APPLICATION OF EMULSION-BASED DELIVERY SYSTEM TO 
ENHANCE BIOAVAILABILITY AND EFFICACY OF 
TANGERETIN 

by 

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Tangeretin (5,6,7,8,4′-pentamethoxyflavone) is a polymethoxylated flavone found predominantly in citrus fruit peels. Numerous bio-functionalities have previously been reported for tangeretin. For chronic intakes, oral ingestion is typically the preferred method since it is the most convenient and non-invasive application route. However, tangeretin exhibits poor oral bioavailability as a result of its hydrophobic chemical structure. Consequently, the required tangeretin oral dosage for many intended therapeutic purposes is difficult to achieve. In this work, we aim to use an emulsion-based delivery system to enhance the bioavailability and efficacy of tangeretin.

In first part of this work, a viscoelastic emulsion system containing >2.5% tangeretin was developed and its physical properties were characterized. In this viscoelastic emulsion system, the emulsion-encapsulated tangeretin was mixed with
homogeneously-entrapped tangeretin crystals, which exhibited remarkable storage stability over six months. Following the development of the tangeretin emulsion system, the processing parameters were further optimized to obtain the required properties that may best enhance the oral bioavailability and efficacy.

The ability of an emulsion-based delivery system to improve the oral bioavailability of tangeretin was examined using in vitro and in vivo models. In vitro lipolysis and TNO gastrointestinal model revealed that emulsion-delivered tangeretin was digested considerably more quickly and was 2.6-fold more bioaccessible than unformulated medium-chain triglyceride (MCT) suspension. In vivo pharmacokinetics analysis on mice confirmed that the oral bioavailability of tangeretin in the emulsion-based system was increased 2.3-fold, with a 23% increase in Cmax when compared with the unformulated suspension.

Moreover, the emulsion-based delivery was proven to be an effective method to increase the oral therapeutic efficacy of tangeretin. The in vitro anti-proliferative activity of tangeretin was first evaluated using MTT essay on colonic carcinoma cell lines and was significantly improved by the use of the emulsion delivery system. The effectiveness of the emulsion system to enhance the in vivo oral efficacy of tangeretin against colorectal cancer development was also evaluated by the AOM/DSS-induced colitis-related colon tumorigenesis model. The tumor incidence, multiplicity, and pathological signs of colorectal adenoma were significantly reduced when tangeretin emulsion was applied. Finally, the related toxicity effects of the tangeretin viscoelastic emulsion were also investigated.
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Figure 7.10 Histological observation of the systemic organs (heart, liver, spleen, lung, and kidney) from female mice used in the subacute toxicity study, including (A) untreated control, (B) control treated with blank MCT, (C) 50 mg/kg of tangeretin in MCT suspension, (D) 100 mg/kg of tangeretin in MCT suspension, (E) control treated with blank VE, (F) 50 mg/kg of tangeretin in VE, and (G) 100 mg/kg of tangeretin in MCT suspension. The histological morphology of liver was examined using H&E staining and microscopy (200× magnification). Bar = 100 μm.
CHAPTER I. INTRODUCTION

“An apple a day keeps the doctor away” is a well-known slogan that emphasizes the importance of consuming an adequate amount of fruits and vegetables as part of the daily diet. In review work by Steinmetz and Potter (1), the positive link between cancer prevention and consumption of fruit and vegetables was evidenced by 206 human epidemiological studies and 22 animal studies. In ancient societies of various origins all over the world, a pharmacological perspective of nature herbal remedies predominated human history until the development of the modern medical system. Even with the exceptional development of medical technology within the last few decades, people are now distanced by various unfavorable toxic side effects that frequently accompany the fast-acting characteristics of therapeutic drug treatment, and start seeking gentler and less invasive alternatives from natural sources. The theory of Hippocrates from 2,500 years ago, “Let food be thy medicine and medicine be thy food,” then became the mainstay of modern belief in protective and disease-preventing dietary practice.

As the link between dietary habits and health conditions has been well-established, bioactive compounds possessing health-promoting functionalities receive significant attention from the scientific community. Nutraceuticals, defined as nutritional components that provide therapeutic or physiological benefits beyond basic nutritional needs, are regarded as an emerging method for preventing chronic diseases. Apart from the modern medical system that targets immediate relief of symptoms and diseases, diet regimens encourage a moderate long-term process for illness prevention or treatment. Being isolated from natural nutritional sources, nutraceuticals are expected to exhibit relatively less toxicity and fewer secondary side effects than drugs used to treat similar
symptoms. The list of nutraceuticals, including bioactive peptides, phenolic compounds, lipids, vitamins, etc., continues to grow as additional new compounds are identified and isolated from various sources (2, 3). Even though the biological mechanisms underlying the functional activities may vary, a wide variety of research has documented that nutraceuticals can be used to promote cognitive health, regulate blood glucose and cholesterol level, reduce inflammation, prevent cardiovascular disease, and inhibit cancer development, among other benefits (2-7).

For chronic intake, oral ingestion is the preferred method by most consumers since it is considered to be the most convenient, non-invasive and cost-effective application route. With diverse environmental conditions along the human gastrointestinal (GI) tract, orally-consumed bioactives face a much more significant to sustain the original chemical structure than other ingestion routes, such as parenteral injection, intraperitoneal injection, transdermal application, etc. The GI tract sets both physiological and chemical barriers for oral drug delivery. Nutraceuticals enter the GI tract through the mouth and from that point they are subject to an aqueous environment of pH fluctuation (mouth pH 5-7, stomach pH 1-3, small intestine pH 6-7.5), temperature deviation (ambient temperature to body temperature), enzymatic degradation, salts or bio-compounds interaction, intestinal uptake, metabolism, and efflux (Figure 1.1). Moreover, the absorbed biological components are subject to rapid intestinal and first-pass metabolism, causing a transformation of chemical structure that may result in a change in bioactivity. The bioavailability of a species is defined by the Food Drug and Administration (FDA) as “the rate and the extent to which the therapeutic moiety is absorbed and becomes available to the site of drug action.” The overall bioavailability is
the conclusive measure of the compound’s concentration that becomes accessible for intestinal absorption, transport, and then left un-metabolized when get into the system circulation (8). To become accessible for absorption, the compound must be solubilized or dispersed in the aqueous intestinal lumen. Lipophilic compounds, in contrast to hydrophilic ones, are poorly soluble in the aqueous environment and thus commonly found to be poorly uptaken by the intestinal lining. On the other hand, the hydrophilic ingredients have a low transport coefficient across the intestinal lining, of which the phospholipid bilayer is the major cell wall component. Once again, after intestinal absorption, bioactives are then subjected to extensive metabolic activities that may change their chemical structure and lead to functionality alteration. In other words, due to various factors, nutraceuticals may have poor bioavailabilities that result in insufficient concentration to produce meaningful therapeutic functionalities.
Figure 1.1 Characteristics of lipophilic compound (Represented by *) that result in low oral bioavailability during common intestinal absorption and within the metabolic pathway. Picture redrawn from Ref. (9).

**BIOLOGICAL FATE OF NUTRACEUTICALS AFTER ORAL INGESTION**

For chronic disease prevention or treatment, oral administration is the favored route for the consumption of bioactive agents since it requires a low level of application skills, reduces the likeliness of disease transmission, lowers medical costs, and allows a flexible consumption schedule. However, upon oral ingestion, the physicochemical environment of the GI tract may partly influence the solubility, stability, bioavailability and efficacy of nutraceuticals, depending on their specific chemical structures (Figure 1.2). Following uptake by the gut wall, first-pass metabolism by intestinal cells and the liver significantly reduce the concentration of bioactives reaching the system circulation. Therefore, delivery systems designed with functional properties that are capable of
improving solubility, protecting and enhancing cellular uptake and transport, altering compound release kinetics, and bypassing rapid metabolic activities have gained much popularity in many investigations as an approach to increase compound oral bioavailability. In this section, a brief overview of the fundamental physicochemical and physiological activities that occur during oral ingestion, digestion, absorption, and metabolism will be provided. Moreover, we will also discuss the factors determining bioavailability, as well as strategies that investigators commonly use to augment the concentration of nutraceutical systems.
Digestion in the gastrointestinal tract

Figure 1.2 Graphical representation of absorption and first-pass metabolism of nutraceuticals in the human body. The active compound enters the system circulation through the paracellular and transcellular intestinal absorption route. While the compounds entering through the paracellular route escape intestinal metabolism, the compound metabolic activity starts in the intestinal epithelial cells when the transcellular route is utilized. After entering the portal vein system, nutraceuticals are then subject to extensive hepatic metabolism.
**Mouth**

Upon oral ingestion, nutraceuticals consumed as dietary supplements can be ingested in the form of tablets, capsules, softgels, gelcaps, liquids, powders, or part of a food matrix that is swallowed with or without mastication. During the stopover time in the oral cavity, mastication breaks down ingested food into small pieces, mixes it with saliva, and converts it to bolus. Depending on their chemical structure and properties, nutraceuticals are variably digested by enzymes while experiencing changes in pH (5.6-7.6), temperature, ionic strength and complex flow profile (10, 11). Even though the residence time in the mouth is relatively short (15-20 seconds), the mingling with saliva initiates the digestion process, allows easier swallowing, and supports improved digestion activities in the stomach (10-12).

**Stomach**

Swallowed bolus passes through the esophagus and then enters the stomach, where it experiences a significant pH drop (pH ≈ 1-3). Depending on the bolus composition and quantity, the stomach pH may be increased by various degrees, for some of which the physiochemical environment is no longer at an optimum for important enzymatic activity. The buffering capacity of gastric juice prevents a gradual pH increase that may cause the loss of enzyme bioactivity. In conjunction with HCl secretion, the stomach pH is regulated and brought to optimum condition (pH 2.0) for gastric enzymatic activities. During digestion in the stomach, the bolus is slowly moved to the small intestine in the form of chyme, which is produced by continuous muscular mixing, gastric enzyme digestion, and hydrochloric acid oxidation. Due to differences in chemical or physical properties, most nutraceuticals are stable in the gastric environment, while
others may be subject to irreversible chemical or physicochemical transformations that result in decreased bioavailability and efficacy. For example, bioactive peptides are especially vulnerable to acid hydrolysis and protease degradation in the gastric compartment. Since therapeutic effectiveness is closely related to certain amino acid sequences, disintegration of peptide integrity may result in the complete loss of activity (13-17). Moreover, probiotics ingested orally to improve the intestinal microbial balance also require survival while passing through the upper GI tract. The extreme gastric pH condition may significantly reduce the probiotics’ viability before they can reach the intestine (18, 19). On the other hand, it has been widely reported that an insufficient gastric residence time of many polyphenols is one of the major causes of low bioavailability (20-24). Sufficient gastric retention time is particularly important for bioactives with slow and incomplete absorption by the upper section of small intestine. Therefore, despite its indispensable role in the digestion, absorption, and metabolism of nutrients, the stomach also poses physiological, physiochemical, and biochemical barriers for the efficient uptake of nutraceuticals.

**Small intestine**

After a short residence period in the stomach, the partially-digested food mass, chyme, continues move toward the next part of GI tract system. The small intestine, which has an average length of 500 cm, is sub-divided into three consecutive sections: duodenum, jejunum and ileum (25). To protect the gut from potentially harmful acid, the acidic chyme is then neutralized by mixing with alkaline pancreatic juice when it enters the uppermost section of small intestine. During the retention period in the gut, the partially-digested nutrients continue to be processed when mixing with sodium
bicarbonate, bile salts, phospholipids, and enzymes by dynamic abduction. With the presence of various digestive enzymes, the small intestine is the major site where most of the nutrients are digested and broken down into smaller molecules made available for absorption. As stated in many previously-published studies, the absorption of nutraceuticals is greatly affected by the composition of the food ingested simultaneously. In particular, the presence of lipids significantly increases the absorption of lipophilic compounds (26-31). Due to the chemical structure, lipophilic compounds, which are poorly soluble in the aqueous intestinal lumen, tend to precipitate and be excreted without being absorbed. Lipase hydrolysis of lipids plays a critical role in determining the uptake of lipophilic bioactive compounds. The resulting products from lipid digestion, fatty acids (FA) and monoglycerides (MG), complex with bile salts and transform into micelle or mixed micelle in the pre-absorption phase. During this phase, lipophilic bioactives dispersed or dissolute in the intestinal lumen are resolubilized and incorporated into the micelle and are then taken up by the gut epithelial cells. Depending on the chemical structure and properties, the absorption and bioavailability of nutraceuticals are also affected by gut physiological and physiochemical factors, such as stability to pH and temperature environment, ionic interaction, susceptibility to enzyme degradation, and retention kinetics within the intestinal lumen. For example, water-soluble tea polyphenol, (−)-epigallocatechin gallate (EGCG), is unstable in the intestinal environment (neutral to alkaline) and is rapidly degraded to lose its bioactivity (32, 33).

Colon

The colon, the final step of the digestive process, absorbs the remaining water and nutrients in digestible food matter coming from the small intestine. At this stage, the
remaining nutrients continue to be digested by the gut microbial community. Digestion of unabsorbed carbohydrates, protein, and short-chain fatty acids contributes to approximately 5-10% of energy requirements (34, 35). The long residence time allows water and residual nutrients to be absorbed by the colonic epithelial linings into the system circulation. In terms of nutraceutical digestion, the inefficient muscular mixing of high-viscosity colonic dietary mass results in the low availability of most nutraceuticals to the absorption membrane. The unabsorbed dietary nutraceuticals then become accessible to the gut microorganisms as they moving along the colon. Even though the effects of microbial conversion on the bioavailability and efficacy of nutraceuticals remain unclear, the importance of such metabolic activities in the gut has been widely discussed by preceding investigations (36, 37).

