different detector was used---an ESA 550 Coularray Detector. The detector potentials were set at -100, 100, 300, and 760 mV. 300 mV was used to determine peak area. The mobile phases consisted of A (1.75% acetonitrile and 0.12% tetrahydrofuran in 30 mM NaH_2PO_4 (pH 3.35)) and B (58.5% acetonitrile and 12.5% tetrahydrofuran in 15 mM NaH_2PO_4 (pH 3.45)), with the following gradient elution program: 4% B at 0.01 min, 4% B at 7 min, 17% B at 25 min, 28%

B at 31 min, 33% B at 37 min, 98% B at 38 min, 98% B at 43 min, and 4% B at 44 min. The flow rate was 1 mL/min, and the injection volume was 50 µL.

Calibration curves

Quantitation of cytosolic flavonoid levels was done by peak area measurements in comparison with calibration curves for naringenin, eriodictyol, apigenin, fisetin, and quercetin. 100 mM stock solutions in dimethylsulfoxide of all flavonoids were diluted in water at different concentrations for the standard preparation. For naringenin and eriodictyol, the 0.01, 0.03, 0.1, 0.3, and 1 µg/mL solutions were prepared to plot the calibration curves. For apigenin, 0.03, 0.1, 0.3, and 1 µg/mL were prepared to plot the calibration curves. For fisetin and quercetin, 0.1, 0.3, 1, and 3 µg/mL were prepared to generate the calibration curves.

Data Analysis

The cytosolic level of each flavonoid (µg/mL) from HPLC analysis was normalized with the protein concentration (µg/mL). This resulted in cellular uptake in the nmol flavonoid/ mg protein unit. All the values are expressed as mean ± SD. The values were analyzed using One-way ANOVA, and the significance of the difference between means was determined by Tukey comparisons using Minitab. A P-value of less than 0.05 was considered as being statistically significant.

Chapter 3: Results

HPLC analysis

HPLC profiles of the purified flavonoids

Using HPLC/UV-Vis (280 nm) analysis, the retention times are 16.15, 17.9, 22.2, 23.4 minutes for eriodictyol, naringenin, apigenin, and chrysin, respectively (Figure 3). HPCL-ECD chromatograms (Figure 4) showed that fisetin and quercetin eluted at 16.23 and 19.0 minutes, respectively.

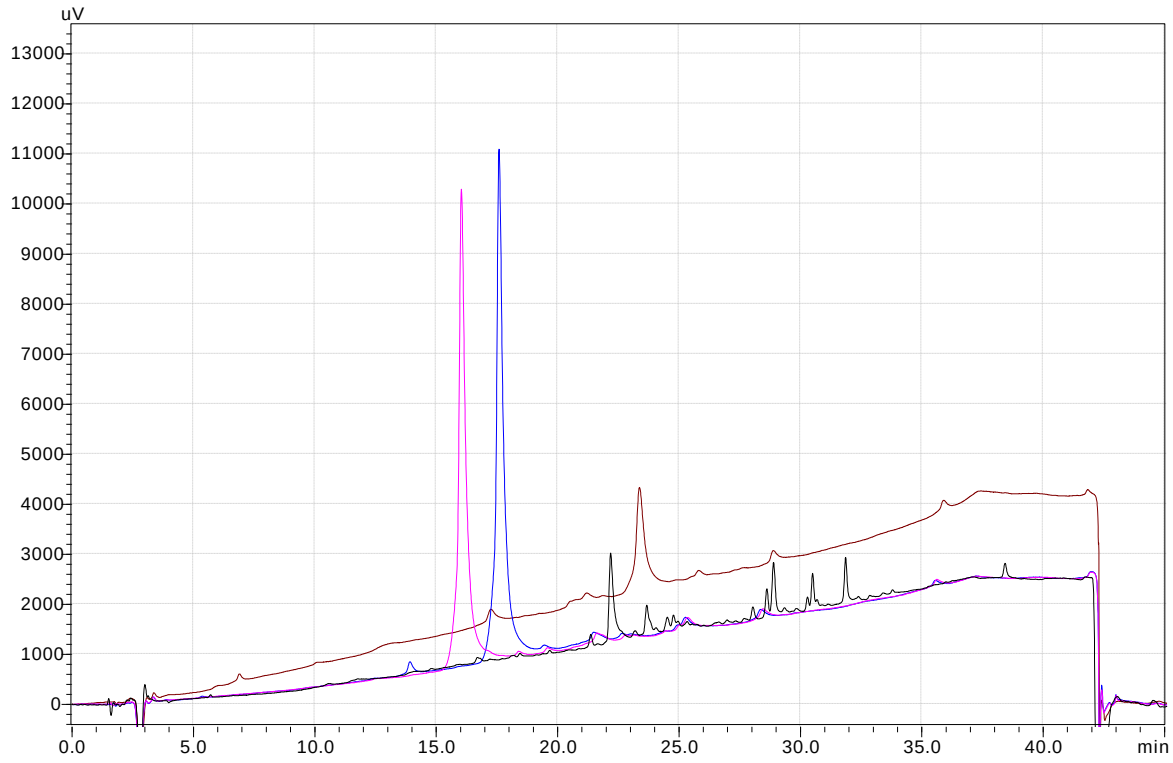

Figure 3: HPLC/UV-Vis (280 nm) chromatograms of the standards (1 µg/mL). Peaks: pink = eriodictyol, blue = naringenin, black = apigenin, and brown = chrysin.

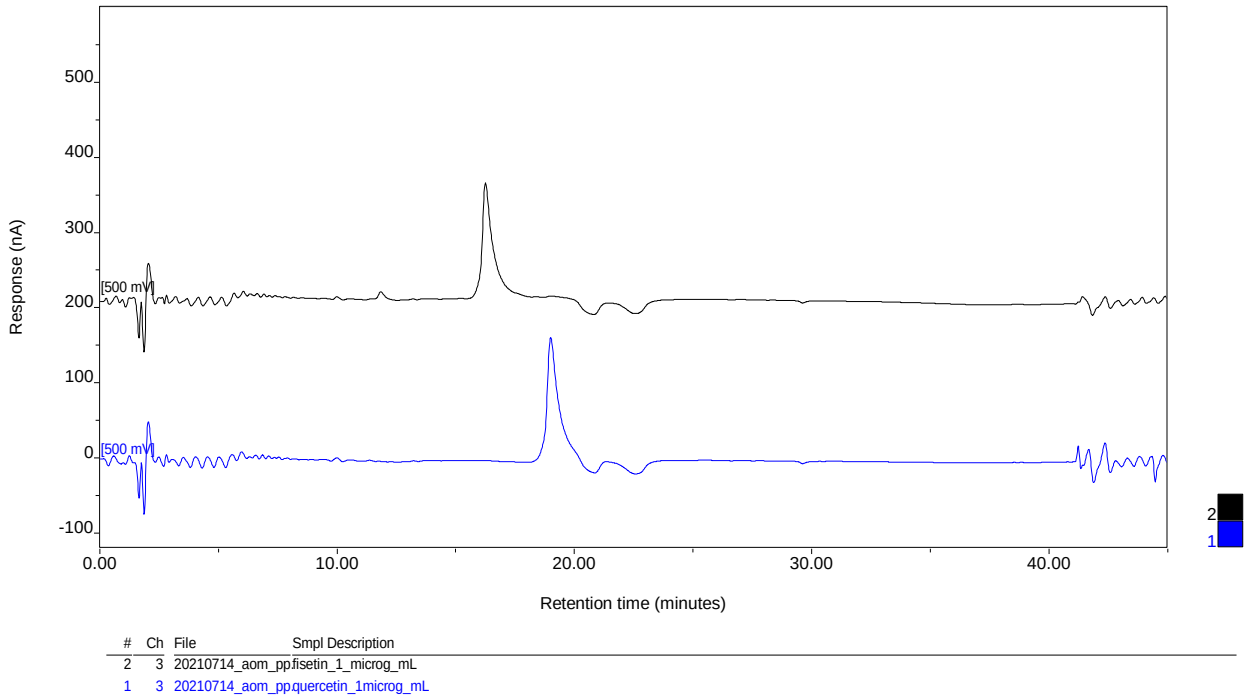

Figure 4: HPLC-ECD chromatograms of the standards. Peaks: black = fisetin, blue = quercetin.

Calibration curves of standard flavonoids and flavonoid-treated SCC-25 cells chromatograms

Naringenin

The calibration curve of naringenin was linear from 0.01 µg/mL to 1 µg/mL with a slope = 218128 (n = 5, S.E. = 1781.2), a y-intercept = -3327.5 (n = 5, S.E. = 27.2), and R^2 0.9998 (Figure 5). Naringenin-treated SCC-25 cells show the peak at 17.9 minutes, indicating the cells took up some naringenin (Figure 6).

