ASSESSING THE IN VITRO BIOAVAILABILITY OF TANGERETIN AND ITS DERIVATIVES IN CACO-2 CELL MODEL

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And approved by

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ABSTRACT OF THE THESIS

Assessing the in vitro Bioavailability of

Tangeretin and Its Derivatives in Caco-2 Cell Model

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Flavonoids are widely distributed in fruits, vegetables and grains. Tangeretin (TAN) is one of the most abundant polymethoxyflavones (PMFs), which is found almost exclusively in citrus peel. In present work, we applied silica gel column chromatography to purify tangeretin from bitter orange citrus peel extracts mixture. Despite of the wide bioactivities documented, tangeretin has relatively low bioavailability due to its low water solubility, which is a major limiting their application as functional neutraceuticals.

Therefore, hydroxylation and acetylation reaction was carried out to improve the solubility of TAN. Moreover, the bioactivity and bioavailability of TAN, 5-OH-TAN and 5-Ac-TAN were evaluated using cell viability assay and transport assay on Caco-2 human colon carcinoma cell line. When compared to TAN, stronger anti-proliferation activities were stronger in 5-OH-TAN and 5-Ac-TAN among which 5-Ac-TAN showed the strongest inhibition. The Caco-2 monolayer transport assays were in good agreement that 5-Ac-TAN had better ability to across through cell membranes. Interestingly, 5-OH-

TAN and 5-Ac-TAN were subjected to cellular transformation when passing through the cellular membrane. While 5-OH-TAN was partially methylated to TAN, 5-Ac-TAN was transformed into both TAN and 5-OH-TAN, which in total was transported in higher concentration from apical compartment to basolateral side than 5-OH-TAN group.

In summery, 5-Ac-TAN had the strongest bioactivity of inhibiting the growth of Caco-2 colon carcinoma cells than TAN and 5-OH-TAN. In addition, 5-Ac-TAN also higher membrane permeability than 5-OH-TAN in Caco-2 monolayer cells. Therefore, functional group on C5 position seems to have critical effect on the compounds' biological abilities as well as the bioavailability.

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To my lab mates, I would like to thank all of them for their help and kindness.

The last but not the least, I would like to express my gratitude in words to my parents for all their love, support, understanding and encouragement.

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CHAPTER 1 INTRODUCTION

1.1 Polymethoxyflavones

1.1.1 Polymethoxyflavones and tangeretin

Flavonoids, a major class of polyphenolic compounds, are widely distributed among fruits, vegetables and grains. Polymethoxyflavones (PMFs) refer to a class of flavonoids containing two or more methoxyl groups on 15-carbon benzo-γ-pyrone skeleton structure (C6-C3-C6) with a carbonyl group at the C4 position. This group of flavones is found almost exclusively in citrus genus, particularly in the peel of sweet oranges (Citrus sinensis) and mandarin oranges (Citrus reticulata).

Citrus originated in Southeast Asia and the most important commercial varieties include oranges, lemons, grapefruit, tangerines and so on. According to the report about world markets and trade of citrus (United States Department of Agriculture, USDA: Foreign Agricultural Service, July 2014), global orange production for 2013/14 is estimated to rise 2% from the previous year to 50.7 million metric tons. There are two main markets for citrus fruit, of which one is fresh fruit market and the other is the processed citrus fruit market (mainly orange juice). It is estimated that United States production is 6.3 million tons, where 95% of the oranges are used for processing juice, which will yield a considerable amount of orange peel by-product. Although there is a small amount of PMFs in commercial citrus juice due to mixing with peel constituents during the industrial process, most PMFs are obtained from the orange peel by-product (Gattuso et al., 2007).

PMFs have been shown to exhibit an expansive range of biological activities, including anti-tumor (Miyata et al., 2008), anti-carcinogenic (Li et al., 2007), anti-inflammatory (Ho et al., 2012), anti-atherogenic (Whitman et al., 2005) and anti-oxidant (Li et al., 2007). Among which, their greater anti-carcinogenic activity have recently drawn a remarkable attention than other flavones.

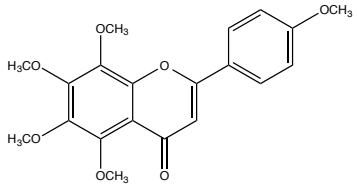
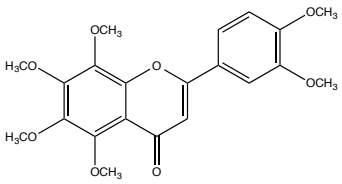
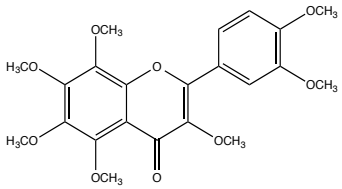
Even though PMFs have higher permeability through the small intestine as compared to polyhydroxylated flavonoids (such as quercetin, narigenin and luteolin), and are readily absorbed into the blood circulation system of the human body because PMFs are more lipophilic due to the hydrophobic nature of methoxyl groups as compared to hydroxyl groups, the bioavailability of PMFs however is still relatively low because of their low water solubility. Since oral administration is a widely applied method for the delivery of foods and drugs, the fact that PMFs have low oral bioavailability limits their application in functional foods. However, there has been a lag in the investigation on the bioavailability of PMFs.

So far, more than 20 polymethoxylated flavonoids have been isolated and identified from different tissues of citrus plants (Li et al., 2006). Table 1.1 shows the list of PMFs mostly found in citrus peel and also closely related to this present study. Among the flavonoids identified from citrus plants, tangeretin (TAN) and nobiletin (NOB) are two most common and abundant PMFs, which also almost exclusively exist in citrus peel. The importance of both compounds is suggested by a voluminous literature.

Tangeretin, which contains 5 methoxyl groups, can be exclusively found in the peel of most citrus fruits, in which contain up to 30 ppm tangeretin. It has white crystalline solid

appearance and stay chemically stable as a solid over extended period at -20□ or 80□. Tangeretin is soluble in methanol or ethyl acetate, yet insoluble in water. Besides these physical property advantages, animal research shows the potential of tangeretin as a cholesterol-lowering agent (Kurowska et al., 2004). In vitro studies show that tangeretin appears to induce apoptosis in leukemia cells while sparing normal cells (Hirano et al., 1995). And tangeretin can block cell cycle progression at the G1 (growth) phase in colon cancer cell lines. Therefore the fact that tangeretin is a great functional ingredient has been widely accepted because of its great anti-carcinogenic activity as well as its stable physical properties.

Table 1.1 Structures of major PMFs isolated and identified from citrus plants closely related to this present study.

Structure	Name	Molecular Formula	Molecular Mass (g/mol)
	Tangeretin (5,6,7,8,4'- penta methoxylflavone)	$C_{20}H_{20}O_7$	372
	Nobiletin (5,6,7,8,3',4' - hexa methoxylflavone)	$C_{21}H_{22}O_8$	402
	HeptaMF (3,5,6,7,8,3',4'-hepta methoxyflavone)	$C_{22}H_{24}O_9$	432

1.1.2 Hydroxylated polymethoxyflavones (OH-PMFs) and 5-demethyltangeretin (5-OH-TAN)

Hydroxylated polymethoxyflavones (OH-PMFs) are a group of novel PMFs containing one or more hydroxyl groups and they are almost exclusively found in citrus species as well.

Same as PMFs, a broad spectrum of biological activities such as anti-tumor, anti-inflammatory and anti-carcinogenic activities were also found in OH-PMFs. In addition, more and more studies have shown that OH-PMFs have significantly better bioactivities than that of PMFs (Ma et al., 2014; Charoensinphon et al., 2013). The number of hydroxyl groups and the ligand binding property may lead to the changes in their hydrophobicity, permeability to biological membranes and metabolic pathways. And all these factors may contribute to them being more bioactive.

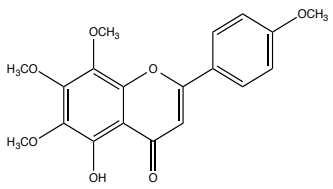
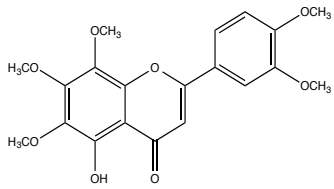
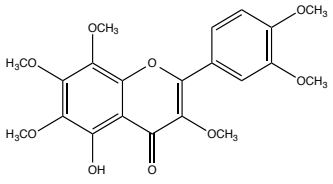
Among these naturally existing OH-PMFs containing different numbers of hydroxyl groups at different positions, 5-OH-PMFs have been widely studied due to their remarkable bioactivities (Zheng et al., 2014). Some major 5-OH-PMFs related to this study are shown in Table 1.2.

5-hydroxy-6,7,8,4'-tetramethoxyflavone (5-OH-TAN) known as gardenin B, was used as an antidote for detoxification of poison; a medicinal herb in Southeast Asia; and is also a taste modifier. It can enhance refreshing flavor, reduce saltiness and the flavor associated with acetic acid, and inhibit unpleasant listing of sweetness (Li et al., 2006).

5-OH-TAN is a special lipophilic PMFs which has been shown to have strong anti-cancer effects. The hydroxyl group at C5 position is important for its anti-cancer activity. However, because of these particular structures, it has poor oral bioavailability due to its low water-solubility and as a consequence, relatively poor bioavailability. Thus this may limit its application.

Although 5-OH-TAN is exclusively exist in citrus fruit peels, its amount naturally contained in orange peels is too scarce and it is too difficult to extract and to purify enough amount for a complete basic research from citrus peel extracts. Thus, in this study, 5-OH-TAN was obtained from tangeretin by hydroxylation reaction.

Table 1.2 Structures of major 5-OH-PMFs isolated and identified from citrus plants closely related to this present study.

Structure	Name	Molecular Formula	Molecular Mass (g/mol)
	5-OH-Tangeretin (5-hydroxy-6,7,8,4'-tetramethoxylflavone)	$C_{19}H_{18}O_7$	358
	5-OH-Nobiletin (5-hydroxy-6,7,8,3',4'-pentamethoxylflavone)	$C_{20}H_{20}O_8$	388
	5-OH-HeptaMF (5-hydroxy-3,6,7,8,3',4'-heptamethoxyflavone)	$C_{21}H_{22}O_9$	418

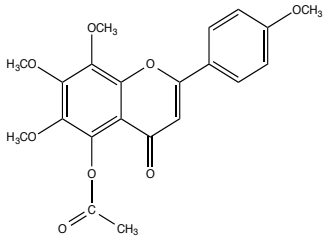
1.1.3 Chemically modified PMFs and 5-acytltangeretin

Since chemical modification is a great method of improving the properties of naturally existing compounds, some study have shown strong interests upon chemically modifying PMFs through synthesizing.