**Absorption and metabolism**

Under fed or fasted conditions, orally-ingested nutraceuticals in the form of solution or solid move along the GI tract with various luminal components at speeds that depend on the overall rheological properties. To provide therapeutic or physiological benefits, nutraceuticals must be absorbed from the GI tract, sustain presystematic metabolisms, enter the vascular circulation, and reach the target site with biologically-relevant concentration. The transfer of nutraceuticals across the intestinal lining can be achieved through a transcellular or paracellular route. In transcellular absorption, the gut epithelial cells take in nutraceuticals from the gastric lumen by either passive or active transport mechanisms. While passive transport across the plasma membrane is mainly driven by concentration gradients, the passage of some nutraceuticals may utilize the active transport route, in which the active compounds are taken up by transcellular
protein channels or phospholipid vesicles. Once the bioactives get into the cell, they are then subject to metabolism by enzymes, such as cytochrome P-450, glycosyltransferases (UGTs), sulfotransferase (STs), and others (38). The intracellular biotransformation sets the physiochemical barrier that reduces the amount of bioactive compounds reaching the portal blood system. In contrast to the transcellular route, paracellular transport allows small molecules (MW < 200) to diffuse across the gut wall through tight junctions located in between the epithelial cells. Nutraceuticals employing the paracellular route bypass intestinal metabolic activities and are absorbed into portal blood with their original chemical structure (38, 39). Even though the portal blood system is the major absorption route in the GI tract, the intestinal lymphatic system appears to be a better alternative for the uptake of some highly lipophilic compounds. Exhibiting high affinity with the enterocyte-derived lymph lipoproteins, the highly lipophilic compounds are absorbed into the intestinal lymphatic system and directly deposited to the systemic circulation without going through the first-pass intestinal and hepatic metabolism (40). Depending on the absorption routes and sites that each nutraceutical has undertaken, the rate and concentration of orally-administered active compounds that become available to the systemic circulation are varied considerably.

In conjunction with intestinal metabolisms, hepatic metabolisms have been confirmed as major mechanisms that contribute to the physiochemical transformation of ingested bioactive compounds. Due to the high density of metabolic-related cytochromes and enzymes, the liver is thought to be the limiting factor responsible for reducing the orally-administered dose that reaches the systemic circulation (41). A family of membrane-associated proteins, cytochrome P-450 (CYPs), is involved in the metabolism
of most endogenous compounds and, specifically, accounts for 75% of the biotransformation of active compounds (42). Within the P-450 enzymes, the CYP3A family is essentially the most important bioactive-metabolizing enzyme. Being widely found in both gut epithelium and liver, activated CYP3A enzymes are capable of metabolizing a wide range of bioactive compounds through oxidation reactions (41, 43). While metabolic activity is an important mechanism that the body utilizes to eliminate any potential toxin, its interaction with nutraceuticals may reduce the relative bioavailability and may alter the therapeutic efficacy of the nutraceuticals.

**Bioavailability**

While nutraceuticals have become a rapidly growing interest in disease prevention and treatment, the bioavailability of their key active ingredients have become an important issue in the development of oral formulations. Since health-promoting abilities are greatly dependent on the dosing efficiency, nutraceuticals must withstand the physiological or physiochemical activities that may alter their chemical structure as they move along the GI tract system. Being susceptible to pre-absorption modification and post-absorption first-pass metabolic conversions, orally-ingested nutraceuticals in general are poorly available to the system circulation and, thus, therapeutically meaningful dosages are difficult to achieve. Prior to absorption, the properties of nutraceuticals could be modified as the digestion environment gradually changed in pH, ionic strength, compositions of polymer and surface-active constituents, enzymatic activities, and complex GI motilities. Moreover, compounds that sustain the gastric modification are taken up by the intestinal enterocytes and, subsequently, are subjected to extensive first-pass metabolic activities that result in further transformation of chemical characteristics.
As the bioavailability of nutraceuticals can be defined as the fraction of the orally-administered dose that eventually reaches the systemic circulation, a simplified equation was used to described the overall bioavailability (F) in relation to the fraction absorbed from the GI tract and the fraction remaining unchanged by first-pass metabolic activities:

\[ F = f_{abs} \times F_M \]  

(Eq. 1.1)

where \( f_{abs} \) is the fraction of bioactives absorbed from the gastric lumen and \( F_M \) is the fraction that remains unchanged by first-pass metabolism. Several physical and chemical characteristics, such as aqueous solubility, particle size, gastric retention time, diffusion rate across the intestinal epithelial wall, etc., may accountably influence the rate and extent of nutraceuticals being taken up by intestinal absorption. Therefore, \( f_{abs} \) can then be expressed mathematically as follows (11, 44, 45):

\[ f_{abs} = f_B \times f_T \]  

(Eq. 1.2)

In this mathematical expression, \( f_B \) represents the amount of compounds released into the gastric lumen and made bioaccessible for cell wall absorption. To be accessible for intestinal absorption, the ingested compound must be either solubilized or dispersed in the aqueous lumen while residing in the human gut system. The other component in the equation (\( f_T \)), the fraction of bioactive compounds being transported across the gut wall, contributes to the intestinal absorption appreciably, depending on the compound transport coefficient and permeability. While absorption from the gastric lumen increases the portal concentration of nutraceuticals, the first-pass metabolic activities occurring in the successive organs considerably reduces the dose that reaches the system circulation. Due to differences in the tissue distribution and interactions, \( F_M \) of nutraceuticals can be viewed separately as (38):
Here, $F_G$, $F_H$, $F_L$ stand for the fraction of remaining bioactives not metabolized by the gut, liver, and lung, respectively. With all factors together, the bioavailability of an orally-ingested nutraceutical is the product of complex uptake and metabolic mechanisms that occur within the biological system. For optimum oral bioavailability, ingested nutraceuticals must possess favorable physical and chemical stability, gastric compatibility, and resistance to various metabolic conversions.

**APPROACHES TO ENHANCE ORAL BIOAVAILABILITY OF NUTRACEUTICALS**

When passing through the human GI tract, orally-ingested nutraceuticals are subject to diverse physiological and physiochemical barriers that reduce the doses reaching the system circulation. To enhance the bioavailability of bioactives, many scientists have formulated various delivery systems with functional properties to overcome these limiting factors. In this section, we will be discussing several approaches that investigators have utilized when designing an optimum system for nutraceutical deliveries.

- **Protection of labile compounds.** Upon oral ingestion, nutraceuticals are subjected to complex digestion processes that involve changes in physiological or physiochemical environments. From the mouth to the colon, the dynamic environmental conditions of the GI tract may cause instability to the chemical structures of active ingredients. The factors, such as pH variations, ionic strength, enzyme degradations, mechanistic motilities, etc., all potentially contribute to the degradation of nutraceuticals. Therefore, delivery systems that are designed with protective mechanisms could
enhance the gastric stability of labile bioactive nutrients and, thus, the oral dosing efficiency.

- **Extension of gastric retention time.** Gastrointestinal digestion is a dynamic moving process, in which nutrients are continuously pushed down to subsequent digestion stages and absorption sites. Insufficient gastric retention time results in the incomplete absorption of nutrients, excessive excretion of target compounds, and reduction in the dose-responsive efficiency for therapeutic purposes. Formulating delivery systems with higher viscosity or ability to slow down the gastric movement of bioactive compounds prolongs the residence time in the GI tract and allows a higher percentage of bioactives to be absorbed before gastric emptying.

- **Increase aqueous solubility.** In order to be effectively taken up by the intestinal enterocytes, the bioactive compound needs to be either solubilized, suspended or dispersed in the aqueous environment. Unlike hydrophilic nutrients, compounds that are lipophilic tend to have poor solubility and frequently precipitate as clusters when added to the aqueous environment. Large compound clusters inhibit intestinal absorptions, which involve a critical particle size requirement, and are rapidly eliminated through excretion mechanisms. Since poor aqueous solubility is a major factor that limits the absorption of lipophilic compounds, delivery systems that are capable of enhancing the solubility or dispersion of such ingredients can effectively augment the concentration entering the target action site within the biological system.

- **Control/delayed release.** The maintenance of constant dosing level within the systemic circulation is one of the important factors to sustain meaningful physiological efficacy between therapeutic intervals. Well-designed control release
mechanisms are capable of sustaining constant release profiles that best comply with the digestion and absorption process. In order for the contained compound to be released from the delivery vehicle, the delivery vehicle will need to be digested or disintegrated by enzymatic activity. The release rate and time of bioactive compounds can be controlled through selecting materials with different digestive sustainability and incorporating layers of digestive protection material on vehicle surfaces.

- **Facilitation of lymphatic uptake.** Most compounds are absorbed by the small intestine, enter the systematic circulation through the portal vein, and are then subject to hepatic metabolism. Lymphatic uptake, as discussed earlier, is a better alternative for systematic transport of certain highly lipophilic compounds. Compounds that are transported through lymphatic uptake are exempt from the first-pass hepatic metabolism, and thus may promote higher bioavailability of the parental compound. This is due to the fact that the rate of lymphatic uptake is determined by the ability of bioactive compounds to associate with lipoproteins within enterocytes (26). The utilization of some lipid-based delivery vehicles with nano-scale particle size is proven to be an effective way to increase the direct intestinal lymphatic uptake of lipophilic compounds.

- **Intestinal permeability enhancement.** A variety of materials has been shown to change the physical barrier function of the intestinal wall (46). Intestinal membrane fluidity can be affected by dietary lipid intake (47) and interaction with muco-adhesive polymers (48, 49). When formulating a delivery vehicle, all components making up the system structure can be designed to support intestinal membrane fluidity. For example, chitosan, as a positively-charged muco-adhesive polymer, was
documented to affect intestinal membrane integrity and tight junction widening, which allows the paracellular absorption of hydrophilic compounds (48-50).

- **Modulation of metabolic activities.** While absorption set the first barrier for bioavailability, the first-pass metabolic activities are the second hurdle that reduces the system dosing level of nutraceuticals. The inclusion of physical or chemical inhibitors of metabolic enzymes in the delivery system may significantly enhance the concentration level of bioactives in the system circulation. However, the utilization of such enzyme inhibitors may require more careful consideration to avoid increased toxicity due to impaired detoxification activity.

**EMULSION-BASED NUTRACEUTICAL DELIVERY SYSTEMS**

Much advancement has been achieved in the field of delivery systems for enhancing the oral bioavailability and, thus, the efficacy of nutraceuticals. Delivery systems such as emulsion have a long-standing history of being utilized as a protective mechanism for many flavor and active ingredients. This is because the effectiveness of nutraceuticals to provide therapeutic or physiological benefits greatly depends on the bioavailability of key active ingredients to the target site of action. Factors such as poor aqueous solubility, insufficient residence time in the GI tract, instability to changing physiological environments, low transport coefficient across the intestinal lining, susceptibility to rapid metabolic transformation, etc., could significantly lower the efficacy of nutraceuticals in disease prevention. Among all systems, emulsion is one of the most exploited and convenient oral delivery approaches to encapsulate, to protect, and to deliver active components having low bioavailability (29, 31, 45, 51). Depending on formulation composition and processing methods, different types of emulsion systems
can be conveniently customized to fit specific delivery needs. Specifically, oil in water (O/W) emulsion is demonstrated to be exceptionally effective to enhance solubility, absorption, transportation, and bio-efficacy of lipophilic functional food ingredients and pharmaceutics using in vitro and in vivo models (29, 45, 52, 53).

**Types of O/W Emulsion System**

With oil dispersed as small spherical droplets in the aqueous phase (usually water but not always), O/W emulsion can easily be incorporated to various aqueous-based environments. O/W emulsion is used in many food and beverage products, such as salad dressings, ice creams, mayonnaise, ready-to-drink coffee, milkshakes, and other items. Moreover, 70% of human body weight is composed of water, with the GI tract mainly filled with aqueous fluid. Therefore, investigation of O/W emulsion as the delivery vehicle for drugs or functional food ingredients is a particularly convenient and efficient method, for consumption as part of a dietary supplement. In much previous research, different emulsion formulations (Figure 1.3) effectively enhanced the bioavailability of lipophilic drugs by increasing drug solubility, promoting intestinal absorption, and mediating control of the rapid metabolic activity within the GI tract (28, 30, 54, 55). Emulsion is a favorably versatile system that can be easily prepared to possess assorted functionalities by altering different types of oil (short, medium, or long chain triglycerides; liquid or solid lipid; purified or crude oil extract), emulsifiers (synthetic or natural; neutral, positively or negatively charged; HLB values), and processing parameters (high speed homogenization, high pressure homogenization, ultrasound homogenization, temperature gradient, etc.).
**Figure 1.3** Different types of O/W emulsion systems.

*Conventional emulsion*

Conventional emulsion, also known as macroemulsion, is composed of two immiscible liquids, with one dispersed in the other by action of mechanical shear. The droplets in the dispersed phase typically have mean droplet diameter (MDD) within the range of 0.1 – 100 μm. Since the size of disperse phase droplets falls into the same order of light wavelength \( (d \approx \lambda) \), conventional emulsions typically appear as cloudy or opaque liquid systems, with the highest light scattering value at \( d \approx 200 \text{ nm} \) \((45)\). Furthermore, due to its relatively large droplet size and thermodynamically unstable nature, conventional emulsion is prone to gravitational separation and droplet aggregation, which eventually leads to phase separation upon storage. The viscosity of emulsion systems can be altered through selection of different emulsifiers, the size and concentration of dispersed droplets, and the presence of foreign particles. Nevertheless, since the
formation of conventional emulsion usually involves lower energy input and simpler processing requirements compared with emulsions having a much smaller droplet size, conventional emulsion is still currently the most commonly utilized form of emulsion in the food industry.