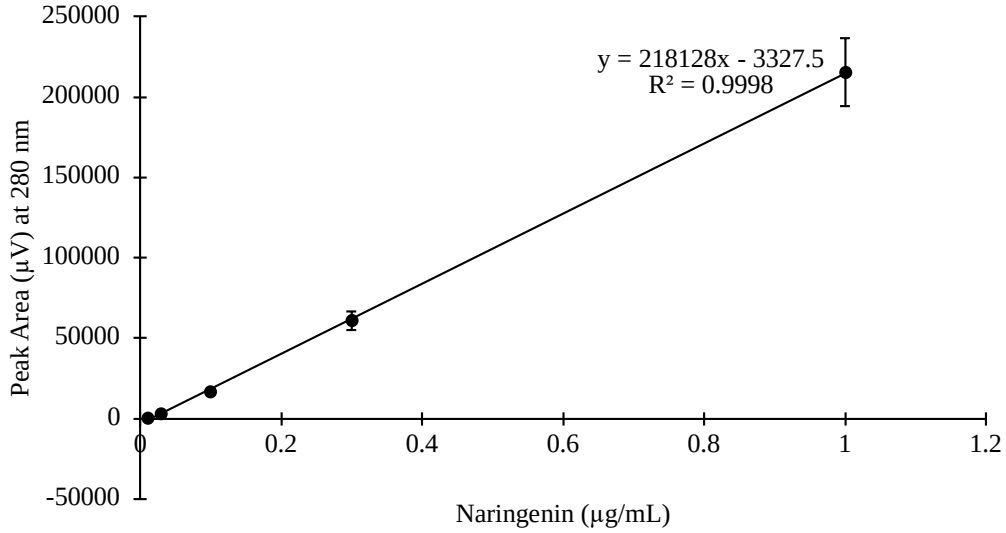

Figure 5: HPLC/UV-Vis (280 nm) calibration curve of standard naringenin

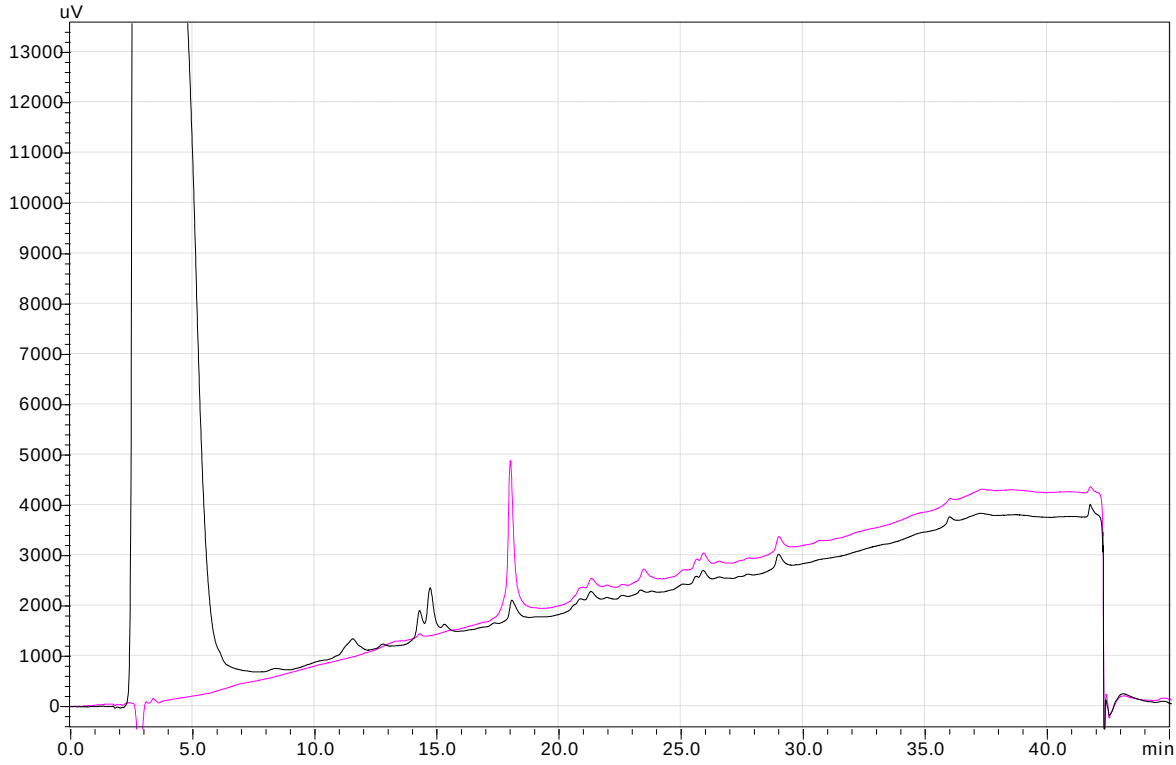

Figure 6: HPLC/UV-Vis (280 nm) chromatogram of naringenin in cytosol of SCC-25 cells treated for 1 h with 50 µM. Peaks: pink = naringenin standard (0.3 µg/mL), black = naringenin-treated SCC-25 cells.

Eriodictyol

The calibration curve of eriodictyol was linear from 0.01 µg/mL to 1 µg/mL with a slope = 224179 (n = 5, S.E. = 2894.9), a y-intercept = -3120.1 (n = 5, S.E. = 40.3), and R^2 0.9995 (Figure 7). HPLC chromatogram of eriodictyol-treated SCC-25 cells (Figure 8) showed the presence of eriodictyol inside the cells as its retention time is very close to that of standard eriodictyol.

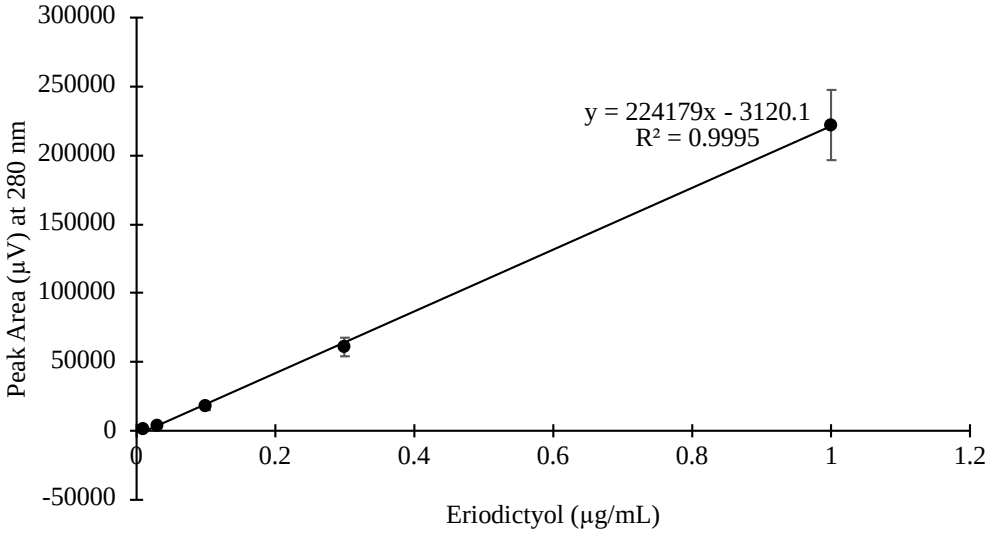

Figure 7: HPLC/UV-Vis (280 nm) calibration curve of standard eriodictyol

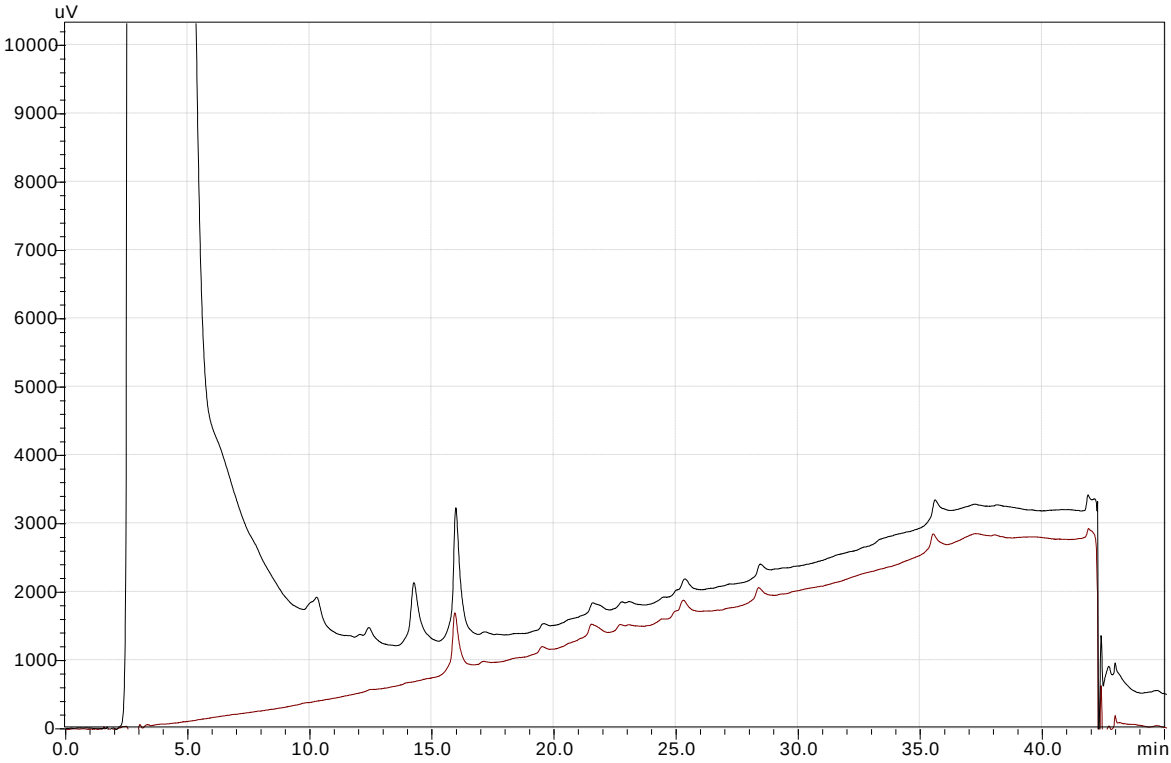

Figure 8: HPLC/UV-Vis (280 nm) chromatogram of eriodictyol in cytosol of SCC-25 cells treated for 1 h with 50 µM. Peaks: brown = eriodictyol standard (0.1 µg/mL), black = eriodictyol-treated SCC-25 cells.