Because of the particular structures of PMFs and OH-PMFs, they tend to have low aqueous solubility and as a consequence, relatively poor bioavailability. This might affect their biological activity in vitro and in vivo. In an effort to improve the bioavailability, some studies have been conducted to derivatize flavonoids by glycosylation and acetylation.

TAN, 5-OH-TAN and 5-Ac-TAN induced significant decrease of cell viability in HCT116 and HT-29 cells. And 5-Ac-TAN treatment displayed more potent and effective cytotoxic effect than that of the two other compounds (Lai et al., 2013). However, the lack of study on 5-Ac-TAN provides us a strong interests in it since it has already shown more potent than tangeretin for inducing cytotoxicity in colon cancer cells.

Table 1.3 Structure of 5-acetyl-tangeretin.

Structure	Name	Molecular Formula	Molecular Mass (g/mol)
	5-Ac-Tangeretin (5-acetyl-6,7,8,4'-tetramethoxylflavone)	$C_{21}H_{20}O_8$	400

1.2 Cancer

1.2.1 Definition

Cancer is a class of diseases arises from an abnormal growth of cells and there are over 100 different types of cancer. Normal cells in the body follow an orderly path of growth, division and death. Cancer begins to form when the programmed cell death process, which is called apoptosis, breaks down. This leads to the abnormal cells uncontrollably grow to from lumps or masses of tissue called tumors and do not die. Then a tumor may spread and invade into other parts of the body and grow.

Utilizing natural compounds has been reported as a plausible approach for cancer prevention.

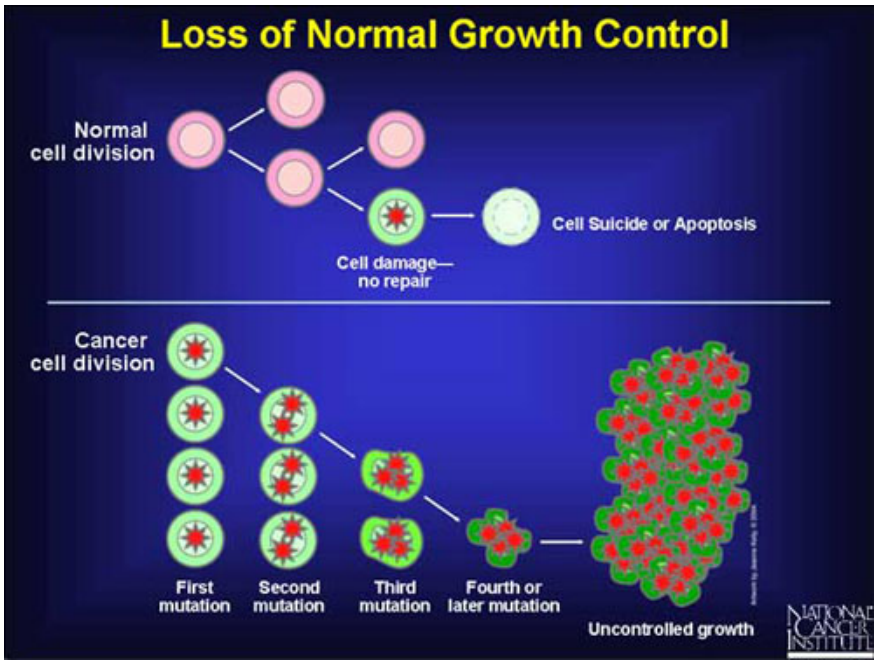


Figure 1.1 Cancer arises from a loss of normal growth control (National Cancer Institute, 2012)

1.2.2 Statistics

According to the World Health Organization (WHO), estimated 8.2 million people worldwide died from cancer (excluding non-melanoma skin cancer) in 2012 and 30% of cancers could be prevented. According to American Cancer Society, there are estimated 1,665,540 new cases and 585,720 deaths from cancer in the United States in 2014.

The information from the US National Cancer Institute's Surveillance Epidemiology and End Results (SEER) Database showed that, from 2009 through 2011, the most current years for which data are available, the risk that a male in United States will develop cancer during his lifetime is one in two while it is one in three for female. Meanwhile, the most common cancer in male is prostate cancer (15.02%) and it is breast cancer (12.33%)

for female in the United States. For both male and female, lung cancer (7.43%, 6.17%) and colon cancer (4.84%, 4.49%) are also two kinds of common cancers.

All these incredible statistics have shown an urgent research and development in cancer prevention and treatment.

1.3 Bioavailability

1.3.1 Overview

The bioavailability is an overall result of absorption, distribution, metabolism and excretion.

(a) Absorption is the capability of a compound passing into the systemic circulation through oral administration.
(b) Distribution describes the efficiency and speed indicating how well a compound reaches the target tissues.
(c) Metabolism explains the rate that a compound is eliminated from the systemic circulation, following its initial absorption.
(d) Excretion is the rate that a compound is excreted from the systemic circulation and ultimately from the body.

Bioavailability is determined by the combination of all these factors above. And Figure 1.2 provides a diagram of the bioavailability process. Therefore, the bioavailability of a compound can be defined as the amount of compound that reaches the blood circulation system and ultimately the intended tissues.

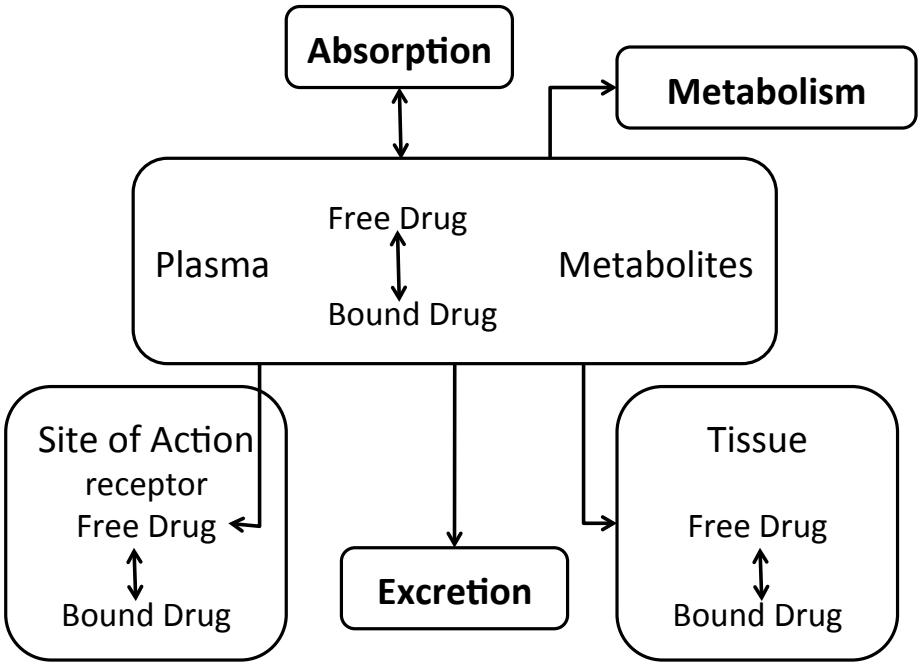


Figure 1.2 Overview of Bioavailability and key factors (Bourne, 2010; Balani et al., 2005; Van de Waterbeemd et al., 2003).

The effectiveness of a compound is dependent on their intestinal absorption to get into blood systemic circulation and ultimately reach the target tissues. Therefore, the intestinal epithelium is a key determinant for the oral consumption of food and pharmaceuticals.

Permeability indicates the velocity of a compound to cross through the intestinal membranes into the blood circulation system. Permeability denotes the overall effects of influx and efflux in the body. Intestine like cells such as Caco-2 cells can be used to predict permeability.

1.3.2 Bioavailability of PMFs

For almost all kinds of foods and drugs, oral administration is the most effective and widely applied method for their delivery. Solubilization, permeation, and metabolism are three important factors affecting the oral bioavailability. Even though there have been a remarkable number of investigations on the bioactive effects of PMFs, only a scarce number of them have been focused on their bioavailability. However, some studies have been shown interests on the solubility of PMFs which is a key factor when accessing the bioavailability but this is way far less to tell a whole story of their overall bioavailability.

1.3.3 Caco-2 cell monolayer model

Caco-2 cell line is a continuous cell of heterogeneous human epithelial colorectal adenocarcinoma cells. This line was isolated from a primary colonic tumor. Upon reaching confluence, the cells express characteristics of enterocytic differentiation. Caco-2 cell monolayers have been widely utilized as a tool for evaluating permeability, assessing the oral absorption levels and studying the absorption mechanism of compounds. In typical permeability and transport assay, Caco-2 cell lines are cultivated on permeable filters. And it usually takes 21 days for Caco-2 cells to grow and differentiate into a monolayer representing and functioning as small intestinal epithelium (Figure 1.3).

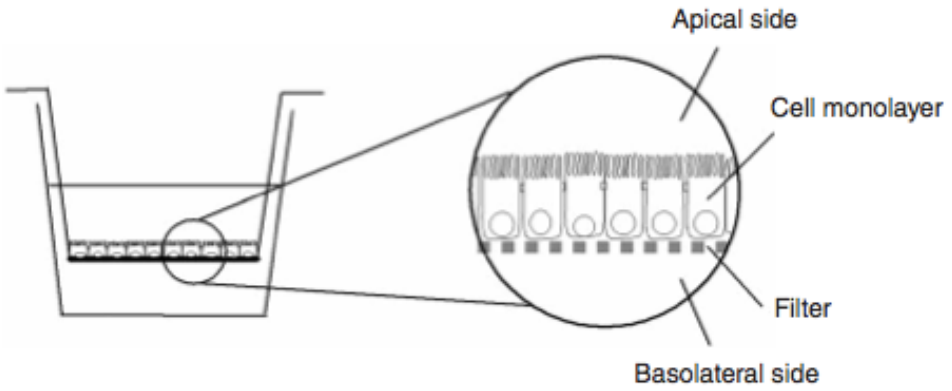


Figure 1.3 Diagram of the Caco-2 cell monolayer cultivated on a permeable filter support. Test compound is place on the apical or basolateral compartments. (Hubatch et al., 2007).

Caco-2 cell monolayer experiments provide important information as follows:

(a) The apparent permeability (Papp) from apical compartment to the basolateral compartment represents the overall effect of transportation carried out by both absorptive transporters and secretory transporters.

(b) The apparent permeability (Papp) from basolateral compartment to apical compartment measures the effects of secretory transporters only.

(c) The ratio of Papp from basolateral compartment to apical compartment over the Papp from apical compartment to the basolateral compartment evaluates efflux. If the ratio is greater than 3, then there is a greater possible of efflux will occur. This means a compound is being pumped out too quickly from the blood circulation system, which will have an effect on the amount of compound in the systemic circulation and thus affect the absorption and bioavailability of the compound.

Therefore, the transport experiment may tell us about the permeability type of a compound as well as its transport mechanisms, including paracellular, transcellar or active carrier (Fig 1.4).