**Micro- and nanoemulsion**

Micro- and nanoemulsions, sometimes together referred to as submicron emulsions, are systems with much smaller dispersed droplets than conventional emulsion. In general, microemulsion has a droplet diameter ranging between 5 – 50 nm, while droplet diameter of nanoemulsion is about 20 – 100 nm (45). The formation of microemulsion occurs spontaneously over time and requires no energy input for particular composition ratio of dispersed phase, continuous phase, and emulsifier (56). The spontaneously-formed microemulsion is resistant to any types of phase separation, even for prolonged storage periods, as it is thermodynamically stable at the lowest free energy state. The nanoemulsion system, on the other hand, is a metastable but not thermodynamically stable system that will eventually phase separate upon long-term storage. Yet, due to the small MDD, nanoemulsion is sustained quite well on separation caused by gravitational force and, thus, can remain kinetically stable for a relatively longer period than conventional emulsion. Moreover, the attractive force between dispersed droplets is smaller with the smaller MDD and aggregation is then reduced. Both micro- and nanoemulsion systems appear as transparent liquid solutions, since MDD is much smaller than the wavelength of light \((d << \lambda)\) and scattering of light is minimum. The rheological property of submicron emulsion can also be altered through selection of formulation materials and processing conditions. However, when the
concentration of the disperse phase is the same for both conventional and nanoemulsion systems, nanoemulsion may have a slightly higher viscosity, since the hydrodynamic interaction between droplets increases as the distance between droplets decreases (57) due to denser packing of smaller droplet unities. Submicron emulsion with various favorable characteristics has become an emerging topic for advanced delivery systems. The small size, stability, and the transparent property of submicron emulsion offer many exciting possibilities in drug/nutraceutical delivery and development of novel functional foods.

**Self-emulsifying drug delivery system**

Self-emulsifying drug delivery systems (SEDDS) are isotropic mixtures of various types of oil, surfactants, and which sometimes contain cosolvents. SEDDS may be considered as an incomplete or halved emulsion system, which only contain the oil phase and surfactant but do not include an aqueous phase. The basic idea of the SEDDS is that it is not considered an emulsion until added to an aqueous environment, where it is spontaneously self-emulsified to form fine O/W emulsion or microemulsions (SMEDDS). The MDD size of SEDDS is typically between 100 – 300 nm, while SMEDDS has a droplet size less than 50 nm (58). Even though SEDDS is a spontaneously-formed, thermodynamically-stable system, it requires mild agitation or mixing to initiate the formation of the emulsion structure. When administered orally, SEDDS can easily be produced by digestive motility of the GI tract (59). SEDDS or SMEDDS generally appears as a bulk mixture of viscous oily mass. However, as a result of the MDD difference, when added to an aqueous environment, SEDDS can then transform into a cloudy/turbid emulsion, whereas SMEDDS is essentially transparent. In
contrast to emulsions that present many storage and processing difficulties, SEDDS may be a convenient and physically stable alternative that possesses many manufacturing advantages in the development of novel drug delivery products.

**Solid lipid nanoparticle**

Instead of containing a liquid disperse phase, a solid lipid nanoparticle (SLN) system includes either solid or semisolid lipid particles as part of its O/W emulsion system. The degree of crystallinity within the dispersed solid core can be altered by varying the compositional liquid/crystalline lipid ratio. Similar to emulsion with liquid disperse phase, SLN has a size range from 50nm - 1μm (60) depending on the lipid concentration, types and amount of emulsifier used, and inclusion of other functional material. During processing of SLN, temperatures above the lipid crystalline melting point should be maintained for ensuring homogeneous droplet dispersion, even coating of emulsifier, and encapsulation of the active compound, if applicable. High-pressure homogenization is a common technique used for the development of SLN. Due to the delicacy of the instrument, it is very important to ensure that all material passing through the system is in melted liquid state to prevent obstruction. Prolonged diffusion kinetics is a common characteristic shared by most of the crystalline matrix. Therefore, when SLN is utilized as the delivery vehicle for lipophilic bioactive compounds, the reduced compound mobility (Figure 1.4) within the crystalline matrix results in a slower drug release rate and higher digestive stability (61-63), which qualifies it as a suitable carrier system for controlled-release purposes. The positive impact on the bioavailability and efficacy of active compounds using SLN as a controlled-release carrier system is demonstrated by much research (60, 64-66). SLN also exerts a dependable protection
mechanism for volatile flavor compounds (67) and easily-degraded environmentally-sensitive compounds (68). Overall, SLN is an emerging method to solve many problems in drug instability, absorption and target delivery.

![Comparison in compound release behavior of emulsion systems containing liquid (left) and solid (right) dispersal phases.](image)

**Figure 1.4** Comparison in compound release behavior of emulsion systems containing liquid (left) and solid (right) dispersal phases.

*Multilayer emulsion*

Multilayer emulsion, also called layer-by-layer emulsion, is considered to be an advanced emulsion system with added functional properties. Through the manipulation of droplet surface composition, multilayer emulsion can be synthesized to possess many desirable properties, such as better digestion, improved storage stability, increased resistance to environmental stress, and enhanced membrane permeation and absorption. The fundamental principle for multilayer development is the electrostatic attraction between oppositely-charged molecules. Briefly, the preparation of multilayer emulsion starts from making a primary emulsion with charged emulsifier (either anionic or cationic) and the subsequent addition of oppositely-charged biopolymer to the system as a secondary coating. If more than two layers of the lamination is desired, polyelectrolyte
with opposite charge from the previous layer will be applied (Figure 1.5). The physical and functional properties of emulsion systems can be effectively improved using the layer-by-layer technique. For example, using a multilayer emulsion system composed of negatively-charged lecithin as the primary emulsifier and positively-charged chitosan as a secondary coating for citral protection has shown improved citral stability and reduced off-flavor compound production (67). Other improvements in physical stability, such as resistance to droplet aggregation and creaming, is also found in emulsion systems with multilayer interfacial coating due to increased repulsive force and particle density (45). Furthermore, the potential of multilayer emulsion for improved functionality has not been fully exploited. Principally, there is much opportunity remaining to research applications in the delivery of active ingredients for improved oral stability, solubility, permeability and absorption properties.

Figure 1.5 Schematic formation of multilayer emulsion system.
**Emulsion preparations and functional mechanisms**

As a result of chemical structure variation among phytochemicals, reasons that lead to low bioavailability may be distinctive and, thus, each may require a different strategy when designing delivery systems. To prepare a successful emulsion formulation not only involves specific material composition and proportion, but also requires a precisely calculated processing method, timeline and set of environmental conditions. In the following section, we will be introducing common methods applied for emulsion preparation and functional mechanisms that improve bioavailability.

**Preparation method**

Homogenization is the process of forming emulsion from mixing two immiscible liquids by homogenizers, which are machines that provide mechanical stress to physically break down the dispersed phase into small droplets (27). The droplet size of an emulsion system depends upon the energy density supplied by various homogenizers. Frequently, homogenization is a two-step process, which involves the formation of a primary coarse emulsion and then a subsequent size reduction, creating a fine secondary emulsion by machines that supply higher energy density.

- **High speed blender/homogenizer (HSH):** HSH is a convenient device that is commonly used by the food industry for directly mixing bulk oil and aqueous solvent to form primary coarse emulsion. The rotating blade of HSH creates a turbulent velocity gradient that interrupts the oil-water interface and converts it into an emulsion, with one dispersed in the other. Depending on the design and rotating speed of the stirrer, the droplet size of emulsion created by HSH can range from several micrometers (μm) to submicron scale.
Colloid mill: Colloid mill is a mechanical device that can efficiently reduce the droplet size of intermediate to high viscosity liquid through high shear force created by a set of stationary and rotating disks. Instead of being added as unmixed bulk oil and aqueous phase, samples that feed into the colloid mill system are usually a preformed coarse emulsion, and use of the colloid mill more efficiently breaks it down to a smaller droplet size. The size of emulsion droplets formed by a colloid mill is typically 1 – 5 μm in diameter and can be adjusted through altering the spinning speed, gap width between the stationary and rotating disks, surface roughness, and processing time.

High Pressure valve homogenizer (HPVH): In HPVH, a high-pressure environment can be created either by an electric motor or a gas/air-driven high-pressure pump up to 30,000 psi. With the application of high-pressure processing, HPVH generates intense shear, cavitation, and turbulent flow conditions that break down large droplets into tiny ones. HPVH is a technique that is commonly used to produce fine droplet particles as small as 0.1 μm. HPVH frequently includes a manually-adjustable gap size. When the gap size is small, higher pressure is required to push the coarse emulsion pre-mix (Figure 1.6) through the narrow valve, where it experiences a tremendous amount of mechanistic stress and becomes a finer emulsion mixture. Besides pressure, the extent of emulsion droplet size reduction is also determined by the number of passes of the sample through the valve. In general, the higher the applied pressure and the more processing passes into the HPVH system, the finer and smaller the emulsion droplets become.
Figure 1.6 General formation process for emulsion with fine droplets using a combination of high-speed homogenizer and high-pressure homogenizer.

- **Ultrasonic Homogenizer (Ultrasonicator):** Ultrasonicators generate high-intensity ultrasonic waves that produce high shear stress and pressure within the contacted sample. The emulsion droplet is broken up mainly by cavitation effect and overwhelming droplet oscillation. Since premixed coarse emulsion is not required for ultrasonic homogenization, ultrasonic processing is a convenient one-step procedure that can create fine disperse droplets when emulsion forms. The ultrasonic homogenizer generates emulsion droplets of similar size in a much more energy-efficient manner than a high-pressure homogenizer. However, the sonication process usually generates excessive amounts of heat, causing the unfavorable transformation of heat-sensitive material. Therefore, it is advisable to combine the ultrasonicator with an effective cooling system and to utilize a short-pulse technique during application.

- **Membrane Filtration:** In the membrane filtration technique, pressure is applied to drive emulsion through a semipermeable membrane. Depending on the membrane pore size, emulsion droplets resulting from membrane filtration can have a size range
from micron (microfiltration) to nano or micro (ultrafiltration) scale. While other homogenization techniques usually generate an appreciable amount of heat, membrane filtration is a preferable alternative for processing emulsions that contain heat-sensitive materials. Membrane filtration is an energy-efficient method to physically reduce emulsion droplet size while causing no chemical or phase change.

- **Microfluidization:** Microfluidizers can be used to produce extremely small emulsion droplets by mixing accelerated oil and aqueous phases when they collide on an impingement surface. The accelerated oil and aqueous droplets bump into one another in high-velocity intermingling and simultaneously break down to fine particles. Microfluidizers are especially useful in small-batch processing of samples with limited quantity or expensive compositional material.

- **Phase inversion:** Phase inversion is a low-energy method for the preparation of emulsion by changing the spontaneous curvature of the surfactant (69). Emulsions formed by the phase inversion method undergo a transition from water-in-oil emulsion to oil-in-water emulsion or vice versa. The inversion of phase can be induced by changes in physical conditions, such as temperature, or by variation in emulsion composition, such as surfactant, lipid, or aqueous phase concentration. The phase inversion method is capable of generating small emulsion droplet sizes in the nano (nm) range and is preferred by energy-conscious manufacturing production.

**TANGERETIN**

Tangeretin (4',5,6,7,8-pentamethoxyflavone) belongs to a sub-group of flavonoids called polymethoxyflavone. Polymethoxyflavones (PMFs) are an emerging category of phytochemicals that are mainly extracted from the peels of citrus fruits. By
definition, PMFs are compounds that have two or more methoxy groups attached to the 15-carbon benzo-γ-pyrone skeleton structure, with a carbonyl group on the C₄ position (Figure 1.7). Owning to the substituted methoxy groups, PMFs have superior metabolic stability and membrane permeability over flavonoids (70). PMFs are documented to show promising anti-inflammatory (71), anti-atherosclerosis (71), and selective anti-proliferative activity to cancer but not normal cells (72, 73). PMFs exist primarily in citrus peel, which is the major by-product from manufactured citrus-origin products. During the year of 2009-2010, a total of 10.9 million tons of citrus were produced in the U.S. Around 34% of these products were used for juice production, yielding approximately 44% of citrus peels as by-products (approximate 4 billion pounds). Therefore, isolation and utilization of PMFs from usually discarded orange peel will not only provide a chance to combat treat human disease, but also to create significant economic value to the society.

![Figure 1.7 Chemical structures of polymethoxyflavone (PMF) and tangeretin.](image)

Being part of the PMF family, tangeretin functions as a potential chemopreventive agent since it exhibits potent anti-inflammatory (74, 75), anti-proliferative (76), and anti-carcinogenesis (77, 78) activities (Figure 1.7). Previous studies have shown that
tangeretin reduced IL-1β-induced cyclooxygenase (COX-2) expression in human lung epithelial carcinoma cells, A549 (75). Gene expression of other pro-inflammatory cytokines such as interleukin TNF-α and IL-6 was also found to be downregulated by tangeretin (79). Moreover, tangeretin inhibited cell proliferation through inducing G1 cell cycle arrest in breast and colon carcinoma cells (80, 81). However, the bioavailability of tangeretin is low due to its low aqueous solubility, arising from the multiple substitutions of methoxy groups on the skeleton backbone when ingested orally. Consequently, the required tangeretin concentration for many intended therapeutic purposes is difficult to reach using an oral delivery route.

LECITHIN

Lecithin, also known as phosphatidylcholine (PC), is not only an important cell membrane constituent, but also plays an essential role during the digestion and absorption of lipophilic compounds. Lecithin can be isolated from a variety of natural sources, such as milk, egg, soybean and rapeseed, sunflower seed, etc. Since lecithin is a naturally-occurring component in living organisms, orally-ingested lecithin is compatible with many physiological activities that can be easily broken down during digestion, metabolized and utilized by living cells while not promoting toxic side effects to humans. In this sense, lecithin as a direct food substance has been granted generally recognized as safe (GRAS) status by the U.S. Food and Drugs Administration (FDA) under Title 21, part 184. Lecithin structurally contains a non-polar tail of two long-chain fatty acids and a polar head with a zwitterion phosphate-choline group (Figure 1.8). It is categorized as an amphiphilic molecule and is widely used as an emulsifying, wetting, and dispersing agent. In our previous investigations, lecithins were used as emulsifiers in the citral
encapsulating system to effectively improve storage stability and applicability (67). With a GRAS status and the ability to provide physiological benefits, lecithin is an emerging material to be used as an emulsifier or surfactant for oral formulations. The effectiveness of phospholipid-based delivery systems has been proven effective to improve the bioavailability of many lipophilic active ingredients (29).