Apigenin

The calibration curve of apigenin was linear from 0.03 µg/mL to 1 µg/mL with a slope = 27794 (n = 4, S.E. = 786.8), a y-intercept = -1076.3 (n = 4, S.E. = 304.7), and R^2 0.9984 (Figure 9). The retention time of apigenin (22.2 min) was found be very close with that of peak present in the chromatogram of apigenin-treated SCC-25 cells (22.35 min) (Figure 10).

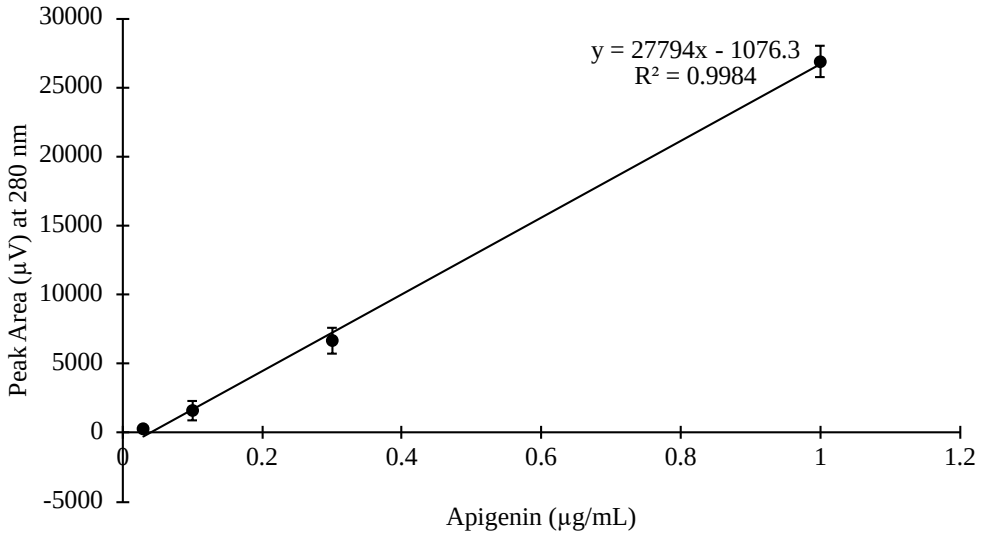

Figure 9: HPLC/UV-Vis (280 nm) calibration curve of standard apigenin

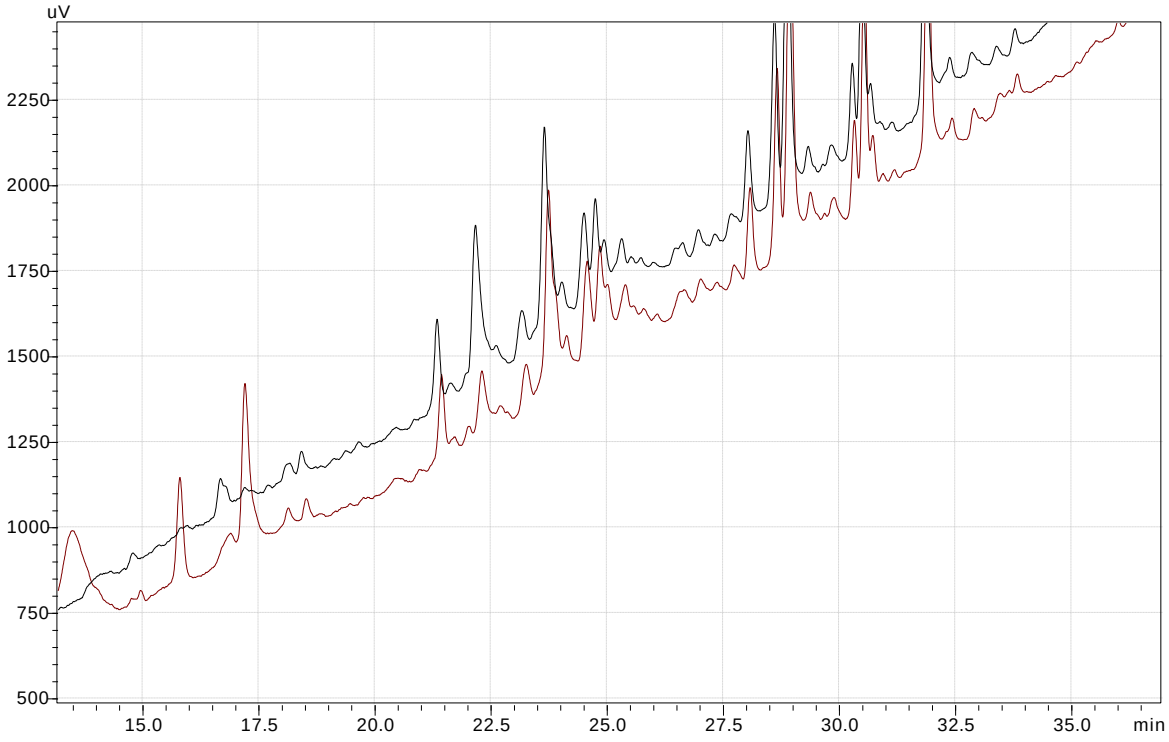

Figure 10: HPLC/UV-Vis (280 nm) chromatogram of apigenin in cytosol of SCC-25 cells treated for 1 h with 50 µM. Peaks: black = apigenin standard (0.3 µg/mL), black = apigenin-treated SCC-25 cells.

Chrysin

The calibration curve of chrysin could not be generated as chrysin because the solubility was poor in water and 5% methanol. A further experiment is needed to develop the method for chrysin standard preparation.

Fisetin

The calibration curve for fisetin revealed significant linearity between concentration and peak area (Figure 11; *slope* = 9.0388, y-intercept = -0.4102, R^2 = 0.9993). HPLC chromatogram of fisetin-treated SCC-25 cells (Figure 12) showed the intracellular fisetin as its retention time is very close to that of standard fisetin.

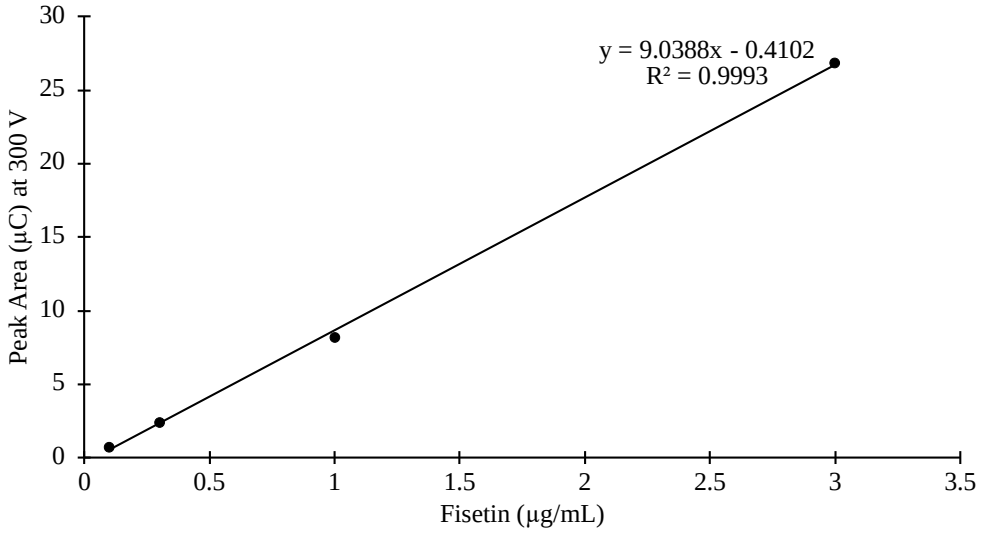

Figure 11: HPLC-ECD calibration curve of standard fisetin

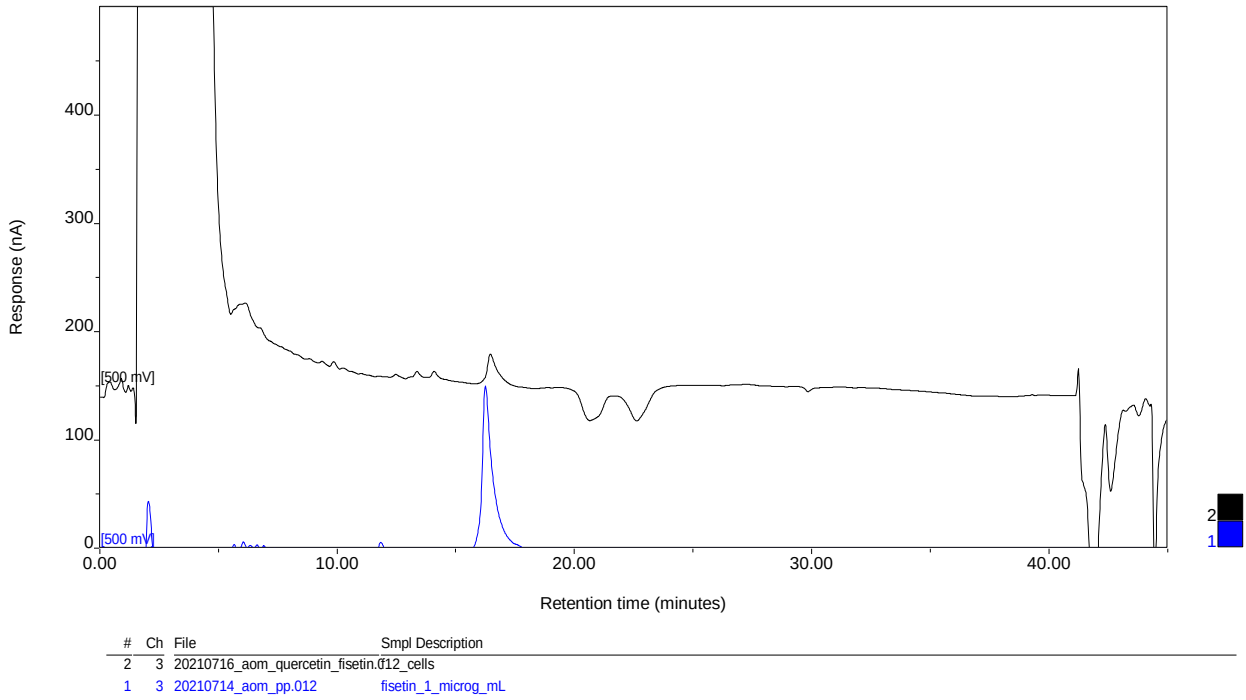

Figure 12: HPLC-ECD chromatogram of fisetin in cytosol of SCC-25 cells treated for 1 h with 50 µM. Peaks: blue = fisetin standard (1 µg/mL), black = fisetin-treated SCC-25 cells.