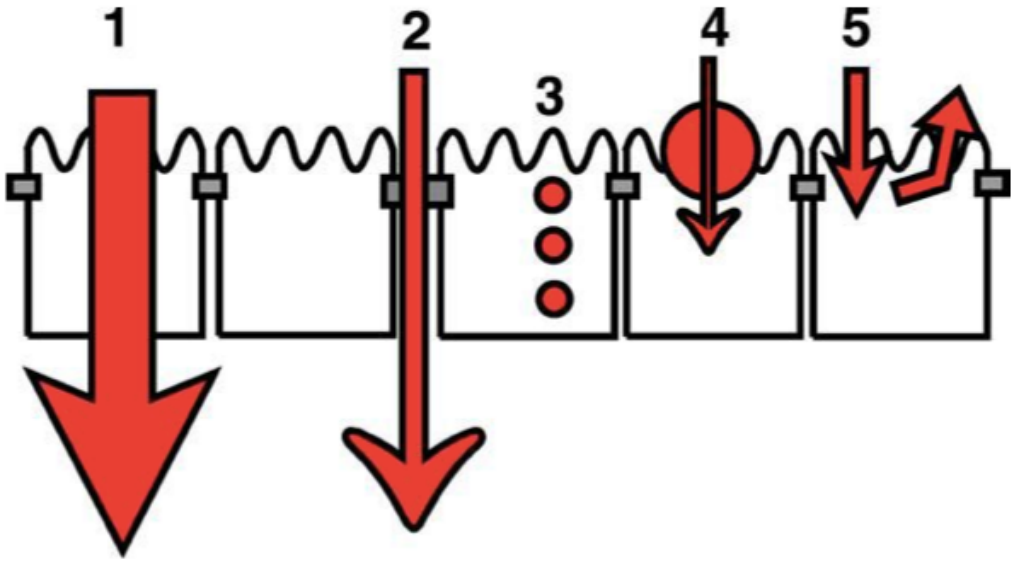


Figure 1.4 Possible compound transport pathways across the intestinal mucosa, illustrating (1) transcellular and (2) paracellular modes of passive transport, (3) transcytosis, (4) carrier-mediated transport, and (5) efflux transport. A combination of these routes often defines the overall transepithelial transport rate of nutrients and drug (Deferme etal., 2008).

Caco-2 cancer cell line model provides deep insight of the preliminary phase of investigation on intestinal permeability, transport, absorption through membranes and overall potential bioavailability of an interested compound.

CHAPTER 2 PREPARATION OF TANGERETIN BY COLUMN CHROMATOGRAPHY TECHNOLOGY

2.1 Introduction

Flavonoids are widely distributed in fruits, vegetables and grains. One class of these compounds is called polymethoxyflavones (PMFs), which is found exclusively in citrus genus, particularly in the peel of sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulata*). So far, more than 20 polymethoxylated flavonoids have been isolated and identified from different tissues of citrus plants (Li et al., 2006). Among these 20 PMFs, tangeretin (TAN) and nobiletin (NOB) are the most common and abundant ones, which almost exclusively exist in citrus peels. Therefore, it is a demanding trend to develop rapid, simple and economical methods to extract major PMFs from different types of citrus peels.

In this study, column chromatography was utilized combined with HPLC to purify and identify the tangeretin and nobiletin from bitter orange peel extracts. The isolation and biological activities of PMFs and their derivatives have gained great attention recently. Many methods have been reported involving the isolation and extraction of PMFs from citrus peels. Although good separation of PMFs was obtained from reversed-phase HPLC were poor. Therefore, it is unfeasible to use reversed phase HPLC to isolate each component in PMFs group.

Column chromatography is utilized to purify individual chemical compounds from mixtures of compounds. The major advantages of this technology come from the relatively low cost as well as the fact that the stationary phase is disposable. Scales used

can be wide, ranging from micrograms to kilograms, which is great for preparation of large scales of products.

Bitter orange is a hybrid from *Citrus maxima* (pomelo) and *Citrus reticulate* (mandarin). It is widely used as essential oil in perfumes and flavors. It is also employed in herbal medicine as a stimulant and appetite suppressant approved by USDA. As a by-product from manufactures, the bitter orange peel was yielded at a large quantity and there will be lots of benefits for the society and economy if it can be effectively utilized.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

The bitter orange peel extracts (without citrus peel oil) used in this study was obtained from China and it was in dry powder form. The components in the mixture were tangeretin and nobiletin. Besides, it contains trace amount of other PMFs, 5-OH-PMFs, brownish pigments and other uncertain impurities as well.

Ethyl acetate (EtAc, ACS grade), hexanes (ACS grade) and acetonitrile (ACN, HPLC grade) were purchased from Pharmco-AAPER (Brookfield, CT, USA). Silica gel (60 Å, 40-63 μm, standard grade) was purchased from Sorbent Technologies (Norcross, GA, USA).

2.2.2 Instrumentation

The glass column (24/40 outer joint, 1 1/2in IDX 18in E.L., 2mm Stpk) was purchased from Chemgalss Life Sciences (Vineland, NJ, USA). The HPLC system was purchased from Dionex (Sunnyvale, CA, USA) and equipped with an UltiMate 3000 Pump, an UltiMate 3000 Variable Wavelength Detector, and an UltiMate 3000 auto-sampler. Data collection, processing and instrument control were achieved using Chromeleon software. Ascentis® RP-Amide C18 HPLC column (15cm X 4.6mm, 3um) was purchased from Supelco Analytical (Bellefonte, PA, USA). The Rotavapor R-114 vacuum evaporator was purchased from Büchi. FT-IR was purchased from Thermo Electron Corporation.

2.2.3 Column chromatographic conditions

This method was preliminary established by Dr. Shiming Li at Food Science Department, Rutgers University. First of all, preparation of a silica gel packed vertical glass column was as follows: the stationary phase (180 g silica gel powder) was suspended in adequate amount of hexane, and carefully poured into a glass vertical column (24/40 outer joint, 1 1/2in IDX 18in E.L., 2mm Stpk). Application of high pressure on the top of the column is highly recommended to avoid the air bubbles and cracks of the stationary phase.

Then 5 g bitter orange peel extract was loaded onto the top of silica gel column followed by covering it with 8 g sea salt to protect the samples from the disturbance of adding mobile phase. All the solid surfaces should be flat.

The tap at the bottom of column was opened before adding fresh eluent into the column. The elute method was designed as gradient elution starting from hexane/ethyl acetate

(V/V) = 100/0 to hexane/ethyl acetate (V/V) = 0/100. Since more polar a component is, the stronger it adsorbs onto the silica gel, and later it is eluted from the column. Therefore, the polarity of the mobile phase is the key factor that affects the retention time and the purity of the interested component. .

The eluate was applied to the thin layer chromatography (TLC) plate and the fluorescence signal was monitored with UV lamp during the process to check when the desired components started to come out and when it was completely collected. Each fraction of eluate was collected with glass beakers or flasks. The volume ratio of mobile phase was changed only when the previous component was completely eluted.

HPLC was used to determine the compounds and their purities in each fraction. The samples were then evaporated by rotary evaporator.

2.2.4 HPLC analysis

Ascentis® RP-Amide C18 HPLC column (15cm X 4.6mm, 3μm) was purchased from Supelco Analytical (Bellefonte, PA, USA) and was used to analyze compounds. The mobile phase consisted of (A) water and (B) acetonitrile. Elution condition, which was developed by Dr. Shiming Li, was as follows: Solvent B increased from 40% to 55% in 10 min, then increased to 70% in 5 min, then increased to 90% in 1 min, then kept for 3 min, then decreased to 40% in 1 min, and then kept for 4 min for equilibrium.

The mobile phases were degassed by sonication for 30 min. The UV-Vis detection wavelengths were set at 214 nm, 280 nm and 326 nm. The flow rate was set at 1.0 mL/min.

2.2.5 UV-Vis analysis

The UV-Vis spectra were collected using Cary 60 UV-Vis Spectrophotometer purchased from Agilent Technologies (Santa Clara, CA, USA). Data collection, processing and instrument control were achieved using the Agilent Cary WinUV software. The scan wavelengths in this study were covered from 200nm to 800nm.

2.3 Results and Discussion

2.3.1 Optimization of column chromatography conditions

The aim of this study was to achieve the separation of tangeretin and nobiletin by column chromatography in a relatively short time with relatively simple gradient profile of the mobile phase.

Mobile phase with different ratios of hexanes to ethyl acetate were evaluated.

After evaluation, the optimal conditions were achieved with gradient elution as follows:

Hexane/Ethyl Acetate (V/V)	Volume (mL)
70/30	500
60/40	500
50/50	500
40/60	1500
30/70	1500

Tangeretin started to come out from the silica gel column at the ratio of hexane/ethyl acetate (V/V) = 40/60, while nobiletin was collected when the mobile phase volume ratio changed to hexane/ethyl acetate (V/V) = 30/70. This is because of their different chemical structures. Tangeretin has only five methoxyl groups while nobiletin has an extra one, which indicates that nobiletin has stronger polarity compared to that of tangeretin. Therefore, nobiletin adsorbed stronger onto the silica gel and needed more polar mobile phase containing more ethyl acetate to be eluted.

We used HPLC to identify each component (refer to chapter 2.3.2) before vacuum evaporating the organic solvent. There were approximately 2.22 g tangeretin and 2.28 g nobiletin in 5 g bitter orange peel extract, accounting for 44.4% and 45.7%, respectively, of the total weight. The final yield of tangeretin and nobiletin after column chromatography purification were 59.9% (1.33 g) and 76.8% (1.75 g), respectively, and their purities were 98% and 99% as determined by HPLC.

The factors affecting the purification efficiency of column chromatography may include: dimension of the column, particle size of the matrix, mobile phases, temperature, pressure, flow rate and packing. In the present study, the purification was conducted under normal pressure, without applying higher pressure on the column, which could be responsible for the moderate yield of tangeretin and nobiletin.

Compared to other isolation and purification methodologies, such as supercritical fluid extraction, column chromatography takes longer time, while it is an easy and cost effective way to purify large amount of compounds, and the purity of the compound isolated by this method is high. Although the volume of the mobile phases required in

this study was large, all the solvents can be recycled and reused, which will reduce the cost of this experiment.

2.3.2 HPLC results

In this study, high performance liquid chromatography (HPLC) was applied to identify the component isolated from the column chromatography by comparing with the purchased standard. The purity of the isolated component was also determined by HPLC.

As shown in Figure 2.1, at the wavelengths of 280nm and 326 nm, there was only one peak shown at the retention time of 11.153 min, which was the peak of tangeretin, as evidenced by comparing the tangeretin standard purchased from Quality Phytochimical LLC (Edison, NJ, USA), which had a peak showing around 11 min. Moreover, the relative area percentage of this peak shown in Figure 2.1 is 97.95%, indicating that the purity of tangeretin isolated from column chromatography is around 98%.