In addition to being used as a functional food additive, lecithin itself was well documented to provide important physiological benefits and was suggested as an oral supplement for daily consumption. Having choline as part of its structure, PC is recognized as an effective dietary supplement for satisfying the daily choline requirement for humans (~550 mg/day). Choline, as a structural labile methyl donor, is critical for cellular signaling, normal neuro-functions, hormone secretion, protein synthesis, and DNA methylation (82-88). Frequent consumption of lecithin has been proven to protect the liver from damage due to alcohol consumption and abnormal fat metabolism (89, 90), to lower the blood cholesterol through increasing the high-density lipoprotein (HDL) formation (89, 90), and to maintain the function of the brain and immune system related to aging (91-93).
Figure 1.8 Chemical structure of lecithin (phosphatidylcholine).
CHAPTER II. DEVELOPMENT OF A VISCOELASTIC EMULSION SYSTEM FOR ORAL TANGERETIN DELIVERY

PROJECT TITLE: DESIGN OF HIGH-LOADING AND HIGH-STABILITY VISCOELASTIC EMULSIONS FOR POLYMETHOXYFLAVONES

The work in this chapter has been published in the title of “Design Of High-Loading And High-Stability Viscoelastic Emulsions For Polymethoxyflavones” in the Journal of Food Research International (Volume 54, Issue 1, Pages from 633 to 640) on November 2013.

Abstract

Polymethoxyflavones (PMF), a class of highly lipophilic phytochemicals found in citrus peels, were documented to possess various potent bio-functionalities. The efficacies of PMFs are greatly limited by their low solubility in aqueous environment and rapid metabolic activities. Moreover, the incorporation of PMFs into pharmaceutical, nutraceutical, cosmetic, and food products has been hindered by the instability of their liquid formulations and crystalline sedimentation at application and storage temperature. In this paper, a new method of forming highly stable viscous emulsions was revealed to suspend and encapsulate high loadings of PMFs (>2.5%) for prolonged storage under ambient temperature. Without utilizing any potentially toxic organic solvent during processing, oil-in-water (O/W) viscous emulsions were constituted solely by GRAS status food ingredients: medium chain triglyceride (MCT), lecithin, and water. The viscous emulsion matrices were homogeneous mixtures of PMFs crystals and saturated emulsion droplets with an average particle size of approximately 400 nm. Depending on
its lipophilicity, the size of PMFs crystals entrapped in the viscous emulsion matrix varied considerably; 9.3±1.3 µm and 3.8±0.6 µm for tangeretin and 5-demethyltangerein respectively. The effectiveness of viscous structures to prevent PMFs crystalline sedimentation was confirmed by studying dissolution kinetics at 0, 15, 30, and 60 times the emulsion dilutions. With PMFs concentrations remaining constant in undiluted emulsion samples during the investigated time frame, PMFs dissolution concentrations decreased to 26.3 ± 2.8% and 52.7 ± 2.6% for tangeretin and 5-demethyltangeretin, respectively.

**Introduction**

The discovery of many potential bioactive compounds from natural plant sources has given the world hope of finding treatments for many age-related disorders such as cancer, chronic inflammation, coronary artery heart disease, osteoporosis and diabetes. Though various injection-based options are available to deliver these active compounds to patients, the most non-invasive and commonly acceptable method is oral delivery. However, the effective dose of these active compounds required for effective therapeutic effect is difficult to reach due to one or more unfavorable chemical or biological characteristics (e.g., temperature sensitivity, UV sensitivity, instability during pH fluctuation, low aqueous solubility, low transportation rate through the intestinal epithelial layer, fast metabolic activity, and rapid and extensive urinary clearance).

To overcome these obstacles, many approaches such as chemical modification, emulsification, dispersion, and micelle encapsulation, have been applied to increase the oral bioavailability of these active compounds. Moreover, these active compounds are not alike. They all have specific chemical compositions and associated favorable or
unfavorable characteristics. In other words, there is no one universal delivery strategy that is applicable to all bioactive compounds. Therefore, for each individual compound, scientists need to identify the specific causes of low bioavailability and utilize the different strategies available to augment biological functionality.

As researchers explore new bioactive compounds from plant sources, a recently emerging bioactive compound category, polymethoxyflavones (PMFs), have been revealed to possess various potent health benefits. PMFs are flavones that have two or more methoxy (-OCH$_3$) groups on the 15-carbon benzo-$\gamma$-pyrone skeleton structure with a C$_4$ position carbonyl group. PMFs are documented to show promising anti-inflammatory (71), anti-atherosclerosis (71), and selective anti-proliferative activity to cancer but not normal cells (72, 73). Comparing with PMFs, another recent isolated variation of PMFs, hydroxylated polymethoxyflavone (hydroxylated PMF), which has hydroxyl groups substituting for one or more methoxy groups on the skeleton structure, has been shown to have even more compelling anti-cancer and anti-inflammatory activities (94-96).

PMFs and hydroxylated PMFs are mostly found and isolated from the peel of citrus fruits. Between 2009 and 2010, utilized citrus production in the United States was 10.9 million tons, and 66% of utilized citrus fruit production was further processed to consumer products, which yields large amounts of citrus peel as byproducts. Therefore, the isolation and utilization of PMFs from typically discarded citrus peel will not only provide a chance to combat human disease, but also to create an increase in economic value to the society. Despite their exceptional biological and economical value, the bioavailability of PMFs and hydroxylated PMFs are greatly limited by their low
solubility in aqueous environments (71) as well as their rapid and extensive metabolic activities (97). In the present study, a PMF, tangeretin (5,6,7,8,4’-pentamethoxyflavone), and the corresponding hydroxylated PMF, 5-demethyltangeretin (5-hydroxy-6,7,8,4’-tetramethoxyflavone), were selected as model compounds for the development of a delivery system (Figure 2.1).

Emulsion systems are the simplest and most common method utilized to encapsulate lipophilic drug ingredients. However, as previously reported by McClements’ group (22), the emulsion system was not sufficient to entrap PMFs because the compound continuously migrated from the emulsion oil phase to the aqueous phase due to supersaturation during processing at elevated temperatures. Taking the compound migration into account, a viscous emulsion matrix is proposed to stabilize the suspension and entrapment of tangeretin and 5-demethyltangeretin. In searching for a food-grade amphiphilic ingredient to emulsify tangeretin and 5-demethyltangeretin, a focus on GRAS (generally recognize as safe) materials is required. Rapeseed-origin PC 75 lecithin, as the only emulsifier applied to stabilize high-oil-ratio (>52.5%) oil in a water (O/W) emulsion gel system was used. Lecithin has drawn significant attention and is widely used as an emulsifying agent for its biologically compatible, non-synthetic, natural origin (98, 99). In this study, the effectiveness of a lecithin-based viscous emulsion system to stabilize the encapsulation and entrapment of PMFs was investigated.
Figure 2.1 Chemical structures of tangeretin and 5-demethyltangeretin.

Materials and methods

Materials

Tangeretin (> 98% pure) was purchased from Quality Phytochemicals, LLC. (Edison, NJ, USA). 5-Demethyl tangeretin was synthesized in the laboratory using previously published methods (Li et al., 2009). Rapeseed PC75 lecithin was provided by American Lecithin Company (Oxford, CT). Neobee medium-chain triacylglycerol (MCT) was donated by Stepan Company (Northfield, IL).

Thermal analysis

The thermal properties of dry tangeretin powder, as well as dry 5-demethyl tangeretin powder, were analyzed using differential scanning calorimetry (DSC, Model 823, Mettler Toledo Instruments, Columbus, OH). Each powder sample was weighed (3-6 mg) in a mechanically sealed pan with a fitted lid made of aluminum. The top of the lid was pinched by a needle to allow the release of gas built up during the heating
measurement. The thermal properties of the samples were recorded by heating from 25 to 200°C at the rate of 10°C/minute and subsequent cooling back to 25°C at the rate of 20°C/minute.

**Saturation loading range estimation in Medium-Chain Triacylglycerol (MCT)**

Initially, MCT oil containing 1% (0.125 g in 12.375 g MCT) of tangeretin or 5-demethyltangeretin was heated and maintained at 130°C with stirring. After complete solubilization, 0.125 g of tangeretin or 5-demethyltangeretin was added repetitively until the added compounds could no longer be solubilized (prolonged turbidity for 1 hour). The ranges of saturation loading were then determined based on the data obtained.

**Viscoelastic emulsion preparation**

To begin, 0.63 g of tangeretin or 5-demethyltangeretin powder was added to 15.12 g of MCT during continuous stirring at 130°C. After the added compounds were completely solubilized, 0.45 g of rapeseed PC 75 lecithin was added and stirred until the solution became clear, indicating that all substances added to the oil phase were completely dissolved.

13.8 g double deionized water was measured and then heated to 70°C before being added to the oil phase at the same elevated temperature. This was to prevent compound crystallization due to sudden temperature drop. After the addition of the aqueous phase, the mixture was stirred and maintained at 70°C for 1 minute to allow the formation of a crude emulsion to prevent excessive bubble formation. In order to reduce emulsion droplet size and viscosity, the pre-formed crude emulsion was then subjected to high-speed homogenization (High-speed homogenizer, ULTRA–TURRAX T-25 basic, IKA Works Inc., Wilmington, NC, USA) at 24,000 rpm for 2 minute. After high-speed
homogenization treatment, the emulsion was further processed using a high-pressure homogenizer (EmulsiFlex-C3, AVESTIN Inc., Ottawa, Canada) at an elevated temperature (55°C) for 30 minutes. After processing, the emulsion samples were stored in dark conditions at room temperature.

**Particle size determination**

Particle sizes were determined using photon correlation spectroscopy (PCS); a BIC 90 Plus particle size analyzer equipped with a Brookhaven BI-9000AT digital correlator (Brookhaven Instrument Corporation, New York, NY, USA) was used. To avoid multiple scattering effects, each emulsion sample was diluted 500 times in double deionized water before placing into a 1-cm path length cuvette for measurement. The light source was a solid-state laser operating at the wavelength of 658 nm with 30 mW of power, and a high sensitivity avalanche photodiode detector was used for signal detection. Measurements were conducted at a fixed scattering angle of 90° at 25 ± 1°C. Results were reported as mean particle size and size distribution in triplicate (n = 3).

**Morphology observation**

Samples were diluted in a 1:1 ratio with double de-ionized water, mixed well using a vortex, dropped onto a microscope slide and then covered by a cover slide. Inverted optical microscopy (Nikon TE 2000, Nikon Corporation, Japan) equipped with a CCD camera (Retiga EXi, QImaging) was used for sample observation. All images were processed by C-Imaging software (SimplePCI, Compix Inc.).
Viscosity measurement

Rheological measurements of the emulsion samples were performed using an ARES rheometer (Rheometric Scientific, Piscataway, NJ) with a parallel plate geometry (diameter 25 mm) at room temperature (25 ± 1°C). About 1 mL of the emulsion sample was deposited onto the plate geometry for each measurement. Prior to each dynamic frequency sweep test, a dynamic strain sweep test ranging from 0 to 100% was performed at 2 rad/s angular frequency, and the strain value of 5% in the linear viscoelastic region was adopted. The angular frequency $\omega$ was varied from 1 to 100 rad/s, with 20 data points per decade. The gap between the two parallel plates was properly selected to prevent sample slip. A small amount of mineral oil was utilized to seal the sample edge in order to prevent solvent evaporation.

Dispersion behavior test

The dispersion test was performed to elucidate the nature of the emulsion formed (W/O versus O/W). One mL of formulated emulsion sample was dropped into two separate glass bottles containing either 10 mL of double deionized water or MCT. After the sample was dropped into the container, the glass bottle was vertexed using an electric vertex at a speed of 10 rpm/s for 10 seconds. The formulated emulsion was determined to be O/W emulsion since it was finely dispersed in water but not in MCT oil.

Suspension loading stability evaluation

Loading of the emulsion sample was measured by light absorbance at 234 nm and recorded by an absorbance microplate reader (Molecular Devices, Sunnyvale, CA). A calibration curve was constructed using a diluted solution prepared by adding 1 mL of empty emulsion (containing no active drugs) into 99 mL of 95% ethanol to diminish the
background scattering effect. The constructed calibration curve encompassed an absorbance range from 0.176-2.571 mg/ml and 0.213-1.862 mg/ml for tangeretin and 5-demethyltangeretin, respectively, with $R^2 > 0.999$. The calibration curve was again verified by measuring the compound weight dissolved in the diluted solution before subjecting to the sample loading evaluation. Evaluation of the emulsion sample loading was done by accurately measuring the weight of sample into a 10 ml volumetric flask and diluting to 10 ml with 95% ethanol. Suspended loading concentrations of undiluted samples as well as samples diluted 15, 30 and 60 times were evaluated over five days.

**Results and discussion**

*Thermal analysis*

![Figure 2.2](image)  
**Figure 2.2** Differential scanning calorimetry (DSC) results of tangeretin and 5-demethyltangeretin powders. Arrows indicate the recrystallization temperatures.
To find the optimal condition for emulsion preparation, the temperatures related to re-crystallization (or precipitation) of tangeretin and 5-demethyltangeretin are critical. Both pure tangeretin and 5-demethyltangeretin were originally in powder form and re-crystallized (or precipitated) upon cooling from a warmed solution. In order to determine the temperatures at which PMFs could be fully dissolved in the oil phase (i.e. MCT), DSC measurements were performed to obtain their thermal properties. As shown in Figure 2.2, during the heating stage, either tangeretin or 5-demethyltangeretin in crystalline form shows a sharp melting peak at 152°C or 174°C, respectively. Moreover, indicated by the arrow in Figure 2.2, during the cooling stage, tangeretin presents a broad recrystallization peak at 65°C, while 5-demethyltangeretin exhibits a sharp recrystallization peak at 136°C. The broad recrystallization peak for tangeretin may have been due to the fast cooling during the measurement. DSC results suggested that it is critical to maintain an elevated temperature during preparation of the emulsion in order to inhibit the negative impacts from recrystallization. Because the difference between the melting temperature and recrystallization temperature of 5-demethyl tangeretin was much smaller than that of tangeretin, the recrystallization in the former system was much faster than in the latter. When dissolved in MCT, 5-demethyl tangeretin exhibited faster recrystallization behavior than tangeretin when the temperature was reduced, which agreed with the result from DSC studies. Upon the removal of the heating source, 5-demethyl tangeretin was recrystallized within 5 minutes, while tangeretin crystals took much longer to form. Based on this observation, the optimal temperature of 130°C to solubilize both PMF compounds was determined for the emulsion preparation.
**Formulation loading determination**

Once the optimum temperature was determined, the optimum loading of target compounds dispersed in stable emulsion was further explored. Metastable saturation concentration varies with different temperatures or the combination of environmental factors. Keeping all other conditions constant, increasing temperature will increase the metastable saturation concentration, which leaves the dissolving media in a supersaturated state. At 130°C, the saturation range of tangeretin and 5-demethyl tangeretin was investigated. As shown in Table 2.1, tangeretin was completely soluble and formed a clear transparent oily solution with loading ranging from 1 to 5% in MCT at 130 °C. Turbidity began to show when the loading was increased to 6%, which indicated that parts of the tangeretin added could not be further solubilized, and the saturation concentration had been surpassed. As for 5-demethyl tangeretin, the metastable concentration occurred at a range between 6-7% at 130°C. Based on these results, it was concluded that the critical loading for tangeretin was between 5%-6%, and 6-7% for 5-demethyl tangeretin. Below the critical loading ranges, the emulsion could stabilize in one phase; above them, precipitation may occur.