Quercetin

Figure 13 shows the calibration curve of standard quercetin (equation: y =11.18x – 0.1153). Each point fits well with the linear regression line, indicating that it is a good description of the relationship between peak area and concentration. The R^2 value is 1, further confirming that the line of regression is suitable for this data set. The retention time of peak on chromatogram of quercetin-treated SCC-25 cells (Figure 14) perfectly matches that of quercetin (19 min).

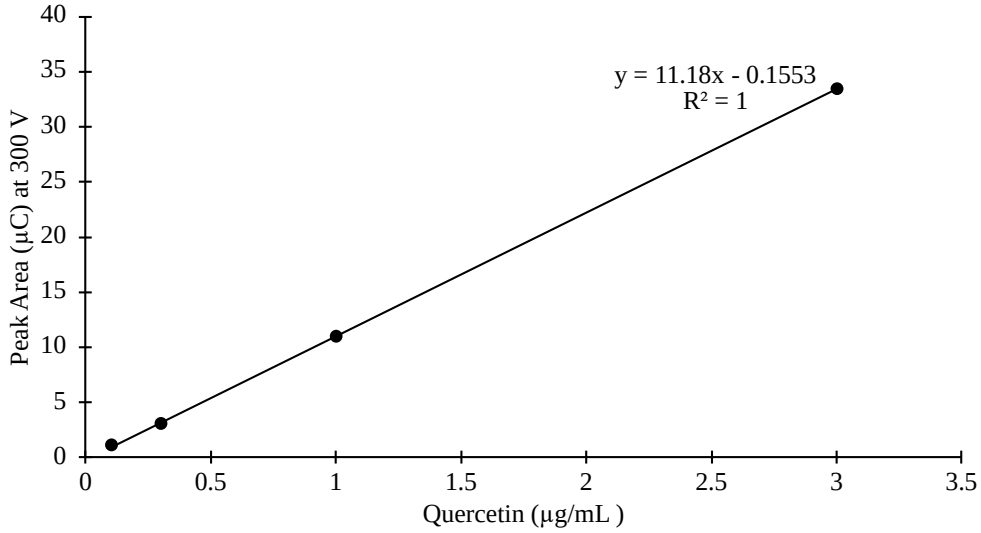

Figure 13: HPLC-ECD calibration curve of standard quercetin

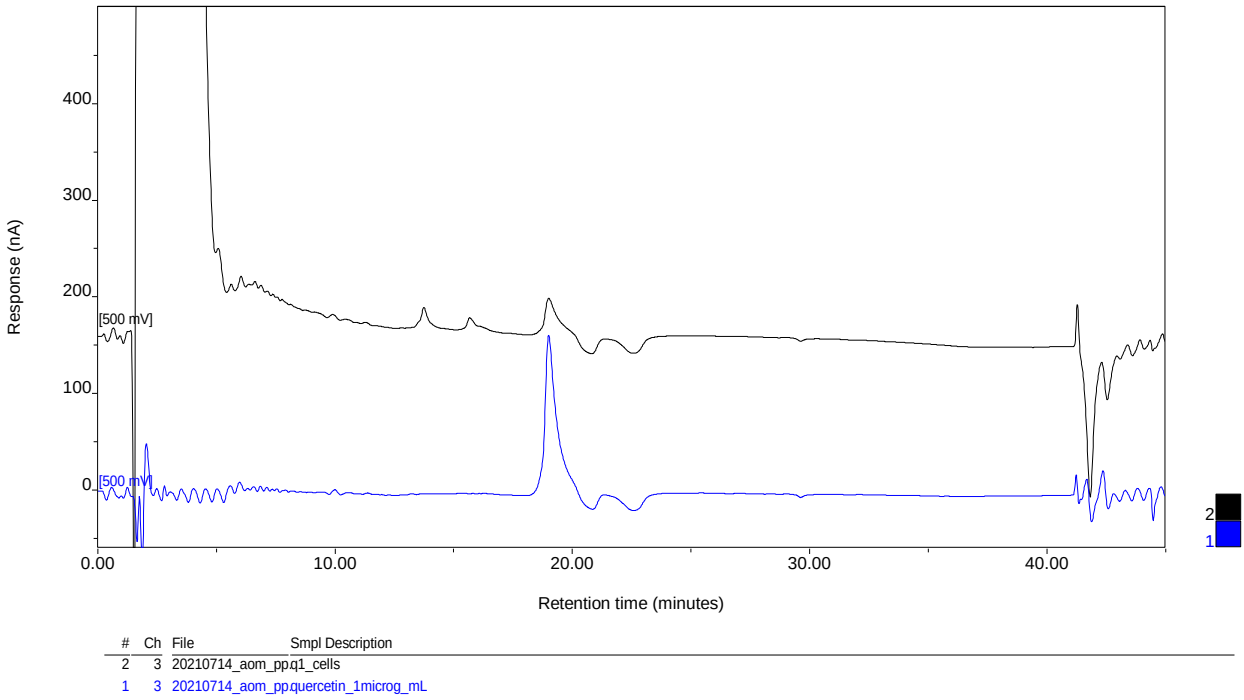

Figure 14: HPLC-ECD chromatogram of quercetin in cytosol of SCC-25 cells treated for 1 h with 50 µM. Peaks: blue = quercetin standard (1 µg/mL), black = quercetin-treated SCC-25 cells.

Standard curve for protein determination

From the Bradford assay protocol from Thermo Fisher Scientific, if using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. [58] And, only blank-corrected 595 nm measurement of BSA concentration at 125, 250, 500, 750, 1000, and 1500 µg/mL should be plotted to generate the standard curve. [58] Therefore, the equation of standard curve for protein determination is $y = -2E\text{-}07x^2 + 0.0008x - 0.0178$.

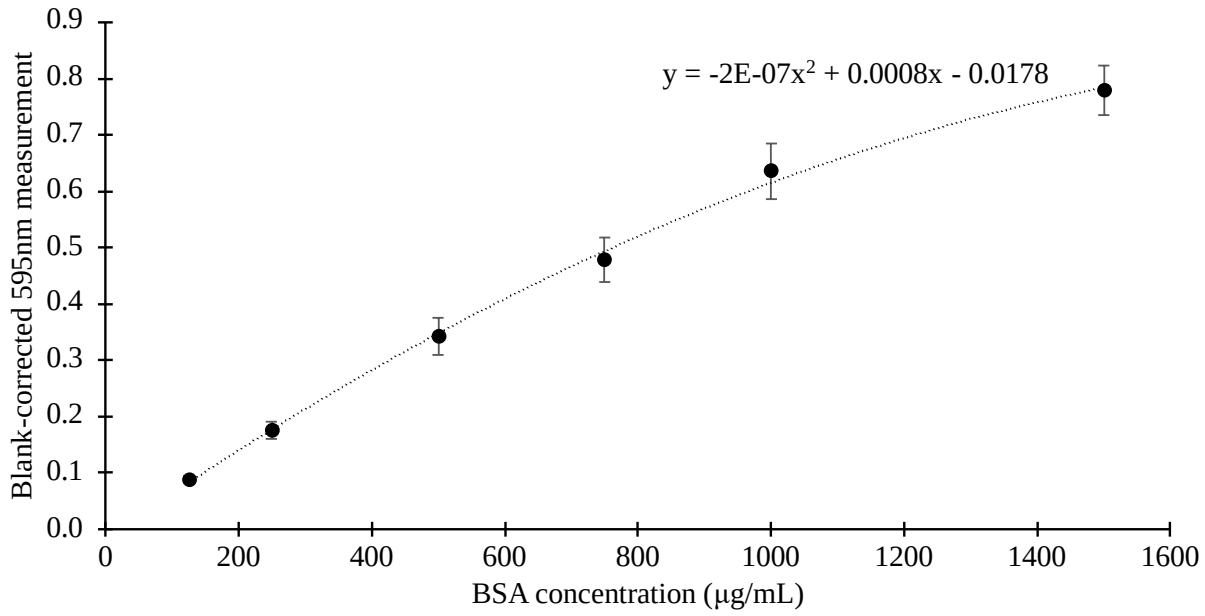

Figure 15: Standard curve for protein determination

Cellular uptake by human oral squamous cell carcinoma cells (SCC-25)

By One-way ANOVA, the p-value is less than 0.05. Therefore, the significant difference of the means cellular uptake of different flavonoids by SCC-25 cells exists. Based on Tukey comparisons, cellular uptake for each compound is significantly different from the others.