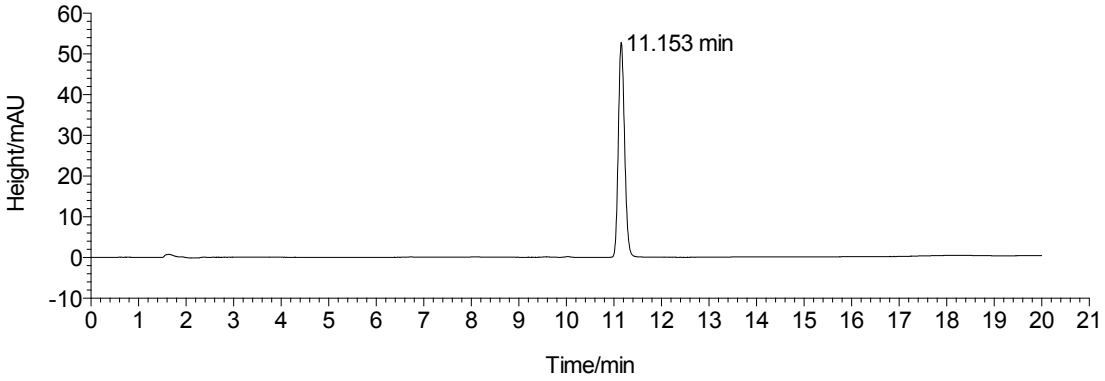


Figure 2.1 HPLC results of TAN at concentration of 10uM at 326nm.

2.3.3 UV-VIS results

By scanning the wavelength range between 200nm and 800nm using Cary 60 UV-Vis Spectrophotometer, the absorption peaks were determined to appear at 268 nm and 329 nm, as shown in Figure 2.2.

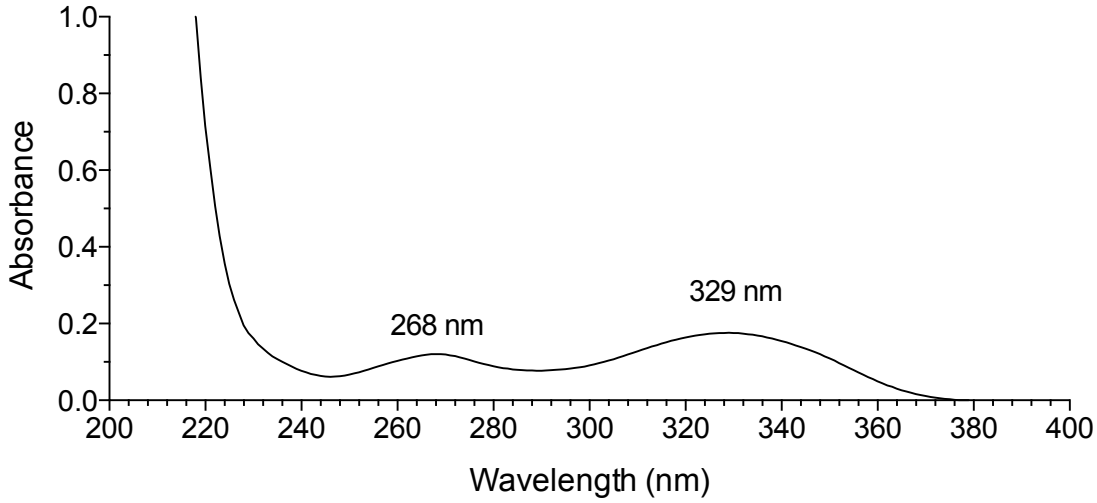


Figure 2.2 UV-Vis graph of TAN at concentration of 10uM

2.4 Conclusion

In conclusion, the method of using column chromatography combined with HPLC for separation and identification of tangeretin and nobiletin was simple, cost-effective, and convenient, and the purity of end product is high.

The column chromatography purification method developed in this chapter can be also used to separate other individual PMFs from a PMFs mixture or to purify an extracts mixture for a need to get rid of the impurity in citrus peel, such as citrus oil. This

technology was applied in the following chapter to purify the desired compound from the reaction products as well.

The HPLC analysis method described above was also used to analyze the trace amounts of tangeretin and its two derivatives in the permeability experiment as well as determine the concentrations of PMFs compounds in various products.

The application of this method allows for obtaining large quantities of tangeretin and nobiletin samples in a relatively short period of time with high purity, and also it is able to determine trace amount of tangeretin for future *in vitro* cell research.

The tangeretin separated from bitter orange peel extract by column chromatography is ready for further chemical modifications, including hydroxylation and acetylation reactions, which generate 5-demethyltangeretin and 5-acetyltangeretin with high purities.

CHAPTER 3 PREPARATION OF DEMETHYLATED AND ACYLATED DERIVATIVES FROM TANGERETIN BY CHEMICAL MODIFICATION

3.1 Introduction

Tangeretin, which contains 5 methoxyl groups, can be exclusively found in the peel of most citrus fruits, which contain up to 30 ppm tangeretin. Hydroxylated polymethoxyflavones (OH-PMFs) are a group of PMFs composed of one or more hydroxyl groups. They are exclusively found in citrus species as well. The number of hydroxyl groups and the ligand binding property may affect their hydrophobicity, permeability to biological membranes and metabolic pathways. And all these factors may contribute to the higher bioactivity of OH-PMFs.

5-hydroxy-6,7,8,4'-tetramethoxyflavone (5-OH-TAN) is one of the characteristic compound in the OH-PMFs group, which exclusively exists in citrus fruit peels. It is also known as gardenin B, which is a medicinal herb in Southeast Asia used for detoxification and serving as a taste modifier. Other functions include enhancing refreshing flavor, reducing saltiness and the flavor associated with acetic acid, as well as inhibiting unpleasant listing of sweetness (Li et al., 2006).

5-OH-TAN has been reported to have stronger anti-cancer effects comparing to tangeretin. The hydroxyl group at C5 position is important for its anti-cancer activity. However, because of this unique structure, it has poor oral bioavailability due to the low water-solubility, which may limit its application. The amount of 5-OH-TAN naturally found in orange peels is so small that it is very difficult to collect enough 5-OH-TAN for

research purpose. Thus, in this study, we synthesized a large amount of 5-OH-TAN from tangeretin by hydroxylation reaction.

In an effort to improve the bioavailability, some studies have been conducted to derivatize flavonoids by glycosylation and acetylation. Lai et al. has demonstrated that acetylation improved cell uptake and growth inhibitory activity of EGCG in human colon cancer cells. Also, 5-Ac-TAN treatment induced significant decrease of cell viability in HCT116 and HT-29 cells and displayed more potent and effective anti-cancer effect than that of tangeretin or 5-OH-TAN (Lai et al., 2013). However, the study on the bioactivity and bioavailability of 5-Ac-TAN is still lacking. So we are motivated to enhance the bioactivity of 5-Ac-TAN to achieve better health benefits.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

Tangeretin (98% purity) was separated from bitter orange peel extract using column chromatography as stated in Chapter 2. Phosphorus pentoxide (certified ACS grade) and acetonitrile (ACN; HPLC grade) were both purchased from Fisher Scientific (Fairlawn, NJ, USA). ACS grade acetyl chloride (>98.5%) and aqueous hydrochloric acid (37%) were purchased from Aldrich Chemical Company Inc (Milwaukee, WI, USA). ACS/USP grade ethyl alcohol (200 proof – absolute, anhydrous), ACS grade ethyl acetate (EtAc) and hexanes were all purchased from Pharmco-AAPER (Brookfield, CT, USA). Standard grade silica gel (60 Å, 40-63 um) was purchased from Sorbent Technologies (Norcross, GA, USA).

3.2.2 Instrumentation

The glass column (24/40 outer joint, 1 1/2in IDX 18in E.L., 2mm Stpk) was purchased from Chemgalss Life Sciences (Vineland, NJ, USA). The HPLC system was purchased from Dionex (Sunnyvale, CA, USA) and equipped with an UltiMate 3000 Pump, an UltiMate 3000 Variable Wavelength Detector, and an UltiMate 3000 auto-sampler. Data collection, processing and instrument control were achieved using Chromeleon software. Ascentis® RP-Amide C18 HPLC column (15cm X 4.6mm, 3um) was purchased from Supelco Analytical (Bellefonte, PA, USA).

3.2.3 Hydroxylation reaction

5-OH-TAN was prepared as follows (Li et al., 2006): 5 g purified tangeretin obtained previously was dissolved in absolute alcohol, and 10 mL 37% aqueous hydrochloric acid was added into the solution. Then the solution was heated and refluxed for 24 h, during which the concentrated hydrochloric acid was added every 4 h.. (Figure 3.1) After the reaction was cooled, the solution was adjusted to neutral by adding sodium hydroxide, and the ethanol was removed in vacuum. The solution was then transferred to a Buchner funnel and washed with de-ionized water several times. The residues in the Buchner funnel were then collected and the light yellow solid 5-OH-TAN was obtained after vacuum drying.

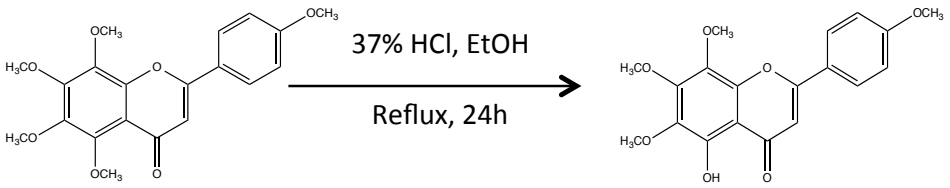


Figure 3.1 The structure and synthesis of 5-demethyltangeretin (5-OH-TAN) from tangeretin (TAN).

3.2.4 Acetylation reaction

All the solvents utilized in this reaction should be anhydrous and more importantly, the complete reaction should be occurring under an anhydrous environment (Li et al., 2006).

CH_2Cl_2 was heated and refluxed with P_2O_5 for 4 hours at 70℃ to remove the water in CH_2Cl_2. Then the anhydrous CH_2Cl_2 was collected by distillation.

0.18 g 5-OH-TAN (0.5 mmol), 0.07 mL CH_3COCl (1 mmol), 0.2 mL pyridine (2 mmol), and 10 mL CH_2Cl_2 were mixed and stirred at ambient temperature for 12 – 14 h. At the end of the reaction, the products were obtained by recrystallization with water. 5-Ac-TAN was purified with a small silica gel column to get rid of 5-OH-TAN and other impurities.