**Table 2.1** Solubilization concentration (% by weight) of PMFs at 130 °C.

<table>
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<tr>
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<th>1%</th>
<th>2%</th>
<th>3%</th>
<th>4%</th>
<th>5%</th>
<th>6%</th>
<th>7%</th>
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<tr>
<td>Tangeretin</td>
<td>CS</td>
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<td>CS</td>
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<tr>
<td>5-demethyltangeretin</td>
<td>CS</td>
<td>CS</td>
<td>CS</td>
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<td>PS</td>
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*CS = Completely Soluble, PS = Partially Soluble

For consistency and ease of formulation design, we decided to keep the loading of two compounds at the same level. With the data obtained from the saturation loading range test, loading concentrations of 4% and 5% were investigated for formulation design.
However, as the loading became closer to the critical concentration, the speed of nucleation and crystal growth also increased (100, 101). In other words, the kinetics of both nucleation and crystal growth are greatly dependent on the degree of supersaturated concentration. 4% solubilizing concentration was observed to have a much delayed recrystallization compared with 5% concentration when removed from heating source. Since 5-demethyl tangeretin has rapid recrystallization characteristics upon cooling, it was more feasible to select 4% compound loading in the oil phase to sustain reasonable time delay due to human action pace limitation.

Dispersion behavior test

Since the emulsion system was designed to enhance oral bioavailability of tangeretin and 5-demethyltangeretin through increasing solubility, it was very important that the delivery system be soluble or dispersed into aqueous environment. Therefore, a dispersion test was conducted to determine whether the formulated viscoelastic emulsion composed of >52.5% oil phase would be an oil-in-water (O/W) or water-in-oil (W/O) emulsion system. One milliliter of formulated samples (tangeretin or 5-demethyltangeretin) was dropped into glass bottles that contained 10 mL of either MCT or DI water as the dispersal medium. After gentle swirling, samples that were deposited into DI water dispersed homogenously, while samples added into MCT beaded up (Figure 2.3). The result from the dispersion test indicated that the surface of emulsion droplets was hydrophilic and that the system was indeed an O/W emulsion that could easily be incorporated into an aqueous environment.
**Figure 2.3** Photographic images of PMF emulsion in MCT or DI water: (A) tangeretin emulsion in MCT; (B) tangeretin emulsion in DI water; (C) 5-demethyltangeretin emulsion in MCT; and (D) 5-demethyltangeretin emulsion in DI water. Emulsions were prepared with 52.5% MCT, 1.5% lecithin, 2.1% PMF compound and 43.9% water.

*Effect of emulsion encapsulation on the crystallization behavior*

Both tangeretin and 5-demethyltangeretin sustain a crystalline structure at room temperature and have a high tendency to return to crystal form after dissolution when cooling from an elevated temperature. Due to the chemical nature of PMFs (see Figure 2.1), both compounds are known to be highly lipophilic and, thus, have low solubility in an aqueous environment (71). This investigation was intended to present the enhanced physical stability of tangeretin and 5-demethyltangeretin by emulsion application in terms of sedimentation and flowability at room temperature. When equally weighted (same
concentration as in the emulsion system) tangeretin and 5-demethyl tangeretin were added separately to DI water and MCT at an elevated temperature with stirring, both compounds precipitated instantaneously in DI water, while both completely dissolved in MCT. Upon cooling from the elevated temperature to room temperature, both compounds precipitated and settled down at the bottom of the glass bottle containing DI water as the dispersing medium (Figure 2.4 A and D). However, when cooling in MCT, tangeretin precipitated at the bottom of the glass bottle (Figure 2.4 B), while 5-demethyl tangeretin rapidly formed a crystal matrix with MCT entrapped within the crystalline structure, forming a chunk of oily crystal mass (Figure 2.4 E). The crystallization behavior observed suggested that both compounds have limited physical stability as homogenous solutions at room temperature, regardless of their solubility differences in water and oil environments.

After encapsulation of tangeretin and 5-demethyl tangeretin in emulsion by high-pressure homogenization treatment, the physical stability and flowability of systems that contain PMFs were greatly enhanced. As shown in Figure 2.4 C and F, both emulsions formed with tangeretin and 5-demethyltangeretin appeared as stable and homogenous emulsions with flowability similar to viscoelastic fluids and showed no sign of sedimentation at room temperature. The observed stability of the PMF emulsion system was remarkable: no sedimentation or phase separation occurred even after more than four months of storage at room temperature (Figure 2.5). Our results suggest that this emulsion system was an effective method to enhance the physical stability of both compound
Figure 2.4 Photographic images of PMFs in DI water, MCT, and emulsion at ambient temperature: (A) tangeretin powder in DI water; (B) recrystallized tangeretin in MCT; (C) tangeretin in emulsion; (D) 5-demethyltangeretin powder in DI water; (E) recrystallized 5-demethyltangeretin in MCT; and (F) 5-demethyltangeretin in emulsion. Emulsions were prepared with 52.5% MCT, 1.5% lecithin, 2.1% PMF compound and 43.9% water.
Figure 2.5 Photographic images of (A) tangeretin emulsion; and (B) 5-demethyltangeretin emulsion after 6 months of storage. Emulsions were prepared with 52.5% MCT, 1.5% lecithin, 2.1% PMF compound and 43.9% water.

Stabilization of viscoelastic emulsion structure

Consistent with the previous literature (102), due to the effect of supersaturation, continuous migration of tangeretin and 5-demethyltangeretin from oil phase to aqueous phase was observed upon cooling. Therefore, without sufficient rheological structure (e.g. gel-like structure), sedimentation as well as phase separation would occur simultaneously. In this experiment, the effect of pressure and crystal size on the emulsion viscosity that inhibited the sedimentation of entrapped compounds was examined. Frequency-dependent storage modulus ($G'$) and loss modulus ($G''$) for empty emulsions (no PMFs
(encapsulated) with and without pressure treatment at 500 bar were evaluated. As shown in Figure 2.6, before pressure treatment, both $G'$ and $G''$ appeared at relatively low values (less than 5 Pa) over the whole frequency range studied; after pressure treatment, $G'$ became much higher than $G''$, which indicated that the rheological characteristics of empty emulsions had changed from more liquid-like to more gel-like behavior after treatment with high-pressure homogenization. The complex viscosity of pressure-treated empty emulsions was about 70 times higher than that of non-pressure treated empty emulsions, whereas both exhibit viscoelastic shear thinning behavior. The possible mechanism of enhanced viscosity was the reduction in particle size (Table 2.2) from 1314.6 nm to 312.8 nm. In previous investigations (103-105), the inverse relationship between droplet size and viscosity was reported. As droplet size decreases, the hydrodynamic interaction between droplets increases as the distance between droplets decreases (57) due to denser packing of smaller droplet unities. Brownian motion of densely packed small droplets also plays an important role at low shear rates (105). Brownian motion is the interaction between particles that randomize their distribution while colliding with one another to form transient clusters as doublet, triplet, or higher entities. At a lower angular frequency, more particles exhibit clustered rotational motion, which in turn contributes to the higher complex viscosity due to the higher energy dissipation than that of single particles. At a higher angular frequency, shear energy becomes large enough to break the interaction between aggregated particles and, thus, causes the drop in complex viscosity (105-108). In summary, pressure treatment reduces the droplet size of the dispersion phase and, thus, significantly augments the complex viscosity.
((A) empty emulsion*, no PMF encapsulated, high speed at 24,000rpm

(B) empty emulsion**, no PMF encapsulated, high speed at 24000rpm, high pressure at 500 bar

**Figure 2.6** (A) Storage modulus (G’) and loss modulus (G”) as a function of angular frequency (ω) at strain = 5% for empty emulsion* without high-pressure treatment; (B) storage modulus (G’) and loss modulus (G”) as a function of angular frequency (ω) at strain = 5% for empty emulsion** with high-pressure treatment. Emulsions prepared contain 54.6% MCT, 1.5% lecithin, and 43.9% water.
As decreasing droplet size increased the viscosity of the emulsion, the length of crystals locked within the viscoelastic emulsion structure exerted an opposite effect on the rheological behavior of the system. That is, the longer the length of the entrapped crystal, the lower the viscosity of the emulsion structure. As seen in Figure 2.7, with all of the processing parameters kept constant, empty emulsions showed the highest complex viscosity, followed by formulations containing 5-demethyl tangeretin with average crystal length at 3.84 μm, and the formulation containing tangeretin with average crystal length of 9.3 μm exhibited the lowest complex viscosity. After high-pressure processing at 500 bars, both tangeretin and 5-demethyl tangeretin were either encapsulated within the oil phase or dispersed homogeneously within the viscoelastic emulsion matrix. The deviation in the crystal length entrapped in the viscoelastic emulsion matrix was counter-related to the lipophilicity of the compound, since larger crystals locked in between the emulsion matrix increase the distance of emulsion droplets. In other words, the less lipophilic the compound is, the smaller the equilibrium partition coefficient (K_{ow}) becomes. Thus, the concentration of compounds outside of the oil phase increases, and these compounds are more likely to interact with each other to form larger (longer) crystals. The chemical structures of tangeretin and 5-demethyltangeretin deviate at 5-position on the aromatic ring, at which tangeretin has an OCH$_3$ group and 5-methyl tangeretin has an OH group. The 5-position OH group of 5-demethyl tangeretin was capable of forming intramolecular hydrogen bonds with the adjacent carbonyl group, which resulted in the formation of a very stable 6-member ring structure (109). When intramolecular hydrogen bonds form, the lipophilicity of the molecule increases as one donor and one receiver of hydrogen bonding function is eliminated (110) since they are
no longer available for hydrogen bonding interactions with water molecules. Therefore, the higher lipophilicity of 5-demethyltangeretin results in lower concentration outside of the oil droplet and forms smaller average crystal size, which contributes to less reduction in complex viscosity than formulations containing tangeretin. In summary, the opposing effects of emulsion droplet size and entrapped crystal length contributed to the overall differences in the viscosity of emulsions.
Figure 2.7 (A) Dynamic viscosities vs. angular frequency ($\omega$) for empty emulsion (×), tangeretin emulsion (Δ), and 5-demethyltangeretin emulsion (ο). Microscopy images (40x) of 1:1 dilution of (B) 5-demethyltangeretin emulsion and (C) tangeretin emulsion, respectively. Emulsions were prepared with 52.5% MCT, 1.5% lecithin, 2.1% PMF compound, and 43.9% water.
Table 2.2 Particle size, polydispersity, and crystal length of emulsion.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Particle Size (nm)</th>
<th>Polydispersity index</th>
<th>Crystal Length (um)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Emulsion*</td>
<td>1315±7.3</td>
<td>0.185</td>
<td>-</td>
</tr>
<tr>
<td>Empty Emulsion**</td>
<td>313±1.2</td>
<td>0.184</td>
<td>-</td>
</tr>
<tr>
<td>Tangeretin Emulsion</td>
<td>435±4.7</td>
<td>0.201</td>
<td>9.33±1.3</td>
</tr>
<tr>
<td>5-demethyltangeretin Emulsion</td>
<td>391±3.2</td>
<td>0.195</td>
<td>3.84±0.6</td>
</tr>
</tbody>
</table>

* no PMF encapsulated, high speed at 24,000rpm, pressure

** no PMF encapsulated, high speed at 24,000rpm, high pressure at 500 bar

To verify whether the viscoelastic structure of emulsion was indeed the most important stabilizing factor to prevent sedimentation of entrapped compounds, the suspended concentration reduction kinetics were evaluated at dilution factors of 0, 15, 30, and 60. Dilution of the viscoelastic emulsions decreased the emulsion droplet concentration and reduced the interaction between emulsion droplets that form a viscoelastic matrix. As the compound concentration of the original viscoelastic formulation remained constant over the experimental time frame, the compound concentration continuously decreased for the diluted samples. During the first 24 hours, more than 40% of tangeretin and 15% of 5-demethyltangeretin was no longer suspended in diluted emulsion samples and settled down at the bottom of the container. The difference in sedimentation speed between these two compounds was partially due to partition coefficient differences as well as size/weight variances, since larger (heavier) objects are prone to a larger gravitational effect. The effect of the viscoelastic structure as a key stabilizing factor was also clearly demonstrated as the sedimentation kinetics gradually slowed down after the first 24-hour incubation. Once the viscoelastic emulsion sample was significantly diluted, the viscoelastic liquid-like structure of the emulsion matrix was no longer sufficient to entrap compounds that were originally located in the
aqueous phase and locked within the emulsion matrix. Therefore, a significant amount of compound sedimentation was observed within 24 hours of incubation (Figure 2.8). After 24 hours, the sedimentation kinetics were mainly dependent upon the compound’s speed of migration from the oil phase to the water phase, as well as the compound’s partition coefficient between the oil and water phases. Since the mechanism involved in the sedimentation within the first 24 hours (Day 0-1) depended significantly on the original PMF compounds entrapped in between the oil droplet and migration of the compounds due to the partition coefficient, the discussion of dissolution kinetics of the compound encapsulated within the oil droplet was mainly focused on the period between day 1 (24 hours) and day 5 (120 hours). From day 1 to day 5, the decrease in the suspended compound concentration patterns was similar for both compounds, with tangeretin slightly faster due to less lipophilic characteristics. Pattern similarity of day 2 to day 6 in dissolution kinetics was analyzed by the vector included-angle cosine method of Fuzzy Cluster Analysis to give similarity values higher than 0.95 among diluted samples and between the two compounds. With a similarity value higher than 0.95, it can be concluded that the dissolution pattern for all samples, regardless of dilution factor, was similar and followed the same mechanism. Moreover, the curve with concentration plotted against time (hours) was exponentially fitted, and the half-life of each sample was calculated according to Equation 1 and reported in Table 2.3.

\[ y = Ae^{-x/t} \]  

(Eq. 2.1)

The calculated dissolution half-life was different among compounds, but was not related to the dilution factor (Table 2.3). From the results of half-time calculation, it can
be concluded that the migration kinetics of compounds entrapped in emulsion were stable upon dilution and may follow the similar reaction order.