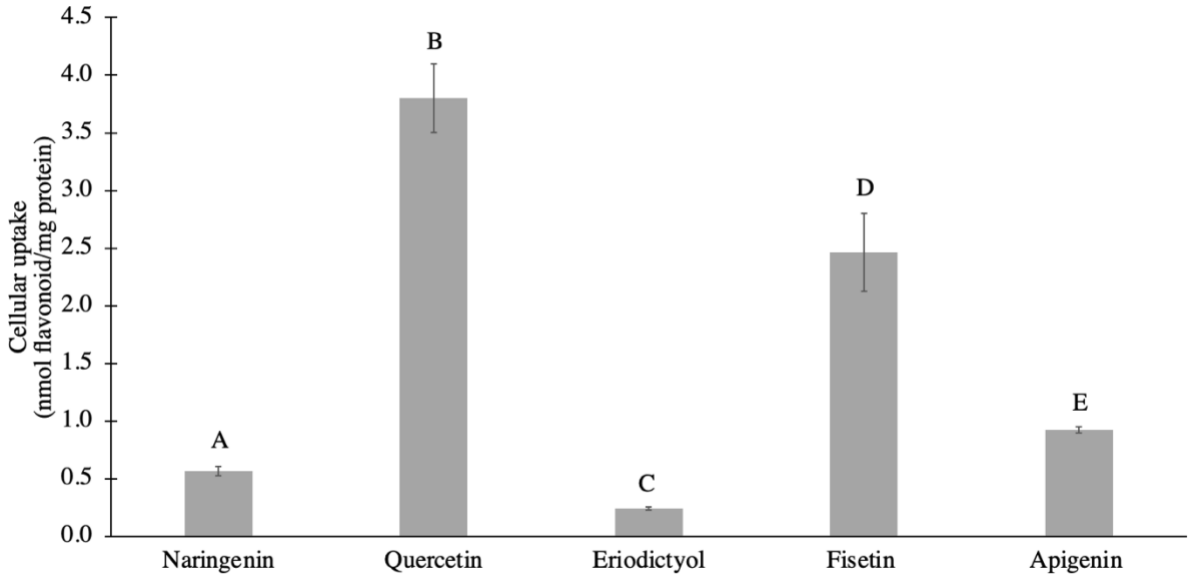

Figure 16: SCC-25 cell uptake of dietary flavonoids (1 hour). Uptake of naringein, quercetin, eriodictyol, fisetin, and apigenin. Mean ± SD values shown (n = 11 for fisetin and apigenin; n = 12 for naringein, quercetin, and eriodictyol). The concentration of the flavonoids used was 50 μM. Different letters indicate significantly different observations ($P < 0.05$).

Chapter 4: Discussion

The development of improved cancer therapies is critical and continues to be a primary focus of cancer research. Flavonoids are getting considerable attention, primarily due to their safety and therapeutic potential. Our lab has been interested in flavonoids' ability to inhibit the growth of oral cancer cells. We found that minor differences in flavonoid structure led to significant differences in anticancer activity---the cytotoxic effects on oral cancer of flavonoids of interest were compared and ranked from the most to the least: Apigenin > Fisetin > Naringenin > Eriodictyol > Quercetin.

The results of the present study indicate that the cellular uptake of five flavonoids by SCC-25 cells is significantly different from each other ($P < 0.05$). The cellular uptake can be ranked from the most to the least: Quercetin > Fisetin > Apigenin > Naringenin > Eriodictyol (Figure 16). These results demonstrate that cellular uptake of flavonoids does not predict cytotoxicity, rejecting the hypothesis. For example, although apigenin did not access the oral cancer cell to the greatest extent, it exhibited the most cytotoxicity. However, flavonoids' anticancer properties are not only determined by the parent flavonoids but also by their metabolites.[59] Thus, one possible explanation for observed results in this study is that SCC-25 cells may metabolize these flavonoids, and these metabolites may have a decrease or increase in cytotoxic characteristics. For instance, Yoon et al. found that naringenin derivatives with methoxy or hydroxy substituent at the C-7 position show greater inhibitory effects on HCT116 human colon cancer cells than naringenin.[60] Another example is the difference in the effect on cell death between apigenin and luteolin, apigenin's metabolite. According to the investigation of apigenin metabolism using liver models of rats *in vitro* (subcellular fractions), phase I metabolism led to the formation of three monohydroxylated derivatives with luteolin as the

major metabolite.[61] Fernández et al. reported that luteolin caused cell death in the HT-29 adenocarcinoma cell line, while apigenin had no effect at all.[62]

So far, there have been no studies on the metabolism of flavonoids by oral cancer and the anticancer activity of those metabolites. Further studies are needed to determine and quantify the metabolites of naringenin, quercetin, apigenin, eriodictyol, fisetin, and chrysin formed by oral cancer cells. To understand the mechanism underlying cytotoxicity, it is important to know what metabolites of flavonoids are formed and whether these metabolites' levels predict cytotoxicity.

This study also demonstrates that standard chrysin solutions could not be prepared by diluting a stock solution chrysin in DMSO into water due to its low water solubility. A further experiment is needed to establish a method to prepare standard chrysin solutions for HPLC analysis in the order to obtain a quantification of cellular uptake of chrysin by SCC-25 cells.

Appendix

Summary Table for Flavonoid Pharmacokinetic and Biotransformation Studies*

Compound	Test Subjects (humans, mice, rats) Number Fasted/ Fed	Dose (mg/kg body weight) ** Dosage form (pill, drink, food, etc.)	Plasma Pharmacokinetic parameters (C_{max}: maximum plasma concentration T_{max}: time to C_{max} AUC: exposure $t_{1/2}$: Elimination half-life)	Major Metabolites in Plasma, Urine, Tissues	Reference
Apigenin	Healthy human n = 11 (5 women) Fasted	0.74 mg/kg Parsley bolus	C_{max} (nmol/l): 192.22 T_{max}: N/A AUC (min•µmol/l): 61.98 $t_{1/2}$: N/A	N/D	Meyer et al., Ann Nur Metab 50 (2006) 167-172
Apigenin	Sprague-Dawley rats n = 4 (all male) Fasted	20 mg/kg Intravenous bolus administration	$AUC_{(0-t)}$ (µg/L h): 3211.54 $AUC_{(0-\infty)}$ (µg/L h): $t_{1/2}$ z (h): 1.75 C_{max} (µg/L): 10933.88	Luteolin (3',4',5,7- tetrahydroxy flavone)- major hepatic metabolite	Wan et al., J. Chromatogr. B 855 (2007) 286-289
Apigenin	Sprague-Dawley rats n = 5 for each treatment group (all male) Fasted	One group was intravenously with 5.4 mg/kg apigenin in form of pure compounds. Another was orally given 100 mg/kg of Flos Chrysanthemi extract (FCE) (equal to 5.4 mg/kg apigenin, dissolved in 0.5%	**Pure compounds (Actual intravenous)** C_{max}: N/A T_{max}: N/A AUC (0-t) (µM•h): 297 AUC (0-∞) (µM•h): 313 t1/2 (h): 9.08 **FCE (Oral doses)** C_{max} (µM): 16.5	N/D	Chen et al., Drug Metab. Pharmaco kinet. 27 (2012) 162-168

		CMC-Na aqueous solution).	T_{max} (h): 3.30 AUC (0-t) (μM•h): 143 AUC (0-∞) (μM•h): 157 $t_{1/2}$ (h): 5.73		
Apigenin	Albino rats n = 6 (does not mention how many male/female) Fasted	100 mg/kg Oral administration of pure apigenin compound	C_{max} (μg mL^{-1}): 0.14 T_{max} (h): 2.0 AUC_{0-t} (μg mL^{-1} h): 0.84 $AUC_{0-\infty}$ (μg mL^{-1} h): 1.27 $t_{1/2}$ (h): 4.80	N/D	Telange et al., Eur. J. Pharm. Sci 108 (2017) 36-49
Apigenin	Wister Albino rats n = 6 for each treatment (all male) Fasted	10 mg/kg Pure compound and commercial capsule	**Pure compound** C_{max} (μg/mL): 21.38 T_{max} (h): 3.60 AUC_{0-24} (μg h/mL): 146.54 $t_{1/2}$ (h): 7.78 **Commercial capsule** C_{max} (μg/mL): 17.48 T_{max} (h): 4.00 AUC_{0-24} (μg h/mL): 141.98 $t_{1/2}$ (h): 19.94	N/D	Alshehri et al., Saudi Pharm J **27** (2019) 264-273
Chrysin	Healthy Humans n = 7 (2 women) Fasted	400 mg oral dose Capsule	Plasma AUC (ng h/mL) Chrysin: 64 Chrysin sulfate: 1490	Chrysin sulfate in plasma Chrysin glucuronide (2-226 mg) in urine, trace amount of Chrysin sulfate	Walle et al., Br J Clin Pharmacol 51 (2001) 143-146