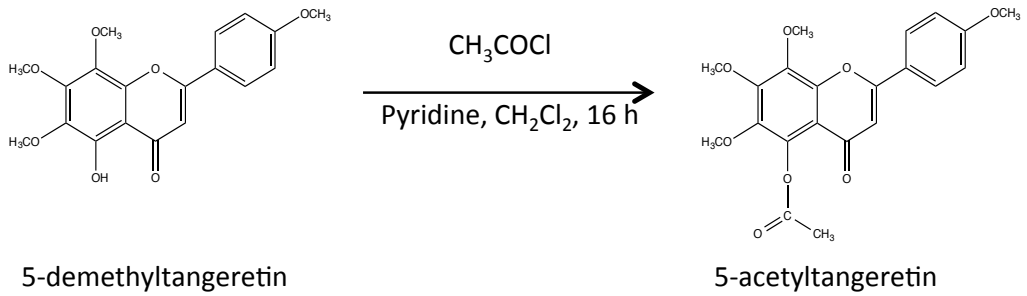


Figure 3.2 The structure and synthesis of 5-acetyltangeretin (5-Ac-TAN) from 5-demethyltangeretin (5-OH-TAN).

3.2.5 HPLC analysis

Ascentis® RP-Amide C18 HPLC column (15cm X 4.6mm, 3μm) was purchased from Supelco Analytical (Bellefonte, PA, USA) and was used to analyze compounds. The mobile phase consisted of (A) water and (B) acetonitrile. Elution condition, which was developed by Dr. Shiming Li, was as follows: Solvent B increased from 40% to 55% in 10 min, then increased to 70% in 5 min, then increased to 90% in 1 min, then kept for 3 min, then decreased to 40% in 1 min, and then kept for 4 min for equilibrium.

The mobile phases were degassed by sonication for 30 min. The UV-Vis detection wavelengths were set at 214 nm, 280 nm and 326 nm. The flow rate was set at 1.0 mL/min.

3.3 Results and Discussion

3.3.1 Hydroxylation reaction

With strong acid treatment, tangeretin can be transformed into 5-OH-TAN, which is scarce in the citrus peel extract. The bitter orange citrus peel extracts mixture utilized in

this study only contained 0.08% 5-OH-TAN. However, 5-OH-TAN reached 24.74% in this citrus peel extracts after 24 h treatment of 37% hydrochloride acid treatment.

With this treatment, we synthesized 3.17g of 5-OH-TAN (purity 94%) as final product from 5.0g of tangeretin (purity 98%).

3.3.2 Acetylation reaction

5-acetyltangeretin does not naturally exist in natural compounds, so in this study, 5-OH-TAN was further transformed into 5-Ac-TAN by acetylation utilizing CH_3COCl. We synthesized 0.10g of 5-Ac-TAN (purity 90%) as the final product from 0.18g of 5-OH-TAN (purity 94%).

3.3.3 HPLC results

In this study, high performance liquid chromatography (HPLC) was applied to identify the compounds after chemical reactions, including hydroxylation and acetylation, by comparing the purchased standards, and the purities of these compounds were determined by HPLC as well.

The purities of the synthesized and purified 5-OH-TAN (Figure 3.3a) and 5-Ac-TAN (Figure 3.3b) were determined by HPLC at 280nm and 326 nm. By comparing the purchased standards, the peak appeared at the retention time of 15.387 min (Figure 3.3a) was 5-demethyltangeretin and the peak at 12.273 min (Figure 3.3b) represented 5-acetyltangeretin, respectively.The relative area percentage of the peak shown in Figure 3.3a is 93.64%, indicating that the purity of 5-OH-TAN synthesized from tangeretin by hydroxylation reaction is around 94%. The relative area percentage of the peak in Figure

3.3b is 90.64%, meaning that the purity of 5-Ac-TAN synthesized from 5-OH-TAN by acetylation reaction is around 91%.

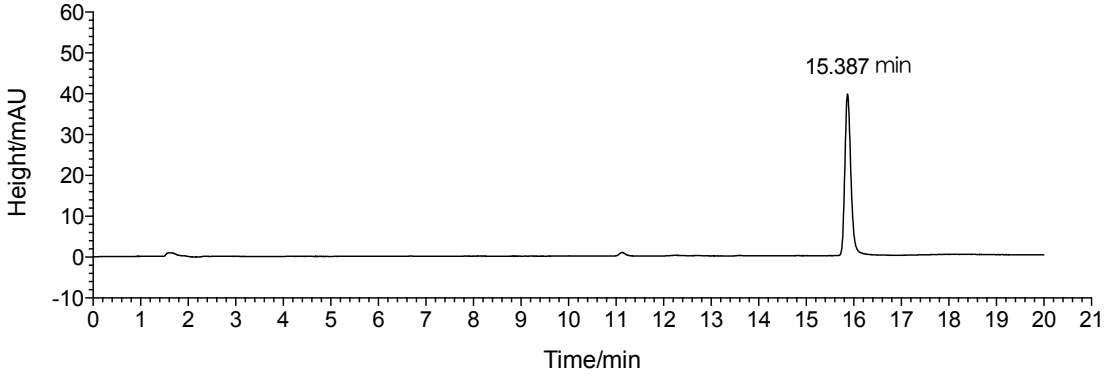


Figure 3.3a HPLC graph of 5-OH-TAN at concentration of 10 uM at 326nm.

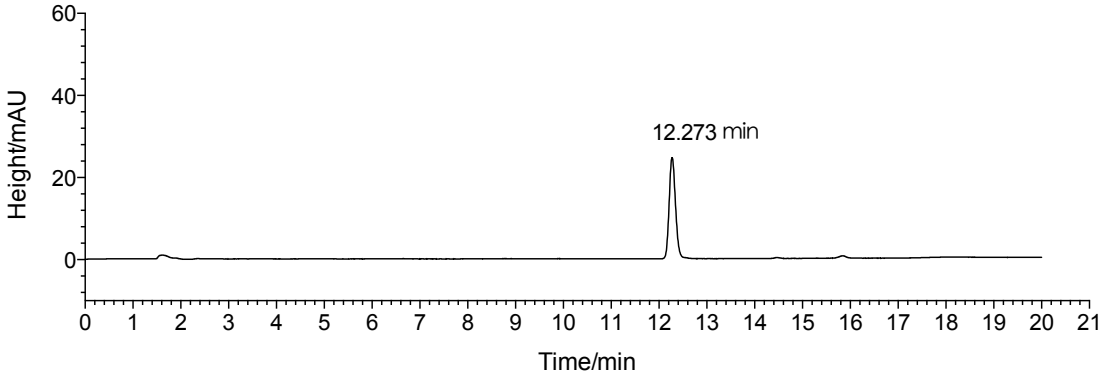


Figure 3.3b HPLC graph of 5-Ac-TAN at concentration of 10 uM at 326nm.

Interestingly, the hydrophilic affinity of 5-OH-TAN is lower than their corresponding tangeretin judging from their dilution order from the C18 reverse phase HPLC system and from normal phase chromatography. This indicates that 5-OH-TAN is less polar than tangeretin. According to Li et al. (2006), this is because a six-member ring intramolecular

hydrogen bond formation between the hydrogen of 5-OH group and the oxygen of the 4-carbonyl group.

3.4 Conclusion

In the present study, the results showed that a reliable chemical and instrumental method and conditions was developed and optimized for rapid preparation of 5-OH-TAN and 5-Ac-TAN.

These two chemical reactions utilized in this study, i.e. hydroxylation and acetylation, were basic methods that were widely used in chemical modification of naturally existing compounds. The mechanism can be easily understood and only a few reagents were utilized to achieve the goal.

Now we already obtained the desired compounds, i.e. TAN, 5-OH-TAN and 5-Ac-TAN, with high purities (98%, 94%, 90% respectively). In the next step, we investigated their bioactivity and bioavailability through *in vitro* cell studies.

CHAPTER 4 COMPARISON OF THE INHIBITORY EFFECTS OF TANGERETIN AND ITS TWO DERIVATIVES ON HUMAN COLON CANCER CELLS

4.1 Introduction

According to the American Cancer Society (2013), cancer was the second most common cause of death in the United States and about one in every fourth deaths occurred is due to cancer. In 2013, the World Health Organization (WHO) stated that cancer would be the most prevailing cause for death worldwide surpassing heart disease and stroke. According to Colon Cancer Alliance (2014), colon cancer is the third most commonly diagnosed cancer and the second leading cause of cancer death in men and women combined in the US. The American Cancer Society estimates that 136,830 people will be diagnosed in 2014 and 50,310 will die from colon cancer in the United States. On average, the lifetime risk of developing colon cancer is about 1 in 20. These statistics indicate that cancer prevention and treatment has become an urgent topic. Healthy diet and lifestyle have drawn great attention since they are effective and easily approached ways to prevent diseases.

The isolation and biological activities of PMFs and their related compounds or derivatives have been of particular interest. PMFs may suppress proliferation while promoting apoptosis. Tangeretin has anti-proliferative, anti-invasive, anti-metastatic, anti-inflammatory, anti-diabetic activities and improves insulin resistance and glucose uptake. 5-demethyltangeretin and 5-acetyltangeretin are two analogues of tangeretin, which have also been shown to exhibit strong inhibitory activity again different cancer

cell lines (Zheng et al., 2014; Lai et al., 2014). That is why we are focused on studies to investigate these compounds for their potential application in chemoprevention. The objective of this study is to evaluate if the structural differences between tangeretin and its two derivatives would affect their inhibitory effects in Caco-2 human colon carcinoma cell lines.

4.2 Materials and Methods

4.2.1 Materials

Highly purified tangeretin was obtained from previous compound preparation using column chromatography technology. 5-OH-TAN and 5-Ac-TAN were obtained from previous compound synthesis by chemical reactions and purification. All organic solvents utilized were of HPLC grade and purchased from Pharmco-AAPER (Brookfield, CT, USA). Caco-2 colon carcinoma cell lines were obtained from Rutgers University. Penicillin and streptomycin, Dulbecco's modified eagle medium (DMEM), McCoy's 5A media, fetal bovine serum (FBS), MEM non-essential amino acid were purchased from Gibco (Grand Island, NY, USA). 96-well cell culture plate and other cell culture supplies were purchased from Corning Incorporated (Corning, NY, USA).

4.2.2 Cell culture treatment

Caco-2 cells were maintained in DMEM containing glucose and L-glutamine, supplemented with 10% fetal bovine serum, 0.1% MEM non-essential amino acid, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

All cells were kept at 37℃ in atmosphere of 5% CO_2. Culture media was replaced every 2 to 3 days and subcultured when the confluency reaches 70-90%.

Caco-2 lines used in this study were between 38 and 50 passages. DMSO was utilized as the solubilizing agent to deliver tangeretin, 5-OH-TAN and 5-Ac-TAN. The final concentration of DMSO in all experiments was kept below 0.1%.