**Figure 2.8** Original tangeretin suspension (A) and 5-demethyltangeretin emulsions (stock emulsions, crosses), with 15 times (filled circles), 30 times (empty circles), and 60 times (triangles) emulsion dilution vs. storage days. The original emulsion stock formulations were prepared with 52.5% MCT, 1.5% lecithin, 2.1% PMF compounds, and 43.9% water.
Table 2.3 Dissolution half-life of different dilution from day 1 (24 hrs.) to day 5 (120 hrs.)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Half Time (t) (hrs.)</th>
<th>Remaining PMFs dissolution concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tangeretin 1:15 dilution</td>
<td>125.00</td>
<td>22.93</td>
</tr>
<tr>
<td>Tangeretin 1:30 dilution</td>
<td>125.00</td>
<td>23.36</td>
</tr>
<tr>
<td>Tangeretin 1:60 dilution</td>
<td>142.86</td>
<td>32.66</td>
</tr>
<tr>
<td>5-demethyltangeretin 1:15 dilution</td>
<td>250.00</td>
<td>58.08</td>
</tr>
<tr>
<td>5-demethyltangeretin 1:30 dilution</td>
<td>166.67</td>
<td>47.78</td>
</tr>
<tr>
<td>5-demethyltangeretin 1:60 dilution</td>
<td>250.00</td>
<td>52.18</td>
</tr>
</tbody>
</table>

Conclusion

In this study, we successfully demonstrated the ability of viscoelastic matrix to stabilize PMF-encapsulated emulsion. PMFs, as represented by tangeretin and 5-demethyltangeretin, tend to recrystallize rapidly upon cooling from melting temperature. Therefore, it is necessary to maintain a high solubilization temperature during sample preparation. However, due to the effect of supersaturation at elevated temperature, PMFs spontaneously migrated out of oil droplets during storage in a lower-temperature environment. Without sufficient construction to entrap the migrated PMF crystal in aqueous phase, the migrated PMFs crystal will quickly separate out of the emulsion system as sedimentation. The viscoelastic structure of this emulsion system is simple, yet very effective to homogeneously disperse PMFs in the mixture.

The viscoelastic emulsion systems developed in this study have dramatically improved many characteristics that enable PMFs to be more bioavailable or bioaccessible to biological systems: 1. High stability for prolonged storage periods at room temperature. Good stability upon storage is very important for future development of consumer products with shelf lives long enough for practical consumer utilization of the intended
biological functionality, as well as providing sufficient economic value for the manufacturers. 2. Flowability at room temperature. Flowability allows easier oral feeding to animals or swallowing by human subjects when clinical studies for the evaluation of toxicity and bio-efficacy are to be conducted. 3. Enhanced solubility and dispersibility in aqueous environment. Upon oral consumption, the compound will be passing through the aqueous gastrointestinal (GI) tract digestion environment, in which non-aqueous-soluble substances will not be absorbed and, thus, cannot be utilized by the animal/human. Moreover, many of the food products contain water as a major component, and therefore, enhanced solubility and dispersibility in aqueous environment will allow PMFs to be incorporated as novel health-promoting ingredients. 4. High loading capacity. Based on a diet consuming 2,000 kcal per day, the effective dosage of PMFs to provide meaningful bio-functionality is approximately 50-250 mg per day for humans (102). According to Manthy et al., the concentration of PMFs found in most citrus peel molasses is lower than 100 μg per mL (111). In other words, to reach the minimum effective dosage, one will have to consume at least 500 ml of citrus peel molasses daily. However, with the loading level of PMFs emulsion, the minimum effective dosage can easily be reached by consuming as little as 1.52 g of the formulation. 5. Potentially enhanced bioavailability. Previous investigation has elucidated that the presence of oil during digestion will enhance the bioavailability of lipophilic compounds through micelle encapsulation (30). Viscoelastic formulations containing >52.5% of MCT and 1.5% of lecithin have high potential to be digested and form micelles to encapsulate the highly lipophilic PMFs and enhance their bioavailability. However, more detailed animal studies are warranted before definite conclusions on bioavailability enhancement can be determined.
Figure 2.9 (A) Microscopic image of 100x 1:1 dilution of tangeretin emulsion and (B) magnified section schematic representation of mixed emulsion matrix composed of saturated emulsion droplet and tangeretin crystals. Possible changes during storage at ambient temperature were suggested as (B) tangeretin continuously migrated from saturated oil droplets to aqueous phase and (C) subsequent crystal growth in the aqueous phase while still locked within the viscous emulsion matrix by emulsion droplets in between. Emulsions were prepared with 52.5% MCT, 1.5% lecithin, 2.1% PMF compound, and 43.9% water.

In summary, it has been proven that the viscoelastic structure is an effective way to stabilize the PMF emulsion system. The emulsion system is a mixture of saturated oil dispersion phase and PMF crystals, rather than a homogeneous emulsion system (Figure
2.9). The mixture of crystal and oil droplets is unavoidable unless the loading of PMFs is below the supersaturating concentration. In the future, we plan to further investigate the effects of bio-functionality enhancement of this formulation on both human cell models as well as in animal trials.
CHAPTER III. PROCESS OPTIMIZATION FOR TANGERETIN VISCOELASTIC EMULSION SYSTEM

PROJECT TITLE: INFLUENCE OF PROCESSING PARAMETERS ON MORPHOLOGY OF POLYMETHOXYFLAVONE IN EMULSION

As of submission of this dissertation, the work in this chapter has been submitted in the title of “Influence Of Processing Parameters On Morphology of Polymethoxyflavone in Emulsion” to the Journal of Agriculture and Food Chemistry for consideration of publication.

Abstract

Polymethoxyflavones (PMFs) are groups of compounds from citrus peels that have been documented as having beneficial bioactivities. Due to their chemical structures, PMFs are very hydrophobic and tend to crystallize at room temperature in either water or oil. As such, PMFs suffer from poor oral bioavailability. To improve the oral efficiency of PMFs, a viscoelastic emulsion system was formulated. Due to the crystalline nature, the inclusion of PMFs into the emulsion system faces great challenges in having sufficient loading capacity and stabilities. In this study, the process of optimizing the quality of emulsion-based oral formulation intended for PMFs oral delivery was systematically studied. By altering the PMF loading concentration, processing temperature and pressure, the emulsion with desired droplet and crystal size can be effectively fabricated. Moreover, storage temperatures significantly influenced the stability of crystal-containing emulsion system. The results from this study are a good illustration of system optimization and serve as a great reference for future formulation design of other hydrophobic crystalline compounds.
Introduction

With the advances in medical sciences and informational technologies, people have become more educated on disease pathologies. Instead of receiving therapeutic treatments after a disease has been diagnosed, engaging in preventive behavior has gained popularity among health-conscious individuals. Many professionals emphasize that maintaining an adequate level of physical activity and a well-balanced diet are the keys leading to healthier lives. However, many people find it difficult to always be “good” and strictly follow healthy doctrines. As a result, consumers are seeking extra help from new technological and scientific advancements to reach their ideal health conditions. The emerging discoveries of many bioactive compounds from dietary sources are the response of scientists to this need. The term nutraceutical is being used to identify these types of bioactive ingredients. Well-known examples of such entities, such as curcumin, quercetin, resveratrol, lutein, β-carotene, epigallocatechin gallate (EGCG), lycopene, etc., are often investigated for their ability to provide physiological or physiochemical benefits to living organisms. For chronic consumption, oral ingestion is probably the most user-friendly method for consumers to receive the advantages from health-promoting bioactivities. However, many of those nutraceuticals are poorly absorbed when consumed orally as a result of their unfavorable chemical characteristics, which include fast degradation, low solubility, poor intestinal uptake and transport, extensive metabolism, and rapid excretion rate. For these reasons, various delivery applications have been developed to augment their oral bioavailability.

When consumed with lipids, the oral bioavailabilities of highly hydrophobic compounds were shown to be significantly improved (28, 29, 31). Thus, many lipid-
based delivery formulations have been developed for the oral consumption of nutraceuticals with poor aqueous solubility\(112\). Emulsion, in this sense, has a long history of being applied as a convenient method to contain, protect, and facilitate uptake and transport of hydrophobic dietary ingredients. Due to this adaptable nature, many derivatives, supplemented with additional functional mechanisms, have been produced to broaden the applications of emulsion-based delivery systems for food and pharmaceutical uses. Moreover, since many studies have suggested an inverse relationship between the particle size and oral absorption of bioactive ingredients, the applications of technologies to physically reduce the emulsion particle to micro- or nano-scale have become a popular trend. However, with the reduction of particle size, emulsion-based delivery systems face the challenge of low compound loading capacity and higher sedimentation rate due to the presence of highly crystalline hydrophobic ingredients \(102\).

PMFs, such as nobeletin, tangeretin, sinensetin, \(3,5,6,7,8,3′,4′\)-heptamethoxyflavone (HMF) and others, contain multiple methoxy (-OCH\(_3\)) groups on their characteristic 15-carbon benzo-\(\gamma\)-pyrone structures and are categorized as a sub-group of flavonoids. PMFs and their hydroxylated derivatives have been proven to be potent therapeutic agents in preventing diseases such as chronic inflammation, tumor development, atherosclerosis, thrombosis, and oxidation damage \(71-73, 94-96\). Multiple methoxyl groups on the structure of PMFs contribute to their highly hydrophobic nature, which results in a low aqueous solubility and poor oral bioavailability. The application of emulsion-based delivery technology has been investigated for PMF oral ingestion with the interest of improving its bioavailability \(102, 113\). However, the high melting point and fast crystallization behavior of PMFs pose great challenges in designing, processing,
storing, and applying emulsion formulations \((102)\). Due to their poor solubility in both oil and water at ambient temperatures, the loading capacity of PMF emulsion systems is generally unsatisfactorily low and the formula is subject to precipitation during storage. In our previous studies, a viscoelastic emulsion was designed to maximize the PMF loading capacity while maintaining a stable system, even if crystallization did occur \((113)\). During the process of optimizing the formulation, interestingly, the crystallization kinetics and the resulting crystal morphology were affected by the processing parameters.

In the present study, we aim to systematically examine the effect of different processing conditions on the crystalline compound in an emulsion system. Moreover, the effect of various storage environments on the crystalline emulsion system was also included as a part of our discussion. Here, for the convenience of the study design, only tangeretin was selected as the model PMF compound in the emulsion system. The knowledge obtained from this study will serve as a useful reference for the future development of oral formulations targeting highly crystalline compounds.

**Materials and methods**

**Materials**

Tangeretin of 98% purity was purchased from Quality Phytochemicals, LLC. (Edison, NJ). Rapeseed PC75 lecithin containing 75% phosphatidylcholine was a gift from American Lecithin Company (Oxford, CT). Neobee 1053 medium-chain triacylglycerol (MCT) was a sample requested from Stepan Company (Northfield, IL). Milli-Q water was used throughout the experiment.
Preparation and processing of PMF emulsion samples

PMF emulsions were prepared according to a previously published method with necessary modifications (113). The distribution of each emulsion phase of the studied samples was kept constant at 46% aqueous, 1.5% emulsifier, and 52.5% oil phase. To evaluate the effect of PMF loading concentration on the resulting crystal in the emulsion, various concentrations (0.3-2.0%) of PMF compound were added to the carrier oil, composed of 100% medium chain triacylglycerol (MCT), at 130 °C with continuous stirring until they were completely dissolved (Table 3.1). After PMF was dissolved in MCT, 0.45 g of lecithin was then added to the 130 °C PMF oil solution as emulsifier, and then stirred until fully solubilized. The aqueous phase (100% double deionized water) was preheated to 70 °C to prevent rapid PMF crystallization induced by impulsive temperature drops. To avoid excessive water loss, the disperse phase solution (MCT with solubilized PMF compound and lecithin) was adjusted to 70°C immediately before mixing with the pre-heated aqueous phase. Once the aqueous phase had been added to the oil phase, the solution was maintained at 70 °C and continuously stirred until one phase of crude emulsion was formed. Since the crude emulsion formed was very viscous and could cause processing difficulty during high-pressure homogenization (EmulsiFlex-C6, AVESTIN Inc., Ottawa, Canada), the crude emulsion was then subjected to high-speed homogenization (ULTRA–TURRAX T-25 basic, IKA Works Inc., Wilmington, NC, USA) at 24,000 rpm. During high-pressure homogenization, all samples used for compound loading and storage studies were processed under 500 bar at 55 °C. However, samples used for comparing the effect of different processing parameters on the emulsion sample quality were produced at various combinations of temperatures (55, 65, and 75 °C).
and pressures (500, 1000, and 1500 bar). Finally, approximately 25-30 g of emulsion sample was collected from each processing batch.