Chrysin	Wistar rats n=6 (all male) Fasted	8 mL/kg *Radix scutellari*e extract (chrysin = 0.23 mg/mL = 1.84 mg/kg) Oral administration	C_{max} (ng/mL): 90.22 T_{max} (h): 0.40 $t_{1/2}$ (h): 9.72 AUC (0-t) (ng •h/mL): 627.8 AUC (0-∞) (ng •h/mL): 746.9	N/D	L. Tong et al., J. Pharm. Biomed 70 (2012) 6-12
Chrysin	FVB wild type mice (all male, n=4) FVB Bcrp knockout mice (all male, n=4) Fasted	20 mg/kg Oral administration	**Wild-type mice** <u>Chrysin</u> T_{max} (h): 4.00 C_{max} (µm): 0.01 AUC_{0-t} (h•µM): 0.06 <u>C-7-G</u> T_{max} (h): 7.00 C_{max} (µm): 0.16 AUC_{0-t} (h•µM): 2.00 <u>C-7-S</u> T_{max}(h): 6.50 C_{max} (µm): 0.13 AUC_{0-t} (h•µM): 1.32 **Bcrp -/- mice** <u>Chrysin</u> T_{max}(h): 3.31 C_{max} (µm): 0.02 AUC_{0-t} (h•µM): 0.11 <u>C-7-G</u> T_{max} (h): 1.81 C_{max} (µm): 0.22 AUC_{0-t} (h•µM): 2.83 <u>C-7-S</u> T_{max}(h): 3.25	Chrysin-7-O-gluconide (C-7-G) Chrysin-7-O-sulfate (C-7-S)	Ge et al., J. Agric. Food Chem. 63 (2015) 2902-2910

			C_{max} (μm): 0.43 AUC_{0-t} (h•μM): 2.62		
Chrysin	Sprague-Dawley rats n = 6 for each group: normal and dementia rats (all male) Dementia rats were injected with Aβ $_{1-42}$ Doesn't mention Fed/Fasted	*Alpinia oxyphylla* fruit ethanol extract (chrysin = 0.25 mg/kg) Oral administration	**Normal rat** AUC (0- t) (ng h/mL): 78.81 AUC (0- ∞) (ng h/mL): 81.6 C_{max} (ng/mL): 18.06 T_{max} (h): 0.25 t ½ (h): 4.51 **Dementia Rat** AUC (0-t) (ng h/mL): 131.1 AUC (0-∞) (ng/mL h): 135.1 C_{max} (ng/mL): 29.28 T_{max} (h): 0.25 $t_{1/2}$ (h): 4.22	N/D	Zhao et al., Molecule 23, 1702 (2018) 1-11
Chrysin	Sprague-Dawley rats n = 6 for each treatment group (all male) Fasted	ASHP group oral dose (9 g/kg) ethanol extract of *Alpinia oxyphylla* Miq. -*Schisandra chinensis* (Turcz.) Baill. Herb pair (ASHP), chrysin = 58.8 mg/kg AOF group oral dose (3 g/kg) ethanol extract of *Alpinia oxyphylla*	**AOF** AUC (0-t) (ng h/mL): 454.02 AUC (0-∞) (ng h/mL): 487.92 C_{max} (ng/mL): 38.45 T_{max} (h): 0.58 $t_{1/2}$ (h): 9.86 **ASHP** AUC (0-t) (ng h/mL): 554.48 AUC (0-∞) (ng h/mL): 631.24 C_{max} (ng/mL): 50.54	N/D	Y. Qi, X. Cheng, H. Jing et al., J Pharm Biomed Anal 177 (2020) 1-12

		Miq. Fructus (AOF), chrysin = 6.3 mg/kg Aβ+ASHP group intracerebroventricular injection (i.c.v.) with Aβ $_{1-42}$ + oral administration (the same as the ASHP group) * Aβ = Amyloid-beta, one of the underlying mechanisms of Alzheimer's disease Aβ $_{1-42}$ induced AD mouse model	T_{max} (h): 0.56 $t_{1/2}$ (h):11.78 **Aβ+ASHP** AUC (0-t) (ng h/mL): 669.21 AUC (0-∞) (ng h/mL): 770.53 C_{max} (ng/mL): 65.45 T_{max} (h): 0.42 $t_{1/2}$ (h): 13.12		
Erodictyol-8-C-β-D-glucopyranoside (EG)	Sprague-Dawley rats n= 6 (all male) Fasted	2.67 mg/kg (Actual intravenous, n=3) 13.4 mg/kg (Oral doses, n=3)	**Actual intravenous** C_{max} (ng/mL): 2980 T_{max} (h): 0.03 AUC (0-t) (h ng/mL): 714 AUC (0-∞) (h ng/mL): 719 $t_{1/2}$ (h): 1.70 **Oral doses** C_{max} (ng/mL): 221 T_{max} (h): 0.50 AUC (0-t) (h ng/mL): 277 AUC (0-∞) (h ng/mL): 277 $t_{1/2}$ (h): 0.65	N/D	Qiu et al., Biomed. Chromatogr. 29 (2014) 220-225
Eriodictyol, Naringenin	Sprague-Dawley rats	20 mg/kg racemic naringenin,	**R (+)-eriodictyol** AUC inf (µg h/mL) serum:	Eriodictyol glucuronide	Yáñez et al., Biopharm. Drug Dispos. 29 (2008) 63-82

	n=18 (all male) Fasted	racemic eriodyctiol intravenous administration	12.367 $t_{1/2}$ (h) serum: 4.163 $t_{1/2}$ (h) urine: 46.688 **S (-)-eriodictyol** AUC inf (µg h/mL) serum: 11.125 $t_{1/2}$ (h) serum: 3.678 $t_{1/2}$ (h) urine: 47.385 **R (+)-naringenin** AUC inf (µg h/mL) serum: 11.643 $t_{1/2}$ (h) serum: 3.400 t ½ (h) urine: 12.769 **S (-)-naringenin** AUC inf (µg h/mL) serum: 13.410 $t_{1/2}$ (h) serum: 3.648 $t_{1/2}$ (h) urine: 12.769	Naringenin glucuronide	
Eriodictyol, Naringenin	Sprague-Dawley rats n=6 (all female) Doesn't mention Fed/Fasted	4 g/kg dried root of *Drynariae rhizoma* extract: eriodictyol (0.66 mg/g = 2.64 mg/kg), naringenin (2.71 mg/g = 10.84 mg/kg) Oral gavage	**Eriodictyol** AUC (0-t) (ng •h/mL): 894.09 AUC (0-∞) (ng •h/mL): 929.83 T_{max} (h): 6 $t_{1/2}$ (h): 2.49 C_{max} (ng/kg)-232.76 **Naringenin** AUC (0-t) (ng	N/D	Xu et al., J Anal Methods Chem. (2018) 1-11

			•h/mL): 946.98 AUC (0-∞) (ng •h/mL): 1164.21 T_{max} (h): 6 $t_{1/2}$ (h): 3.21 C_{max} (ng/kg)-153.87		
Fisetin	Sprague-Dawley rats n= 12 (all male) Fasted	10 mg/kg, dissolved with tetraglycol and filtered through a 0.22 µm membrane filter, Intravenous administration (n=6) 50 mg/kg, dissolved with tetraglycol, Oral administration via gastric gavage (n=6)	**Intravenous administration** free form AUC_{0-720} (nmol ·min/mL): 970.5 $t_{1/2}$ (min): 2.7 Sulfates/Glucuronides (S/G) AUC_{0-720} (nmol ·min/mL): 13226.9 $t_{1/2}$ (min): 90.0 Glucuronides (G) AUC_{0-720} (nmol ·min/mL): 2891.3 $t_{1/2}$ (min): 40.7 **Oral administration** Sulfates/Glucuronides (S/G) C_{max} (nmol/mL): 72.1 AUC_{0-2880} (nmol ·min/mL): 31726.6 Glucuronides (G) C_{max} (nmol/mL): 27.3 AUC_{0-2880} (nmol ·min/mL): 14355.0	Sulfates/Glucuronides, Glucuronides	Shia et al. J. Agric. Food Chem. 57, 1 (2009) 83-89
Fisetin	Sprague-Dawley rats	30 mg/kg dissolved in PEG 400	PK in Rat Plasma and Bile **1) Plasma**	Glucuronide and Sulfate	Huang et al. J. Agric. Food

		1.1 Control group	Chem. 66 (2018) 6300-6307
The control group (n=6, all male) was given fisetin only, and the CsA-treated group (n=6, all male) was given a pretreatment with cyclosporine (CsA, 20 mg/kg, iv) before being administered fisetin. *Use CsA = P-glycoprotein (P-gp) inhibitor to investigate the role of P-gp on the binary excretion of fisetin Doesn't mention Fed/Fasted	i.v. injection	<u>Free form</u> AUC (min · μg/mL): 275.9 C_{max} (μg/mL): 73.94 T_{max} (min): - $t_{1/2}$ (α) (min): 1.72 $t_{1/2}$ (β) (min): 11.29 $t_{1/2}$ (min): - <u>Glucuronide</u> AUC (min · μg/mL): 1719 C_{max} (μg/mL): 27.06 T_{max} (min): 6.67 $t_{1/2}$ (α) (min): - $t_{1/2}$ (β) (min): - $t_{1/2}$ (min): 134.4 <u>Sulfate</u> AUC (min · μg/mL): 6429 C_{max} (μg/mL): 29.10 T_{max} (min): 16.67 $t_{1/2}$ (α) (min): - $t_{1/2}$ (β) (min): - $t_{1/2}$ (min): 208.8	
		1.2 Treatment group <u>Free form</u> AUC (min · μg/mL): 894.5 C_{max} (μg/mL): 92.45 T_{max} (min): - $t_{1/2}$ (α) (min): 5.94 $t_{1/2}$ (β) (min): 436.7 $t_{1/2}$ (min): -	