4.2.3 Cell viability assay

Caco-2 cells were seeded in 96-well plates at a density of 10,000 cells/well. After 12 hours of incubation, media was aspirated and experimental groups were treated with 100μL culture media containing TAN, 5-OH-TAN or 5-Ac-TAN at concentrations of 0.5, 1, 5, 10, 25, 50μM. Separate group of cell treated with only culture media were used as control. After treating for 12 hours, treatment media was replaced with 100 uL of complete culture media containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After 2 hours of incubation at 37℃, the media containing MTT was removed and the reduced formazan dye was solubilized by the addition of 100uL of DMSO per well. Upon complete dissolution in DMSO, the absorbance (570nm) for each well was determined using the spectrophotmetric microtiter plate reader. Results were expressed as percentage of viable cell in relative to the control cells treated with only culture media.

4.2.4 Statistical analysis

Data were expressed as mean ± standard error mean (SEM). Statistical significance of mean difference between two groups was determined by student t-test. Analysis of

variance (ANOVA) model was used when more two groups were to be compared. A significance level of $p < 0.05$ was used for all tests.

4.3 Results and Discussion

In order to investigate the growth inhibition effect of TAN, 5-OH-TAN and 5-Ac-TAN, MTT assay was conducted to determine the cell viability of Caco-2 cells. The level of cell viability is quantified by the absorbance intensity at 570 nm from each well, in which higher the intensity indicate higher cell viability.

The results from this study showed that all three compounds, TAN and its two derivatives 5-OH-TAN and 5-Ac-TAN, performed growth inhibition activity in Caco-2 colon cancer cells in a dose-dependent manner (Figure 4.1).

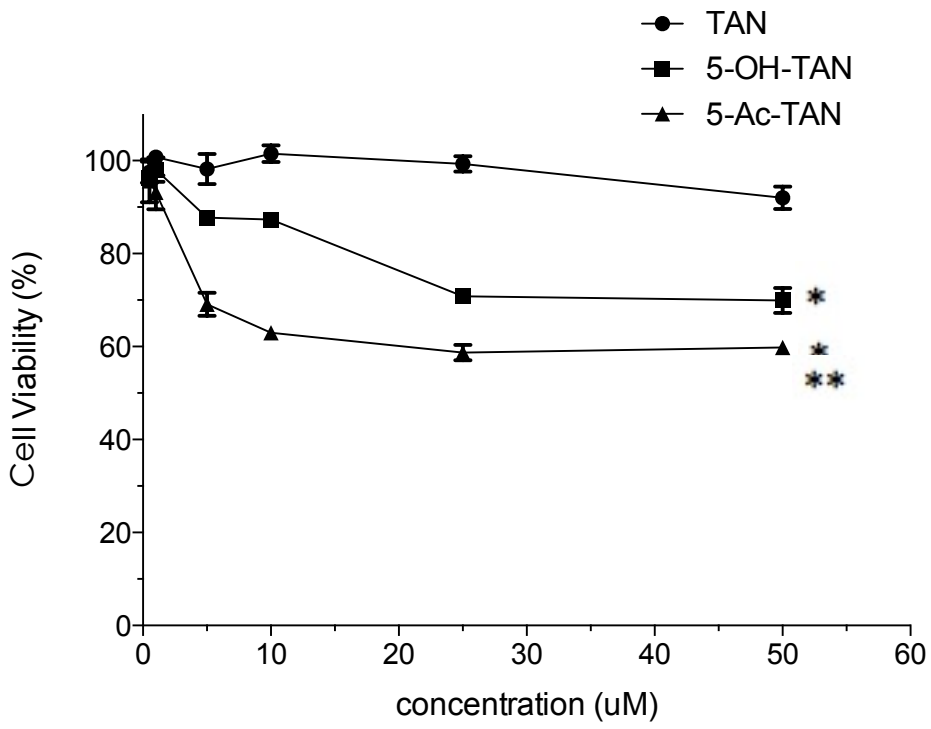


Figure 4.1 Growth inhibitory effects of TAN, 5-OH-TAN and 5-Ac-TAN on Caco-2 human colon adenocarcinoma cells. Cells were seeded on 96-well plates for 12 hours. After this period of time, cells were treated with serial concentrations of TAN, 5-OH-TAN and/or 5-Ac-TAN. After 12 hours of treatment, growth inhibition was measured by MTT assay as described in material and method section. Each point represents the mean ± SEM (n=4). *$p < 0.001$ indicates statistically significant differences from the control group. **$p< 0.05$ indicates statistically significant differences from TAN group.

The cell viability was 92.0 ± 2.4, 70.0 ± 0.9 and 59.8 ± 2.7% at concentration of 50 μM for TAN, 5-OH-TAN and 5-Ac-TAN, respectively. There was significant decrease in the cell viability of 5-OH-TAN and 5-Ac-TAN treated groups ($p < 0.001$ when compared to control group). However, there was no significant growth inhibition effect in TAN treated

group. The results showed that 5-OH-TAN and 5-Ac-TAN were more potent than TAN to reduce the growth of Caco-2 cells. In particular, 5-Ac-TAN exhibited significantly higher anti-cancer effect than TAN ($p < 0.05$) indicates that substitution of acetyl group on the C5 position of the benzo-γ-pyrone skeleton structure plays critical role in the anti-cancer efficacy of such compounds.

Reduction of cell viability to lower than 80% was achieved by treatment with 5 μM of 5-Ac-TAN whereas 25 μM of 5-OH-TAN was require to induce similar level of inhibition. On the other hand, TAN failed to show meaningful cell growth inhibition effect even at μM, the highest treatment concentration used. Overall, 5-Ac-TAN had the best anti-proliferative activity followed by 5-OH-TAN and then TAN. Substitution of hydroxyl and acetyl groups at C5 position of tangeretin significantly enhanced the anti-cancer bioactivity.

The lower cell viability in 5-Ac-TAN treated group was most likely attributed to its higher efficacy to induce apoptosis and cell cycle arrest in cancer cell lines.

According to Lai et al., tangeretin inhibited the cancer-initiation stage by modulating hepatic enzymes, thus affecting xenobiotic activation and detoxification in the liver. 5-Ac-TAN intends to target multiple molecules involve in cell cycle regulation. It can not only induce cell cycle arrest in HT-29 cells, possibly through a p53-independent mechanism, but also induce both S and G2/M phase cell arrest. Both 5-OH-TAN and 5-Ac-TAN induced higher cytotoxicity and apoptosis-inducing effect in HCT116 cells than in HT-29 cell, possibly due to the difference of p53 gene expression in these two cancer cell lines. 5-Ac-TAN stimulated cell cycle arrest in HT-29 cells through a p53-

independent mechanism. In addition, Li et al. suggested that 5-OH-TAN has very strong apoptosis activity, whereas its corresponding tangeretin lack apoptosis property.

Since Caco-2 cells are also a type of colon cancer cell similar to HT-29 and HCT-116 cells, the mechanism of anti-proliferative activities of these compounds should be similar. According to previous publication, we can assume that 5-OH-TAN and 5-Ac-TAN induced apoptosis but not TAN in human colon cancer cells. All three compounds are capable to induce cell cycle arrest in colon cells.

4.4 Conclusion

In this study, we examined the effects of TAN, 5-OH-TAN and 5-Ac-TAN on human colon Caco-2 cancer cell lines. All these three compounds have the basic benzo-γ-pyrone skeleton structure but differ by their functional groups on the C5 position. While TAN has a methoxyl group on the C5 position, 5-OH-TAN and 5-Ac-TAN contain hydroxyl and acetyl on C5 position of A ring, respectively.

MTT viability assay was used to determine the growth inhibition rate of the TAN, 5-OH-TAN and 5-Ac-TAN. The assay indicated that 5-Ac-TAN had the strongest inhibitory effect than the other two compounds on Caco-2 whereas TAN had the lowest inhibitory effect among all. These results were in good agreement with previous reports that showed the demethoxylated derivative have higher bioactivity and increased cytotoxicity. Moreover, in other study, the inhibitory effects of TAN and 5-OH-TAN on other human colon cancer lines has been also explored and similar results were observed (Lai et al., 2014).

In conclusion, on the basis of the above findings, 5-OH-TAN had stronger anti-carcinogenic effect when compares to tangeretin in Caco-2 human colon cancer cells. Highest anti-cancer activity was produced by 5-Ac-TAN than the other two compounds. This study highlighted these compounds deserve additional investigation as a chemopreventive agent in humans against colon cancer.

CHAPTER 5 COMPARISON OF PERMEABILITY OF TANGERETIN AND ITS TWO DERIVATIVES IN HUMAN COLON CANCER MONOLAYER CACO-2 CELLS

5.1 Introduction

Polymethoxyflavones (PMFs) exist exclusively in the citrus fruits in nature, particularly in the peel of sweet oranges and mandarin oranges (Li et al., 2006). Currently, more than 20 PMFs have been isolated and identified. PMFs have been well documented on their broad array of biological activities, such as anti-atherogenic (Whitman et al., 2005), anti-carcinogenic (Ikeda et al., 2006; Manthey and Najla, 2002) and anti-inflammatory (Li et al., 2006) effects.

For almost all kinds of foods and drugs, oral administration is the most effective and widely applied method for their delivery. Solubilization, permeation, and metabolism are three important stages that affect the oral bioavailability. Even though there have been a remarkable number of investigations on the bioactive effects of PMFs, only a scarce number of them have been focused on their bioavailability. The term bioavailability is defined as the fraction of an ingested component that eventually ends up in the blood systemic circulation system and target tissue (Van de Waterbeemd et al., 2003). Since oral administration is a widely utilized method for the delivery of drugs and foods, the effectiveness of a compound depends on their intestinal absorption to get into blood systemic circulation and ultimately reach the target tissue. And the intestinal epithelium is one of the key factors that determine the oral absorption of food ingredients and drugs.

Caco-2 cell monolayers model is well-accepted to evaluate intestinal permeability, examine the oral absorption levels and predict the absorption mechanism of compounds. It is of human colonic origin, and when grown in culture, the cells exhibit properties of small intestinal epithelium as they form a polarized monolayer of well-differentiated columnar absorptive cells expressing a brush border on their apical surface with typical small intestinal enzymes and transporters. They have similar morphological characteristics of human intestinal epithelial cells such as forming polarized monolayers in cultures and differentiating into cells (Fossati et al., 2008; Lind et al., 2007). Therefore, Caco-2 cell monolayer is a great model to examine *in vitro* absorption. The objective of this study was to compare the permeability and transport of tangeretin and its two analogues, 5-OH-TAN and 5-Ac-TAN, in Caco-2 human colon cancer cells that were cultured as monolayers on transwells. Samples were analyzed using high-performance liquid chromatography (HPLC).

5.2 Materials and Methods

5.2.1 Materials

Dimethyl sulfoxide (DMSO) that purchased from Sigma life science (St. Louis, MO, USA) was used as the vehicle to deliver compounds and the final concentration of DMSO in all experiments was 0.1% in culture media.