**Table 3.1** Formulations for emulsion samples of various Tangeretin (PMF) loading.

<table>
<thead>
<tr>
<th>PMF Loading concentration (%)</th>
<th>oil phase (52.5%)</th>
<th>Emulsifier (1.5%)</th>
<th>Aqueous phase (46%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMF (g)</td>
<td>MCT (g)</td>
<td>Lecithin (g)</td>
</tr>
<tr>
<td>0.30</td>
<td>0.09</td>
<td>15.66</td>
<td>0.45</td>
</tr>
<tr>
<td>0.50</td>
<td>0.15</td>
<td>15.60</td>
<td>0.45</td>
</tr>
<tr>
<td>0.60</td>
<td>0.18</td>
<td>15.57</td>
<td>0.45</td>
</tr>
<tr>
<td>0.70</td>
<td>0.21</td>
<td>15.54</td>
<td>0.45</td>
</tr>
<tr>
<td>0.80</td>
<td>0.24</td>
<td>15.51</td>
<td>0.45</td>
</tr>
<tr>
<td>0.90</td>
<td>0.27</td>
<td>15.48</td>
<td>0.45</td>
</tr>
<tr>
<td>1.10</td>
<td>0.33</td>
<td>15.42</td>
<td>0.45</td>
</tr>
<tr>
<td>1.30</td>
<td>0.39</td>
<td>15.36</td>
<td>0.45</td>
</tr>
<tr>
<td>1.50</td>
<td>0.45</td>
<td>15.30</td>
<td>0.45</td>
</tr>
<tr>
<td>2.00</td>
<td>0.60</td>
<td>15.15</td>
<td>0.45</td>
</tr>
</tbody>
</table>

**Particle size measurement**

Since particle size is an important quality parameter for an emulsion system, the average particle size was determined. A BIC 90 Plus particle size analyzer equipped with a Brookhaven BI-9000AT digital correlator (Brookhaven Instrument Corporation, New York, NY, USA) was used for determining the average particle sizes from all emulsion samples produced. Emulsion samples were diluted in DI water by a dilution factor of 5,000 to avoid multiple scattering effects. The light source was a solid-state laser operating at the wavelength of 658 nm with 30 mW of power, and a high-sensitivity avalanche photodiode detector was used for signal detection. Measurements were conducted at a fixed scattering angle of 90° at 25 ± 1 °C. Results were reported in triplicate (n = 3) as mean particle size and size distribution.
**Moisture content analysis**

The moisture contents of emulsion samples were calculated as the percent sample weight loss when dried by an IR-200 Moisture (Denver Instrument Co., Arvada, CO, U.S.A.). To determine the moisture content, emulsion samples (≤ 0.2 g) were placed on a balanced aluminum weight pan (diameter = 100 mm) located on the scale section of the instrument. The samples were continuously heated by four parallel quartz infrared heaters at 200 °C until completely dried. The moisture content was then calculated using the following equation:

\[
\text{% Moisture} = \left( \frac{W_i - W_f}{W_i} \right) \times 100
\]  

(Eq. 3.1)

where \( W_i \) is the initial sample weight being placed on the scale and \( W_f \) is the final sample weight after drying.

**Crystal size and morphology evaluation**

In order to obtain higher loading concentrations of hydrophobic crystalline compounds, the resulting emulsion formulations are sometimes a system containing heterogeneous particles. In a mixed emulsion system, compounds are either solubilized within the emulsion droplet or present as entrapped small solid crystals. Variation of the processing parameters and storage conditions may result in different droplet and crystal characteristics. In this part of the study, crystal size and morphology were monitored using a direct microscopic observation. Undiluted emulsion samples were placed on a microscope slide and then mounted using a cover glass. An inverted optical microscope (Nikon TE 2000, Nikon Corporation, Japan) equipped with a CCD camera (Retiga EXi, QImaging) was used for crystal morphology observation. All images were processed by C-Imaging software (SimplePCI, Compix Inc). The average sizes of crystal (\( n = 100 \))
were measured using Java-based image processing software (Image J) with corrected reference scaling.

**X-ray diffraction**

The information on the crystal characteristics from emulsion samples containing various concentrations of PMF was studied using an X-ray diffraction method. Powder X-ray diffraction experiments were conducted using a D/M-2200T automated system (Ultima+, Rigaku) with Cu Kα radiation ($\lambda=1.5406$ Å). The PXRD patterns were collected at 2θ angles from 3° to 40° at a scan rate of 2 deg/min. A graphite monochromator was used and the generator power settings were 40 kV and 40 mA.

**Study of rheological characteristics**

The rheological properties of the emulsion samples were performed using an ARES rheometer (Rheometric Scientific, Piscataway, NJ) with parallel plate geometry (diameter 25 mm) at room temperature (20.0 ± 0.2 ºC). About 1 mL of the emulsion sample was deposited onto the plate geometry. Prior to each dynamic frequency sweep test, dynamic strain sweep test ranging from 0 to 100% was performed at 2 rad/s angular frequency and the strain value of 5% in the linear viscoelastic region was adopted. The angular frequency $\omega$ was ranged from 1 to 100 rad/s with 20 data points per decade. The gap between the two parallel plates was properly selected to prevent sample slip. A small amount of mineral oil was utilized to seal the sample edge to prevent solvent evaporation.

**Storage stability evaluation**

It is desirable to have a stable emulsion system for long-term storage. In this part of the study, the stability and quality changes under different storage conditions were
evaluated. The emulsion sample quality was examined by monitoring the changes of emulsion droplet size and crystal length over 30 days of storage at 4, 25, and 60 °C. All emulsion samples used in this study were processed in one single batch with a composition of 2.0% PMF, 50.5% MCT, 1.5% lecithin, and 46% DI water.

Statistical analysis

A single-tailed student’s t-test was performed using Sigmaplot 10.0 software.

Results and discussion

Effect of PMF loading concentration on the emulsion property

For oral formulation, it is equally desirable to have adequate stability as well as sufficient loading of the active ingredient so that a therapeutically meaningful dosage can be reached in a practical serving portion. Depending on the purpose and form of consumption, the requirements for a qualified oral formulation can vary significantly. For example, it is essential for an oral formulation to avoid creaming and precipitation when incorporated into a beverage product. On the other hand, when consumed as a soft gel or tablet, it is desirable for the oral system to have higher concentration and sophisticated storage stability of the active ingredient. Therefore, it is necessary for investigators to explore the highest possible loading concentrations of the target compound in the designated oral applications. In our previously published paper, we had successfully developed a viscoelastic emulsion system to contain PMF with sufficient loading capacity and storage stability. Here, in this part of experiment, we aim to systematically examine the effect of incorporating various tangeretin concentrations (0.3%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1.1%, 1.3%, 1.5%, and 2.0%) on the physical characteristics and
related application potentials of the emulsion system. As suggested by previous studies, the particle size of oral formulation was a critical factor in determining the digestion, absorption, and bioavailability of hydrophobic active ingredients (51, 52, 115-119). That is, smaller emulsion droplets often resulted in higher bioavailability due to faster digestion kinetics and potential direct uptake by gut wall and lymphatic systems. When the amount of emulsifier was held constant, the emulsion droplet size increased progressively (378.4 ± 5.3 nm to 565.2 ± 3.3 nm) as the percentage (0.3% to 2.0%) of tangeretin increased in the system, while no significant change in the moisture content was observed (Table 3.2). In general, the concentration of the emulsifier in the system was inversely related to the particle size. As the loading of PMF increased, the larger particle size may have been the result of the various degrees of emulsifier consumed in order to support the PMF solubility of the system.

Table 3.2 Physical characteristics information for the emulsion of different tangeretin (PMF) loading.

<table>
<thead>
<tr>
<th>Loading Concentration (%)</th>
<th>0.3</th>
<th>0.5</th>
<th>0.6</th>
<th>0.7</th>
<th>0.8</th>
<th>0.9</th>
<th>1.1</th>
<th>1.3</th>
<th>1.5</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle Size (nm)</td>
<td>378.4±5.3</td>
<td>388.7±4.4</td>
<td>385.8±4.2</td>
<td>401.4±6.1</td>
<td>463.0±8.6</td>
<td>499.3±5.9</td>
<td>507.6±7.7</td>
<td>513.5±7.0</td>
<td>550.8±5.4</td>
<td>565.2±3.3</td>
</tr>
<tr>
<td>Moisture Content (%)</td>
<td>46.08</td>
<td>46.14</td>
<td>46.81</td>
<td>46.93</td>
<td>45.81</td>
<td>46.17</td>
<td>45.65</td>
<td>46.18</td>
<td>48.12</td>
<td>46.07</td>
</tr>
<tr>
<td>Length (Crystal μm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>196.17±42.44</td>
<td>109.69±24.60</td>
<td>55.89±12.68</td>
<td>23.52±5.88</td>
<td>18.13±6.13</td>
<td>13.38±3.95</td>
</tr>
</tbody>
</table>

Moreover, the inclusion of crystalline hydrophobic compounds into emulsion formulations could sometimes result in a mixed system containing bioactives solubilized as dispersed droplets or present as entrapped crystalline particles. To maximize the loading of hydrophobic crystalline components in an emulsion system, the carrier oil was usually supersaturated using high processing temperatures. As a result, the reduction in temperature caused the compound to be expelled from the supersaturated oil phase and
crystalized in the continuous phase. When the compound concentration (<0.6%) in the system was below the saturation concentration, no crystal formation was observed in the emulsion systems (Figure 3.1 Aa and Ab). As the loading concentration increased to the critical concentration (0.7%) where it was only supersaturated, low amounts of tangeretin crystals appeared in bundles, with a maximum crystal size of 196.17±42.44 μm scattered among the emulsion droplets. With the continuous increase in supersaturation, the number of tangeretin crystals increased (Figure 3.1 Ac to Ai) as the size decreased (Figure 3.1 B). As has been established by others, the size of a crystal produced is inversely proportional to the number of nuclei formed (120-122). The number of nucleation sites considerably increased with higher degree of supersaturation, since the number of molecules required for the formation of nucleating clusters decreased (120). Therefore, smaller crystals were produced as the number of nuclei expanded more rapidly than the crystal growth. In order to be absorbed in the gastrointestinal (GI) tract, a hydrophobic crystalline compound has to be either solubilized or dispersed in the aqueous GI environment. Upon oral ingestion, large clusters of crystalline hydrophobic components are prone to gravitational precipitation, which causes them to be excluded from the body. On the other hand, the bioavailability of crystalline hydrophobic compounds may benefit from smaller size. Small crystals are more advantageous for GI absorption since they are more easily incorporated into mixed micelles or directly absorbed by the gut wall.
Figure 3.1 (A) Microscopic picture of emulsion samples with different tangeretin (PMF) loadings: (a) 0.5%; (b) 0.6%; (c) 0.7%; (d) 0.8%; (e) 0.9%; (f) 1.1%; (g) 1.3%; (h) 1.5%; (i) 2.0%. (B) Quantitative plots of crystal lengths as function of PMF loading concentrations.
Due to the crystalline structure, the variation in the PMF loading concentrations changed the physical characteristics of the viscoelastic emulsion systems, affecting the potential consumer applications. Specifically, the rheological properties of the viscoelastic emulsion are the most important factor in sustaining and stabilizing the PMF-incorporated system. Here, we explore the effects of different PMF loads on the rheological properties in relation to its crystalline characteristic. All the samples containing different degrees of PMF loading had higher storage modulus ($G'$) than lost modulus ($G''$) values, indicating a gel-like elastic behavior. Figure 3.2-A shows $G'$ as a function of frequency for viscoelastic emulsion samples with PMF loading of 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, 1.1, 1.3, 1.5, and 2.0%. To elucidate the effect of PMF loading on the $G'$ value, Figure 3.2-B presents the changes to the $G'$ value at 0.1 rad/s frequency as a function of PMF concentrations. The increase in PMF concentration from 0.3 to 2.0% in emulsion formulation is matched with a decreasing trend in $G'$ values. As the $G'$ value is usually used as an indicator of network interaction, the lowering $G'$ values suggested the weakening of the gel-like structure in the emulsion. As reported in our previous investigation, the interactions between emulsion droplets contribute to the viscoelastic property of emulsion systems (113). The weakening of the network interactions can be mainly attributed to the increasing number of crystals entrapped within the system. The increases of the emulsion crystalline structure were further confirmed by XRD analysis. In Figure 3.3, there was no characteristic peak observed in the emulsion sample with lower PMF concentration (0.5 and 0.7%). However, the characteristic peak for PMF was detected in emulsion samples with higher PMF concentrations.
Figure 3.2 (A) The storage modulus (G') vs. frequency curve of emulsion containing various tangeretin (PMF) concentrations from 0.3 - 2.0%. (B) The plot of G' value at 0.1 rad/s frequency as a function of PMF concentrations.
Since smaller particles may have higher oral bioavailability, it is desirable to reduce the particle size in oral formulations containing hydrophobic components. High-pressure homogenization (HPH) is a common technique for reducing the droplet size in an emulsion-based system. As has been previously reported, smaller droplet sizes were obtained when higher processing pressure and temperature were applied (123). To investigate the influence of HPH on the particle sizes of the crystalline emulsion system, different combination of temperatures and pressures were used for producing PMF
viscoelastic emulsions. Since PMF crystallization is sensitive to temperature variation, it is critical to maintain an elevated processing temperature during emulsion production.