Glucuronide
AUC (min · μg/mL): 5806
C_{max} (μg/mL): 54.84
T_{max} (min): 10.33
$t_{1/2}$ (α) (min): N/A
$t_{1/2}$ (β) (min): N/A
$t_{1/2}$ (min): 164.0
Sulfate
AUC (min · μg/mL): 7858
C_{max} (μg/mL): 42.59
T_{max} (min): 32.50
$t_{1/2}$ (α) (min): N/A
$t_{1/2}$ (β) (min): N/A
$t_{1/2}$ (min): 130.6

2) Bile
2.1 Control group
Free form
AUC (min · μg/mL): 402.5
C_{max} (μg/mL): 38.30
T_{max} (min): 5.00
$t_{1/2}$ (min): 24.83
Glucuronide
AUC (min · μg/mL): 1810
C_{max} (μg/mL): 114.4
T_{max} (min): 6.67
$t_{1/2}$ (min): 24.59
Sulfate
AUC (min · μg/mL): 29170

			C_{max} (µg/mL): 1052 T_{max} (min): 8.33 $t_{1/2}$ (min): 50.97 2.2 Treatment group <u>Free form</u> AUC (min · µg/mL): 278.2 C_{max} (µg/mL): 18.47 T_{max} (min): 5.00 $t_{1/2}$ (min): 12.81 <u>Glucuronide</u> AUC (min · µg/mL): 1460 C_{max} (µg/mL): 60.42 T_{max} (min): 17.50 $t_{1/2}$ (min): 59.48 <u>Sulfate</u> AUC (min · µg/mL): 50120 C_{max} (µg/mL): 1787 T_{max} (min):10.00 $t_{1/2}$ (min): 80.04 *Fisetin is absorbed rapidly and go through extensive phase II conjugation via sulfation and glucuronidation.		
Naringenin Hesperitin	Healthy Humans n = 6 (1 female) Fasted	1.95 mg/kg Capsule	**Naringenin** C_{max} (mg/mL): 2009.5 T_{max} (h): 3.67 AUC (ng h/mL): 9424.5 $t_{1/2}$ (h): 5.52	N/D	Kanaze et al., Eur J Clin Nutr 61 (2007) 472 - 477

			Hesperitin C_{max} (mg/mL): 825.78 T_{max} (h): 3.67 AUC (ng h/mL): 4846.20 $t_{1/2}$ (h): 6.64 h		
Narigenin-7-rutinoside Narigenin-7-glucoside	Healthy humans n = 16 (8 women) Fasted	0.83 mg/kg narigenin-7-rutinoside 0.52 mg/kg narigenin-7-glucoside orange juice	**N-7-R** C_{max} (µmol/l): 0.12 T_{max} (min): 311 AUC (0-600 min, µmol/l min): 18 $t_{1/2}$: N/A **N-7-G** C_{max} (µmol/l): 0.77 T_{max} (min): 93 AUC (0-600 min, µmol/l min): 70 $t_{1/2}$: N/A	Narigenin-7-O-glucuronide Narigenin-4'-O-glucuronide	L. Bredsdorff et al., Br. J. Nutr 103 (2010) 1602-1609
Naringenin	Sprague-Dawley rats n= 10 (all male) Fasted	20 mg/kg (S/R enantiomers ratio = 50/50) Oral and Intravenous administration (n=5 for each administration)	**Oral administration - with enzymatic hydrolysis** <u>R-naringenin</u> AUC_{0-t} (µg h/mL): 9.055 $AUC_{0-¥}$ (µg h/mL): 10.155 C_{max} (µg/mL): 3.38 $t_{1/2}$ z (h): 3.932 <u>S-naringenin</u> AUC_{0-t} (µg h/mL): 8.802 $AUC_{0-¥}$ (µg h/mL): 10.377 C_{max} (µg/mL): 3.211 $t_{1/2}$ z (h): 4.638 **IV administration**	N/D	Wen et al., J. Chromatogr. 49 (2011) 316-320

			- with enzymatic hydrolysis R-naringenin AUC_{0-t} (µg h/mL): 24.643 $AUC_{0-¥}$ (µg h/mL): 26.766 C_{max} (µg/mL): 81.791 $t_{1/2}$ z (h): 2.924 S-naringenin AUC_{0-t} (µg h/mL): 19.145 $AUC_{0-¥}$ (µg h/mL): 19.764 C_{max} (µg/mL): 78.097 $t_{1/2}$ z (h): 2.684 **- without enzymatic hydrolysis** R-naringenin AUC_{0-t} (µg h/mL): 2.258 $AUC_{0-¥}$ (µg h/mL): 2.263 C_{max} (µg/mL): 30.923 $t_{1/2}$ z (h): 0.132 S-naringenin AUC_{0-t} (µg h/mL): 2.139 $AUC_{0-¥}$ (µg h/mL): 2.0827 C_{max} (µg/mL): 27.49 $t_{1/2}$ z (h): 0.109		
Naringenin	Sprague-Dawley rats n=6 (all male) Fasted	100 and 300 mg/kg Intraduodenal administration	PK in Rat Jugular Plasma, Portal Plasma, and Lymph Fluid **Dose = 100 mg/kg**	N/D	Tsai and Tsai, J. Agric. Food Chem. 60, 51 (2012) 12435-12442

Portal plasma
AUC (min µg/mL): 1340
T_{max} (min): 26.3
C_{max} (µg/mL): 15.87
$t_{1/2}$ (min): 43.3
Lymph fluid
AUC (min µg/mL): 24.1
T_{max} (min): 60.0
C_{max} (µg/mL): 0.18
$t_{1/2}$ (min): 46.2
Jugular plasma
AUC (min µg/mL): 39.2
T_{max} (min): 180
C_{max} (µg/mL): 0.30
$t_{1/2}$ (min): 63.2
Bile
AUC (min µg/mL): 367.9
T_{max} (min): 157
C_{max} (µg/mL): 0.78
$t_{1/2}$ (min): 844

Dose = 300 mg/kg
Portal plasma
AUC (min µg/mL): 1750
T_{max} (min): 15.0
C_{max} (µg/mL): 12.9
$t_{1/2}$ (min): 105
Lymph fluid
AUC (min µg/mL): 46.5
T_{max} (min): 60.0
C_{max} (µg/mL): 0.51
$t_{1/2}$ (min): 116

			Jugular plasma AUC (min µg/mL): 45.9 T_{max} (min): 15.0 C_{max} (µg/mL): 0.51 $t_{1/2}$ (min): 89.4 Bile AUC (min µg/mL): 426 T_{max} (min): 86.7 C_{max} (µg/mL): 0.66 $t_{1/2}$ (min): 263		
Naringenin	Wistar rats n=3 (all male, per each group) fasted	naringenin: 92 mg/kg Mixture of hesperidin and naringenin (Mix-160): 161 mg/kg Intragastric gavage	**Naringenin** $t_{1/2}$ (h): 5.13 T_{max} (h): 1.67 C_{max} (ng/mL): 310.35 AUC_{0-24} (ng/mL h): 1006.11 **Naringenin in Mixture** $t_{1/2}$ (h): 4.09 T_{max} (h): 2.00 C_{max} (ng/mL): 472.31 AUC_{0-24} (ng/mL h): 1538.14	N/D	Araujo-León et al., Molecule. 25, 4241 (2020) 1-14
Naringenin	Sprague-Dawley rats n=6 (all male) Doesn't mention Fed/Fasted	Citrus x aurantium L./Fructus Aurantii (FA) extract (10.8 g/kg of the original medical material), 10.8 mL kg, naringin=171.6 mg/g raw herb, So, dose of naringenin in the extract = 1,853.28 mg/kg	C_{max} (µg/L): 2448.08 T_{max} (h): 9.33 $t_{1/2}$ z (h): 2.56 AUC (0-t) (µg•h/L): 19617.11 AUC (0-∞) (µg•h/L): 19651.18	N/D	Yuan et al., Front. Med. 11,933 (2020) 1-12

		Oral administration			
Naringenin	Healthy Humans n=18 (female = 12) Fasted	150 mg and 600 mg naringenin from an extract of the whole oranges Capsule	**NAR150** $AUC_{0\text{-}24h}$ (μM × h): 67.61 C_{max} (μM): 15.76 T_{max} (h): 3.17 $t_{1/2}$ (h): 3.0 **NAR160** $AUC_{0\text{-}24h}$ (μM × h): 199.05 C_{max} (μM): 48.45 T_{max} (h):2.41 $t_{1/2}$ (h): 2.65	N/D	Rebello et al. Diabetes Obes Metab. 22 (2020) 91–98.
Naringenin (NAR) a physical mixture of naringenin and nicotinamide (NAR+NCT) naringenin-nicotinamide cocrystal (NAR-NCT)	Sprague-Dawley rats n=18 (half make and half female) fasted	NAR: 30 mg/kg NAR+NCT molar ration of NAR: NCR=1:2, in terms of NAR 30 mg/kg NAR-NCT (57 mg/kg), NAR does= 30 mg/kg Oral administration	**NAR** T max (h): 0.49 C max (ng/mL): 120.8 t ½ (h): 5.37 AUC (0- t) (ng h/mL): 509.4 AUC (0- ∞) (ng h/mL): 567.6 **NAR+NCT** T max (h): 0.11 C max (ng/mL): 494.0 t ½ (h): 5.50 AUC (0- t) (ng h /mL): 393.4 AUC (0- ∞) (ng h/mL): 413.8 **NAR-NCT** T max (h): 0.09 C max (ng/mL): 1018.3 t ½ (h): 8.24 AUC (0- t) (ng h/mL): 891.9	N/D	Xu et al., J Anal Methods Chem. (2020) 1-10