Highly purified tangeretin was obtained from previous compound preparation using column chromatography technology. 5-OH-TAN and 5-Ac-TAN were obtained from previous compound synthesis by chemical reactions and purification (Li et al., 2006). All organic solvents utilized were HPLC grade and purchased from Pharmco-AAPER

(Brookfield, CT, USA). Caco-2 colon carcinoma cell lines were obtained from Rutgers University (NJ, USA). Penicillin and streptomycin, Dulbecco's modified eagle medium (DMEM), McCoy's 5A media, fetal bovine serum (FBS), MEM non-essential amino acid were purchased from Gibco (Grand Island, NY, USA). 12-transwell insets cell culture plate and other cell culture supplies were purchased from Corning Incorporated (Corning, NY, USA).

5.2.2 Cell culture treatment

Caco-2 cells were cultured in DMEM containing glucose and L-glutamine, supplemented with 10% fetal bovine serum (FBS), 0.1% MEM non-essential amino acid, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. All cell lines were kept at 37℃ in an atmosphere of 5% CO2. The cells were subcultured when 70-90% confluent was reached, using trypsin, and media changed every 2 to 3 days for Caco-2 cells.

Cell lines used were between 38 and 50 passages. The culture medium was changed three times a week after seeding onto the semi-permeable membrane. Experiments were performed after 21-29 days of post-seeding. DMSO was utilized as the agent to solubilize tangeretin, 5-OH-TAN and 5-Ac-TAN. The final concentration of DMSO in all experiments was not higher than 0.1%.

5.2.3 Transport experiments

The transwell inserts was seeded with 0.5 mL of $6X10^5$ cells/mL of Caco-2 cells. The culture medium was changed three times a week after seeding and experiments were performed after 21 to 29 days post seeding. Culture medium was changed 12 to 24 hours

prior to performing experiments to prevent sufficient nutrition induced variation. The culture medium was carefully aspirated from both chambers and the cells were washed twice for 30 minutes by pre-incubation with (37℃) Hank's balanced salt solution (HBSS). The pH of HBSS was 7.2.

Fo4 the experiment, 20 μM TAN, 5μM 5-OH-TAN and /or 5μM 5-Ac-TAN (by DMSO dispersion) was added into the apical compartment. At the beginning of the experiment, initial dosing solution were immediately withdrew from the apical compartment as reference for the initial concentration. At specific time intervals (20min, 40min, 60min, 90min), samples from basolateral compartments were collected and the volume take from each compartment was replenished to the initial volume with HBSS.

Each collected transport sample was extracted using ethyl acetate and then dried using gentle flow of nitrogen. For HPLC analysis, each dried sample was re-suspended with 100 μL of DMSO.

5.2.4 HPLC analysis

Samples were analyzed on he HPLC system was purchased from Dionex (Sunnyvale, CA, USA) and equipped with an UltiMate 3000 Pump, an UltiMate 3000 Variable Wavelength Detector, and an UltiMate 3000 auto-sampler. Data collection, processing and instrument control were achieved using Chromeleon software.

Ascentis® RP-Amide C18 HPLC column (15cm X 4.6mm, 3μm) was purchased from Supelco Analytical (Bellefonte, PA, USA) and was used to analyze compounds. The mobile phase consisted of (A) water and (B) acetonitrile. Elution condition, which was

developed by Dr. Shiming Li, was as follows: Solvent B increased from 40% to 55% in 10 min, then increased to 70% in 5 min, then increased to 90% in 1 min, then kept for 3 min, then decreased to 40% in 1 min, and then kept for 4 min for equilibrium.

The mobile phases were degassed by sonication for 30 min. The UV-Vis detection wavelengths were set at 214 nm, 280 nm and 326 nm. The flow rate was set at 1.0 mL/min.

5.2.5 Statistical analysis

Prism 6.0 was used to perform the statistical analysis. Data were expressed as mean ± standard error mean (SEM).

5.3 Results and Discussion

The level of a compound can be effectively absorbed by human intestine is a critical factor in determining its bioavailability. The permeation rate of TAN, 5-OH-TAN and/or 5-Ac-TAN was investigated using Caco-2 cell monolayers that mimic the small intestine epithelium. The transports of these three compounds were monitored over a 90-minute time period with concentration at 20 μM . Samples from both compartments were taken at each time and replaced with buffer. Samples were subsequently extracted by ethyl acetate, re-suspended in DMSO, and then analyzed by HPLC.

According to the findings, the chemical structures of two tangeretin derivatives changed right after the stock solutions was added into the apical chamber. As shown in HPLC (Figure 5.1), at 0 min, 5-OH-TAN was partially transformed into TAN meaning that the hydroxyl group on C5 position was methylated back to methoxyl group. On the other

hand, in group treated with 5-Ac-TAN, both TAN and 5-OH-TAN in the samples taken from basolateral chamber indicating bio-transformation was not unilateral. All stock solutions were also analyzed by HPLC (Figure 2.1 and Figure 3.3) giving that compounds purity were relatively high (98%, 94% and 91% relatively). This indicates that the transformation of functional groups on these two derivatives occurred inside or on the surface of the Caco-2 monolayer cells.

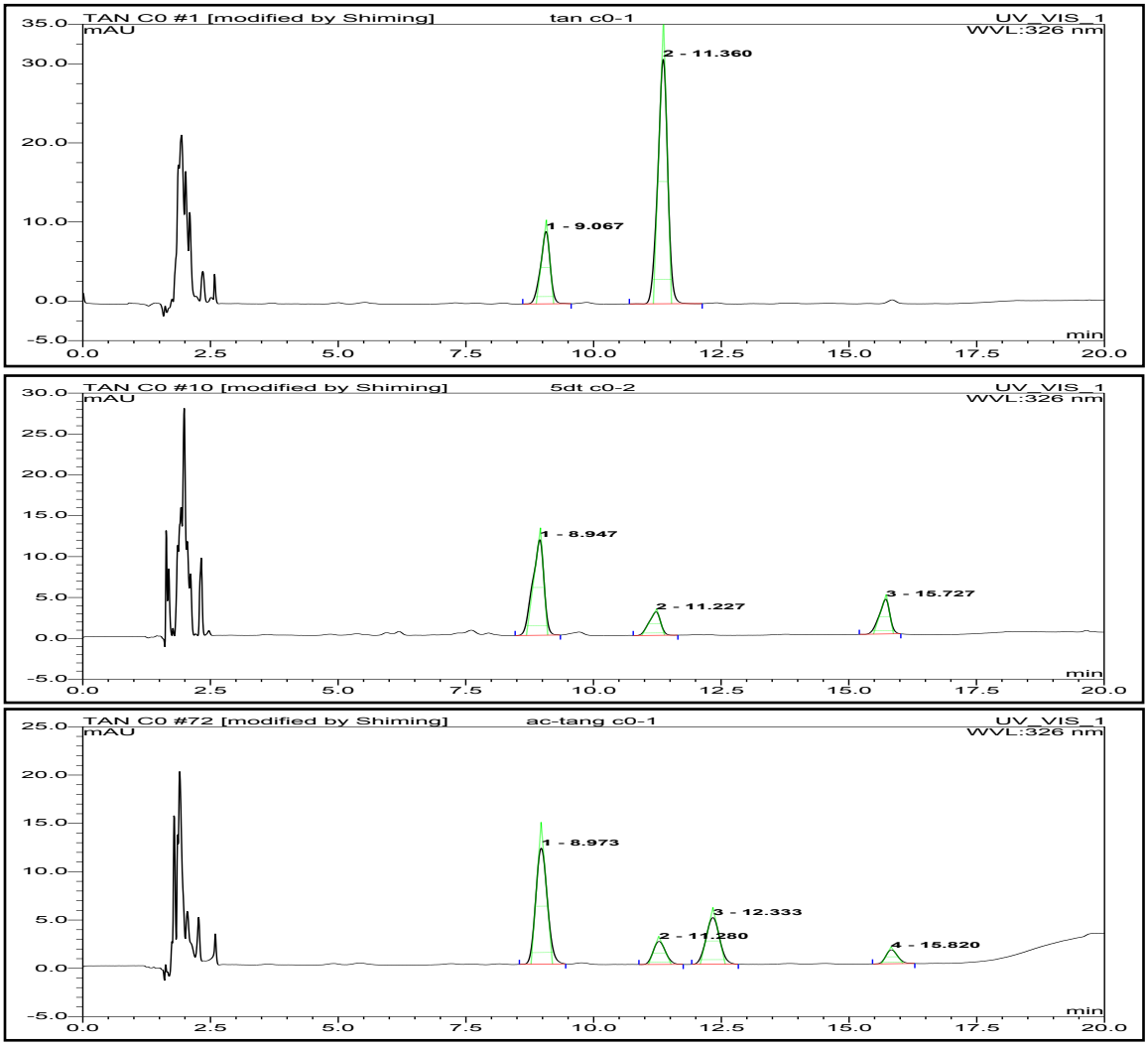


Figure 5.1 HPLC graphs of TAN, 5-OH-TAN and 5-Ac-TAN taken from apical chamber at 0min.

As shown in Figure 5.2, samples taken from basolateral compartment at 20min showed the similar transformation compared to what was shown at 0min in apical compartment.