			AUC (0- ∞) (ng h/mL): 936.4		
Quercetin	Healthy Humans n = 16 (7 women) Fasted	Quercetin aglycone capsule (selected doses = 8 mg, 20 mg, and 50 mg and the mean body weight of the subjects = 65.2 kg. So, doses = 0.123 mg/kg, 0.307 mg/kg, and 0.768 mg/kg)	**Dose = 8 mg** $AUC_{(0-24)}$ (µg h/l): 527 $AUC_{(0-32)}$ (µg h/l): 630 C_{max} (µg/l): 41.4 T_{max} (h): 1.9 $t_{1/2}$ (h): 17.1 **Dose = 20 mg** $AUC_{(0-24)}$ (µg h/l): 882 $AUC_{(0-32)}$ (µg h/l): 1058 C_{max} (µg/l): 66.1 T_{max} (h): 2.7 $t_{1/2}$ (h): 17.7 **Dose = 50 mg** $AUC_{(0-24)}$ (µg h/l): 1138 $AUC_{(0-32)}$ (µg h/l): 1323 C_{max} (µg/l): 86.1 T_{max} (h): 4.9 $t_{1/2}$ (h): 15.1	N/D	Erlund et al., Eur J Clin Pharmacol 56 (2000) 545-553
Quercetin	Healthy Humans n = 10 (4 women) Fed	Quercetin-500 Plus Capsules containing 500 mg Quercetin	Quercetin aglycone AUC (ng/mL h): 62.5 C_{max} (ng/mL): 15.4 T_{max} (h): 3 $t_{1/2}$ (h): 3.47	N/D	Moon et al., Biopharm. Drug Dispos. 29 (2008) 205-217
Quercetin	Healthy Humans n=16 (8 women) Fasted	3 different dietary treatments: apples (apple sauce + apple peel), onions (apple sauce + onion power), or a mixture of the	**Apples** C_{max} (ng/mL): 63.8 T_{max} (h): 2.9 $t_{1/2}$ (h): 65.4 AUC_{0-24h} (ng h/mL): 843	N/D	Lee J., Mitchell A. J. Agric. Food Chem. 60 (2012) 3874-3881

		two (apple sauce + apple peel + onion powder) each deliver ~100 mg of quercetin aglycone equivalents per study: 100 mg/60.7 kg= 1.65 mg/kg Women: 100 mg/54.9 kg= 1.82 Men: 100mg/66.5 kg= 1.50 mg/kg	**Onions** C_{max} (ng/mL): 273.2 T_{max} (h): 2.0 $t_{1/2}$ (h): 14.8 AUC_{0-24h} (ng h/mL: 2340 **Mixture** C_{max} (ng/mL): 136.5 T_{max} (h): 2.4 $t_{1/2}$ (h): 18.7 AUC_{0-24h} (ng h/mL): 1415		
Quercetin	Healthy Humans n=18 (not mentioned how many male/female) Fasted	500 mg of quercetin from 3 different quercetin fortified oral food products: RealFX™ Q-Plus™ Chews, Quercetin fortified Tang® suspension, and First Strike™ Bar RealFX™ Q-Plus™ Chews= 500 mg/60.8 kg= 8.22 mg/kg quercetin Quercetin fortified Tang® suspension= 500mg/ 65.7kg= 7.61 mg/kg quercetin	**RealFX™ Q-Plus™ Chews** C_{max} (µg/L): 1051.9 T_{max} (h): 3.3 AUC total (µg/L•h): 4147.1 $t_{1/2}$ (h): 5.5 **Quercetin fortified Tang® suspension** C_{max} (µg/L): 354.4 T_{max} (h): 4.7 AUC total (µg/L•h): 3845.9 $t_{1/2}$ (h): 8.3 **First Strike™ Bar** C_{max} (µg/L): 698.1 T_{max}(h): 2.3 AUC total (µg/L•h): 5314.8 $t_{1/2}$ (h): 8.0	N/D	Kaushik et al., J. Food Sci. 77 (2012) H231-H238

		First Strike™ Bar= 500mg/ 71.7kg= 6.97 mg/kg quercetin n=6 in each group			
Quercetin (QR), Quercetin-3-O- β-glucuronide (Q3G: a glucuronide conjugate of quercetin)	Sprague-Dawley rats n=34 Fasted	100 mg/kg Q3G 100 mg/kg QR oral administration, n=5	**Q3G** <u>Q3G</u> AUC (0-t) (mg •h/L): 24625.1 AUC (0-∞) (mg •h/L): 31328.4 $t_{1/2}$ (h): 2.6 T_{max} (h): 3.1 C_{max} (mg/L): 4964.8 <u>QR</u> AUC (0-t) (mg •h/L): 1394.6 AUC (0-∞) (mg •h/L): 1400.7 $t_{1/2}$ (h): 1.1 T_{max} (h): 2.9 C_{max} (mg/L): 316.1 **QR** <u>Q3G</u> AUC (0-t) (mg •h/L): 39529.2 AUC (0-∞) (mg •h/L): 42730.3 $t_{1/2}$ (h): 2.4 T_{max} (h): 5.0 C_{max} (mg/L): 6694.5 <u>QR</u> AUC (0-t) (mg •h/L): 1583.9 AUC (0-∞) (mg •h/L): 1597.3 $t_{1/2}$ (h): 0.8 T_{max} (h): 0.3 h	Major metabolite Q3G	Yang et. al., Sci. Rep. (2016) 1-9

			C_{max} (mg/L): 842.1		
Quercetin	Sprague-Dawley rats n=5 (all male) Doesn't mention Fed/Fasted	10 mg/kg Single oral gavage	C_{max} (µg/mL): 2.91 T_{max} (h): 0.6 AUC last (µg h/mL): 10.41 $t_{1/2}$ α (h): 0.25 $t_{1/2}$ β (h): 10	N/D	Abdelkaawy et al., Biomed. Chromatogr. 31 (2017) 2-8

*N/D = Not Determined

**If they only give a dose in mg or g, you can calculate the dose in mg/kg bw by dividing the dose by the mean body weight of the subjects (if they give that)

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ACADEMIC VITA

Pornpat (Aom) Jantip

EDUCATION

The Pennsylvania State University — University Park, PA
Anticipated graduation: *May 2022*

- Bachelor of Science in Food Science, Nutritional Sciences Minor
- Schreyer Honors College

RESEARCH EXPERIENCES

Department of Food Science, College of Agricultural Science — University Park, PA
Undergraduate Teaching Assistant — *January 2022 – Present*

- Assist teaching instructor in training undergraduate students with laboratory works for FDSC 410: Food Chemistry and Analysis (II) and grade lab quizzes and reports.
- Hold weekly office hours to answer any course-related questions from students.

Department of Food Science, College of Agricultural Sciences — University Park, PA
Undergraduate Researcher — *February 2020 - Present*

- Supervised by Dr. Joshua Lambert
- Currently writing an Honor Thesis on *in vitro* bioavailability of the dietary flavonoids by oral cancer cells.
- Currently investigating the cellular uptake and metabolism of Naringenin, Quercetin, Apigenin, Eriodictyol, Fisetin, and Chrysin by the SCC-25 oral cancer cells.

Department of Food Science, College of Agricultural Sciences — University Park, PA
Undergraduate Researcher — *August 2020 – December 2021*

- Associate with Dr. Darrell Cockburn
- Reviewed four human clinical trials performed using potato starch, a resistant starch, as a dietary intervention and combined their microbiome data, and analyzed it using the R program.
- Assisted and observed Andrew Puff, a graduate student, in analyzing the impact of pulses on the gut microbiome by using *in vitro* fermentation of fecal samples.

LEADERSHIP AND ACTIVITIES

Thai Student Association (ThSA) — University Park, PA
Event Coordinator — *April 2020 - 2021*

- Planned and organized the club's internal and external events to make a close-knit community among Thai students and to spread cultural awareness and diversity to the Penn State community.

Royal Thai Government Program Brattleboro, VT
Head Chef Assistant *December - January 2018 - 2020*

- Planned the daily menu and ordered supplies and ingredients by regarding nutrition, portion, and vegetarians.
- Delegated work and responsibility to the team to ensure efficiency in the kitchen.

Centre County PAWS State College, PA
Volunteer *January - March 2019*

- Took care of 20+ dogs in the shelter by feeding and walking them.

HONORS AND AWARDS

- Royal Thai Scholar Award recipient *May 2017 - Present*
  - 10 year-financial support through Ph.D. in Food Science
- Spring 2022 College of Ag Sciences Undergraduate Research Award *December 2021 - Present*
- Oswald Scholarship from the College of Agricultural Sciences *August 2021 - Present*
- Mario W D'Alessioio Scholarship in Agricultural Sciences from the College of Agricultural Sciences *August 2021 - Present*
- Chester D., Agnes H., and Robert D. Dahle scholarship from the College of Agricultural Sciences *August 2021 - Present*
- Sharkasi Endowment in Food Science *May - August 2021*
  - 2021 Summer Research Scholarships
- Forbes Chocolate Leadership Award from Food Science Department *August 2020 - May 2021*
- Kelly Shane Quinn Scholarship in Agricultural Sciences from College of Agricultural Sciences *August 2020 - May 2021*
- Ministry of Science and Technology Scholarship, SCIUS *May 2015 - March 2017*

LANGUAGES

- Languages: Thai (native), English (moderate)

SKILLS

- Statistical program: R program (beginner), Minitab
- Lab skills: Mammalian cell culture, Cellular uptake studies with flavonoids, Bradford method, High-performance liquid chromatography (HPLC) analysis (using UV-Vis and ECD detectors)