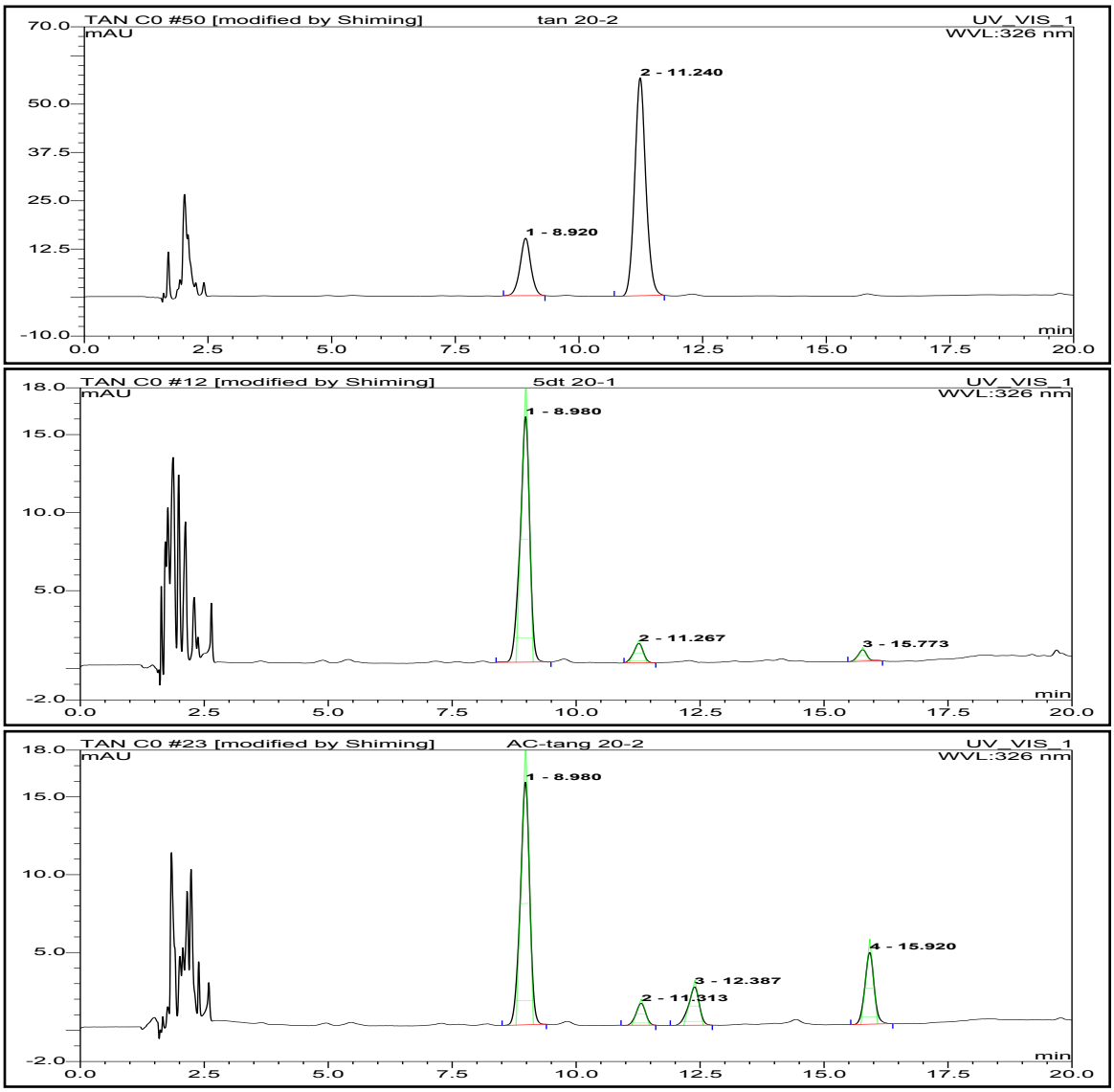


Figure 5.2 HPLC graphs of TAN, 5-OH-TAN and 5-Ac-TAN taken from basolateral compartment at 20min.

Then we analyzed the concentration of each compound as a function of time and presented in Figure 5.3. To clarify, data with the label 'DT-T' represent the amount of TAN recovered from groups that originally treated with 5-OH-TAN group. Similarly,

'AT-T' represented the amount of 5-Ac-TAN transformed into TAN after transfer cross the Caco-2 monolayer cells as a function of time.

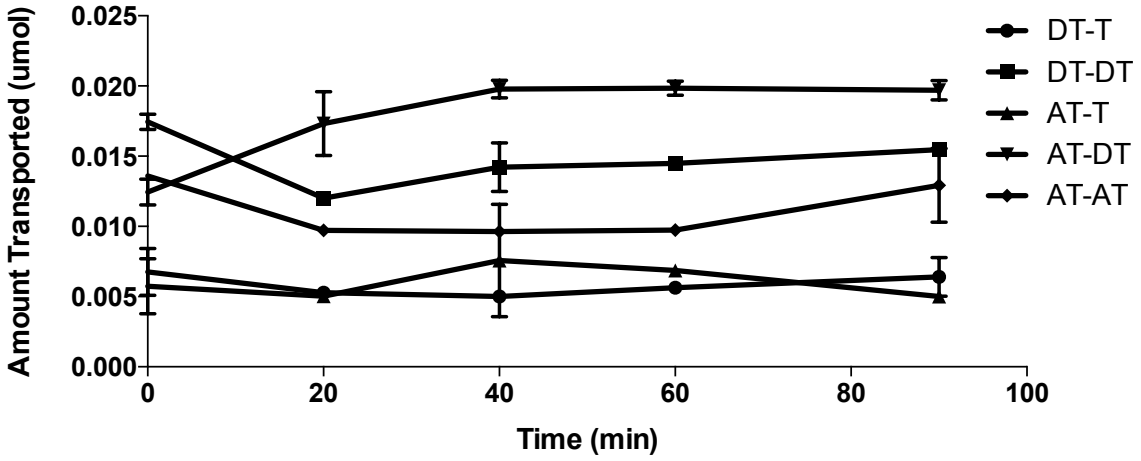


Figure 5.3 Determination of 5-OH-TAN and 5-Ac-TAN transformation at basolateral compartment across the Caco-2 monolayer. Data at each point are presented as mean ± standard deviation (SEM) (n=3).

Since the dosing solution of TAN (20 μM) was four times higher than the concentrations of 5-OH-TAN and 5-Ac-TAN dosing solutions (5 μM), only the Caco-2 monolayer permeability rate of these two compounds were comparable. As shown in Figure 5.3, all the compounds, except for TAN in 5-Ac-TAN group, the amounr transported from apical side to basolateral side increased as a function of time.

At 90 min, the amount of TAN transformed from 5-OH-TAN group was almost comparable to the amount recovered from 5-Ac-TAN group ($0.0064 \pm 8.0X10^{-4}$ μmol and $0.0050 \pm 9.9X10^{-5}$ μmol respectively). Therefore, most of the compound 5-OH-TAN was transported across the cells in its original form ($0.0155 \pm 6.4X10^{-5}$ μmol) without further conversion to TAN.

Meanwhile, TAN, 5-OH-TAN, and 5-Ac-TAN were recovered from the basolateral chamber of Caco-2 monolayer treated with 5-Ac-TAN in concentration of $0.0050 \pm 9.9X10^{-5}$, $0.0197 \pm 4.0X10^{-4}$, and $0.0129 \pm 1.5X10^{-3}$ μmol, respectively. This indicated that most 5-Ac-TAN was transformed into 5-OH-TAN, in which the acetyl group was substituted by hydroxyl group.

When comparing to the amount of 5-OH-TAN recovered from the basolateral chamber was higher in groups originally treated with 5-Ac-TAN group than 5-OH-TAN at level of $0.0155 \pm 6.4X10^{-5}$ and $0.0197 \pm 4.0X10^{-4}$ μmol, respectively. This result suggested that that large part of 5-Ac-TAN was transformed into 5-OH-TAN when crossing through the Caco-2 monolayer cells indicating majority acetyl group is removed upon transportation through monolayer and this functional group modification could serve as permeability enhancing mechanism for 5-OH-TAN.

In conclusion, the 5-acetyl group on the flavonoid structure is relatively unstable than 5-OH group and tends to be replaced by hydroxyl group. Potential explanation for the fact that only small amount of 5-hydroxyl group was replace by methyl group and converted to TAN is due to the hydrogen bond formed between the hydrogen on the 5-OH-group and oxygen on the 4-carbonyl group. More importantly, 5-Ac-TAN had a better potential to get transported across Caco-2 monolayer cells. The result from this study indicated that functional groups on the C5 position alter the hydrophobicity of polymethoxyflaovnes and plays critical role in determining the membrane permeability.

These results achieved good agreement with previous cell viability study giving that 5-Ac-TAN had better potential to get transported and act on Caco-2 colon cancer cells.

Thus, it may become a novel neutraceutical processing technique to better enhance the anti-cancer activity as well as bioavailability.

5.4 Conclusion

PMFs have sparked interest due to their wide spectrum of biological effects including anti-inflammatory and anti-carcinogenic activities. In spite of their potent anti-cancer activity, the investigation on the bioavailability of PMFs showed that the poor dosing efficient could potentially set a gap for it to be used as cancer prevention agent. The objective of this study was to examine the transport of TAN, 5-OH-TAN and 5-Ac-TAN across Caco-2 human colon cancer cell monolayer. Even though they have the similar flavone structure; they are different in their functional groups. 5-OH-TAN contains a 5-hydroxyl group on A ring, while 5-Ac-TAN has a 5-acetyl group instead at C5 position.

The utilization of human intestinal cell line Caco-2 cultivated on permeable membranes is a widely studied model for human intestinal absorption. It is critical to understand the bioavailability of compounds in order to figure out their potential actions *in vivo*.

Walle demonstrated that the methoxylation of PMFs have higher hepatic metabolic stability and intestinal absorption compared to unmethoxylated polyphenols. The low bioavailability and poor absorption of these demethyl flavones is due to extensive conjugative metabolism in the intestine and liver because of the free hydroxyl groups which gives rise to rapid intestinal/heptic conjugation and/or sulfation and excretion (Wen and Walle, 2006).

According to our findings, one can expect that 5-Ac-TAN are have beeter membrane permeability and, thus, being absorbed by the human intestines than 5-OH-TAN. However, more research is necessary in order to determine the permeability when higher dosing concentrations are applied. Moreover, the efflux from basolateral compartment to apical compartment is also interesting.

Tangeretin and its derivatives are promising agents in cancer therapy and prevention but future research efforts are needed to understand their mechanism of intestinal transport.

CHAPTER 6 FUTURE RESEARCH

To make the work done in this thesis more meaningful, future work should continue on exploring the potential of hydroxylated and/or acetylated polymethoxyflavones as cancer prevention agent. In addition, future work should continue to utilize *in vivo* and/or *in vitro* research model to investigate the bioavailability of these compounds as well.

Based on the previous work done on the anti-cancer activity study comparing tangeretin, 5-OH-TAN and 5-Ac-TAN on Caco-2 human colon cancer cell lines, 5-Ac-TAN had the strongest inhibition on the cell viability than the other two compounds. Meanwhile, tangeretin had the weakest inhibition on Caco-2 cell viability as compared to others. However, compared to Caco-2 cells that spontaneously express differentiation characteristics of mature enterocytes by forming a polarized monolayer, other types of human colonic carcinoma cell lines such as HT-29 and HCT-116 cells grow as undifferentiated cells. In general, less differentiation means a greater likelihood of malignant behavior. Therefore, in future study, investigation of inhibitory effects and intracellular uptake study ought to be conducted on other types of human colon cell lines, including HT-29 and/or HCT-116, which are more similar to malignant cancer cells and the results will be more persuasive about the anti-cancer activities of these three The permeability experiment preformed on tangeretin, 5-OH-TAN and 5-Ac-TAN exhibited some new insight on their bioavailability. Since transport assays on these compounds only investigated the permeability of them from the apical side to the basolateral side, the transport experiment evaluating the rate of those compound transported from basolateral compartment to apical compartment should be conducted in the next step. Therefore, it

may help to determine what types of transport mechanism were undertaken. As a result, we can get a better understand on bioavailability and absorption of these compounds.

More importantly, for the two tangeretin derivatives, determining the exact mechanism by which they inhibit cancer cells viability and by which they effect intestinal absorption and modulates the bioavailability is an important and attractive subject for future research. Cell cycle study and apoptosis assays observed with the flow cytometer could also be conducted.

Bioavailability is a key factor dictating the efficiency of bioactive dietary components. Therefore, some other application can be also investigated to improve their bioavailability, such as encapsulating and developing a delivery system to more effectively transport them into our body system and reach the target tsite of action.

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