During HPH, temperatures lower than 50°C promoted massive PMF crystallization and caused blockage of the HPH instrument. To prevent rapid crystallization, three temperatures (55, 65 and 75 °C) were selected for emulsion optimization trials. The three processing temperatures were applied in combination with three different pressure levels of 500, 1000, or 1500 bar, giving a total of 9 production combinations. However, samples processed at 75°C were excluded from the discussion, since it had excessive moisture loss and did not form a stable one-phase emulsion system (data not shown). Under all processing conditions, emulsion samples had an average droplet size around ~500nm with no significant inter-sample deviation. However, the size of crystalline structures in the emulsion system was greatly influenced by different processing parameters. Under the same processing pressures, samples processed at 65 °C produced larger crystal size than samples processed at 55 °C (Figure 3.4 A). The results indicated that the rate of PMF crystallization in the viscoelastic emulsion was positively associated with the temperature (ν ∝ T). The production of larger crystals at higher processing temperatures is likely due to the higher temperature providing the system with more free energy (ΔG), which is required for nucleation as well as for fostering frequent collisions of crystalline molecules (124, 125). Besides processing temperature, the pressure under which the sample is produced also plays a critical role in determining the PMF crystal size. Under similar processing temperatures, crystal sizes became larger when the applied pressure increased (Figure 3.4 B). The phenomenon of pressure application on the elevation of crystallization kinetics has been well studied by previous investigations. Under high
pressure, the melting temperature shifted towards higher temperature, which increased the degree of undercooling and resulted in higher nucleation rates (125-127). Moreover, the crystal growth was promoted since the physical distance of crystalline components was decreased under high-pressure conditions (127, 128). From our study, the crystal was smallest with a temperature/pressure combination of 55°C/500 bar and largest at 65°C/1500 bar with sizes of 7.58 ± 2.48 and 47.72 ± 13.94 μm, respectively.
Figure 3.4 (A) Microscopic pictures of emulsion samples processed at different combinations of temperature/pressure level: (a) 55°C/500bar; (b) 55°C/1000bar; (c) 55°C/1500bar; (d) 65°C/500bar; (e) 65°C/1000bar; (f) 65°C/1500bar. (B) Plot of crystal length at 55°C (empty circle) and 65°C (filled circle) as a function of processing pressure.
The influence on the gel-like structure of the PMF viscoelastic emulsion by the pressure and temperature is given in Figures 3.5 A, B, and C. For all pressure conditions, higher processing temperatures reduced the network interaction more than lower temperatures did. While the network interaction of samples processed under 500 and 1000 bar was not significantly affected by temperature variation, the gel structure was greatly weakened when the temperature was raised at 1500 bar. The results from this study suggested that larger crystals in the emulsion system disrupt the gel-like structure more by interfering with emulsion droplet interaction. In Figure 3.6, the viscoelastic characteristics of emulsion samples under different pressure treatments are compared. Interestingly, the viscoelastic property of the emulsion system was not significantly changed by the pressure variation when temperature was kept constant (55 or 65°C). The results from the rheological study indicated that all emulsion samples sustained a viscoelastic structure even though the network strength was variable.

**Figure 3.5** The storage modulus ($G'$) versus angular frequency curves for emulsion samples processed at different combinations of temperature/pressure level: (A) 55 and 65°C at 500 bar pressure. (B) 55 and 65°C at 1000 bar pressure. (C) 55 and 65°C at 1500 bar pressure.
Figure 3.6 The complex viscosity $\eta^*$ of emulsion samples processed at different combinations of temperature/pressure level: (A) 55°C at 500, 1000, and 1500 bar pressure; and (B) 65 °C at 500, 1000, and 1500 bar pressure.

**PMF emulsion stability under different storage temperatures**

In order for the general population to benefit from oral formulations, it is important for products to have an adequate shelf life. In this part of the experiment, the storage stability of crystalline PMF viscoelastic emulsion was studied under 3 storage temperatures (4, 25, and 60°C) over one-month period. Since the emulsion system was a mixed system, any change in particle size of both emulsion droplets and crystalline components were monitored as indicators of potential instability. To minimize the variability, all emulsion samples were produced in one single batch, distributed between three bottles, and stored in the selected temperature environments. During the one-month study, the emulsion droplets of all three samples remained stable one-phase systems and did not have noteworthy changes in particle size (Figure 3.7 A). Upon closer examination of the droplet particle size, the droplet particles of the 25 °C sample were slightly larger than those of the other 2 storage conditions (Figure 3.7 Ba and Bc). The results from this
observation indicated that the stability of viscoelastic emulsion might not only benefit from storage in lower temperatures where the molecular diffusion rate was limited, but also in higher temperatures where the gel-like surface interaction was stronger. In Figure 3.8 A, the observed microscopic crystal morphology under 3 storage conditions is presented in chronological order over a one-month period. To clarify the effect of storage temperature on crystal growth, the crystal length from all temperature environments as a function of days elapsed is plotted in Figure 3.8 B. According to our analysis, the crystal growth rate at 4 and 25 °C was minimal and showed no significant variability. However, at 60 °C storage temperature, the crystal growth rate was significantly accelerated. The growth rate of the crystalline structure is highly dependent on the molecular diffusion rate and activation energy in order for the crystalline component to fit into the crystal lattice (125). In this sense, higher storage temperature provided all factors required for rapid crystal growth and, in our case, caused higher instability for the crystalline emulsion system.
Figure 3.7 (A) The particle sizes of emulsion samples stored under 4, 25, and 60 °C. (B) Enlarged plots for particle size analysis at each storage temperature: (a) 4 °C (b) 25 °C (c) 60 °C. All emulsion samples used in storage study contain 2.0% tangeretin (PMF).
Figure 3.8 (A) Microscopic pictures of emulsion samples stored at 4, 25, and 60 °C over one month. (B) The crystal length of PMF in emulsion samples stored at 4 (upward empty triangles), 25 (filled circles), and 60 °C (filled downward triangles) as a function of storage days.
Conclusion

In summary, the tangeretin viscoelastic emulsion system provides a good illustration of an oral formulation designed for hydrophobic crystalline compounds. In this study, we systematically demonstrated the process of optimizing an oral formulation that includes crystalline components. Formulations with PMF loading concentrations less than and equal to 0.6% showed no existence of crystal structures in the system and may potentially be a good oral formulation for incorporation into beverage products. However, when higher loading and smaller particle size are the main considerations, loading concentrations higher than 1.5% should be selected. Processing parameters also play a critical role in determining the sample quality when smaller particle size is desired. Since lower temperatures and pressures may create samples with smaller crystalline structure, the processing temperature and pressure should be set at the lowest processible combination. During storage, higher temperatures promoted faster growth in the size of both emulsion droplets and crystals. Therefore, to better preserve the quality, a sample containing a crystalline component should preferably be stored in cooler conditions. The study results clearly illustrate how the controlling of loading concentration, processing parameters, and storage conditions is essential for producing crystals with desired sizes and specifications. The methods from this study can be used for future reference when designing and fabricating oral delivery formulations for crystalline hydrophobic ingredients.
CHAPTER IV. POTENTIAL INTERACTION WITH THE DELIVERY VEHICLE AFFECTING THE BIOLOGICAL FUNCTIONALITY OF TANGERETIN

PROJECT TITLE: EFFECT OF LABILE METHYL DONOR ON THE TRANSFORMATION OF 5-DEMETHYL TANGERETIN AND RELATED IMPLICATION ON BIOACTIVITY

The work in this chapter has been published in the title of “Effect of labile methyl donor on the transformation of 5-demethyl tangeretin and related implication on bioactivity” in the Journal of Agricultural and Food Chemistry (Volume 61, Issue 34, Pages from 8090 to 8097) on August 2013.

Abstract

Polymethoxyflavones (PMFs) belong to a subgroup of flavonoids that particularly exist in the peels of citrus fruits. Despite of their many health beneficial biofunctionalities, the lipophilic nature of PMFs limits their water solubility and oral bioavailability. To investigate the effect of delivery system on the improvement of PMFs bioavailability, a lecithin-based emulsion was formulated for the delivery of two PMF compounds, tangeretin and 5-demethyl tangeretin. While the emulsion system improved the digestion kinetics and the total solubilized PMFs concentrations in in vitro lipolysis studies, the concentration of 5-demethyl tangeretin decreased due to chemical transformation to its permethoxylated counterpart, tangeretin. The emulsifier lecithin used in this emulsion formulation contained a choline head group as a labile methyl group donor. The presence of methyl donor potentially caused the transformation of 5-demethyl tangeretin and
reduced its anti-cancer activities. Moreover, the transformation from 5-demethyl tangeretin to tangeretin in lecithin-based emulsion during lipolysis was the first report in literature, and the mechanism underlying this phenomenon has also been proposed for the first time.

**Introduction**

The benefits of consuming fruits and vegetables beyond the basic nutritional requirements were not recognized until recent decades. Due to advancements in research technologies, scientists can now distinguish tens of thousands of natural compounds that possess health-promoting biofunctionalities. These compounds may potentially serve as advantageous alternatives to synthetic drugs in disease prevention and even treatment at an appropriate dosage. The bioactive phytochemicals vary dramatically owing to differences in species, growth locations and conditions, sections of plants, and other factors. Polymethoxyflavones (PMFs) are an emerging category of phytochemicals that are mainly extracted from the peels of citrus fruits. By definition, PMFs are compounds that have two or more methoxy groups attached to the 15-carbon benzo-γ-pyrone skeleton structure with a carbonyl group on the C₄ position. PMFs, such as nobiletin, tangeretin, sinensetin, and 3,5,6,7,8,3',4'-heptamethoxyflavone, have been well documented to possess anti-inflammatory (71), anti-atherogenic (71), anti-carcinogenic (78, 129), and selective anti-proliferative activity to cancer over normal cells (72, 73, 76). In addition to permethoxylated PMFs, another group of PMFs that include single or multiple hydroxy groups on various positions of the C6-C3-C6 flavonoid skeleton, have also been isolated from aged extract of citrus peel and have been shown to exhibit stronger efficacies than their permethoxylated counterparts (130-135).
Due to the multiple substitutions of methoxy groups on the skeleton backbone, PMFs and hydroxylated PMFs (OH-PMFs), similar to many other lipophilic bioactives, have poor aqueous solubility and, consequently, low bioavailability when ingested orally. In order to augment the oral dose efficacy, many people have relied on a strategy that assumes that improving the aqueous solubility of a compound will allow the enhancement of its bioavailability and bioefficacy. Among many approaches aiming to increase solubility, emulsion encapsulation is one of the convenient and versatile methods that have often been employed by many investigators. Recently, emulsions of different sizes (from >100 nm to <1 μm) and forms, such as solid lipid nanoparticles, self-emulsifying delivery systems, and surface modified particles have been acknowledged to significantly improve bioavailability (102, 119, 136-146) and bioefficacy (24, 52) in both in vitro and in vivo models.

Being granted the status of generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (USFDA), biocompatible lecithin, or phosphotidylcholine (PC), is a common emulsifier used when developing emulsion systems for oral delivery. The structure of lecithin includes a non-polar tail of two long-chain fatty acids and a polar head with a zwitterion phosphate-choline group. Having choline as part of the structure, PC is recognized as an effective dietary supplement for satisfying the daily choline requirement (~550 mg/day) for humans. Choline is a precursor molecule for important neurotransmitter compounds and serves as an active labile methyl (-CH₃) donor during methyl metabolism. It has been well investigated that the increased consumption of PC will boost the concentration in biological systems of choline and labile methyl groups as a consequence. In the present study, an emulsion system using PC as the emulsifier has
been developed. Two key compounds of the PMFs, tangeretin (5,6,7,8,4′-pentamethoxyflavone) and 5-demethyl tangeretin (5-hydroxy-6,7,8,4′-tetramethoxyflavone) were incorporated into emulsion-based delivery systems of identical compositions. Emulsion systems containing either tangeretin or 5-demethyl tangeretin were subjected to \textit{in vitro} bioaccessibility and bioactivity evaluation. In the assessment of bioaccessibility using an \textit{in vitro} lipolysis model, transformation of 5-demethyl tangeretin to its permethoxylated counterpart was observed and confirmed by high performance liquid chromatography (HPLC). To the best of our knowledge, this phenomenon has not been reported in previous literature and the mechanism underlying the transformation has not yet been elucidated.

\textbf{Materials and methods}

\textit{Materials}

Tangeretin of 98% purity was purchased from Quality Phytochemicals, LLC (Edison, NJ, USA). 5-Demethyltangeretin (5-OH tangeretin) with purity >98% was synthesized in our laboratory using a previously published method (71). PC75 rapeseed lecithin containing 75% phosphatidylcholine was a gift from American Lecithin Company (Oxford, CT, USA). Neobee 1053 medium chain triacylglycerol (MCT) was a gift from Stepan Company (Northfield, IL, USA). Pancreatin with 8X USP specification and Tris maleate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium taurodeoxycholate (Na TDC) was purchased from CalBiochem (La Jolla, CA, USA). HPLC-grade acetonitrile (ACN) and HPLC-grade water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sterile filtered, cell culture compatible dimethyl sulfoxide (Sigma–Aldrich, MO, USA) was used for HPLC sample solvent. Minimum essential
medium (MEM) was purchased from HyClone Laboratories, Inc. (Logan, UT, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals were of reagent grade and used without further purification. Milli-Q water was used throughout the experiment.

**Preparation of PMFs emulsions and MCT suspensions**

Tangeretin and 5-OH tangeretin emulsions were prepared using the method previously described in our recent paper (113). Briefly, the oil phase containing MCT, lecithin, and either tangeretin or 5-OH tangeretin was prepared separately from the aqueous phase. The emulsion oil phase was maintained at 130 °C until all materials were completely dissolved. The temperature of the resulting oil phases was then reduced to 70 °C before the aqueous phase (double deionized water, 70 °C) was added in under magnetic stirring to form a crude emulsion. The emulsion was then subject to high-speed homogenization (ULTRA–TURRAX T-25 basic, IKA Works Inc., Wilmington, NC, USA) to reduce sample viscosity before processing using a high-pressure homogenizer (EmulsiFlex-C3, AVESTIN Inc., Ottawa, Canada). The components of the PMF emulsion systems are MCT, lecithin, PMFs (tangeretin or 5-demethyltangeretin), and double deionized water in percentages of 50.4%, 1.5%, 2.1%, and 46%, respectively.

MCT suspension was prepared by adding 0.05 g of either tangeretin or 5-OH tangeretin into 1 g of MCT. The PMF-MCT suspension was subjected to ultrasonication for 2 minutes to break up large clumps of precipitated compound. The suspension samples were vortexed for 5 minutes before the lipolysis study was performed.
To better mimic the digestion activity in the human small intestine, an \textit{in vitro} lipolysis study was conducted using the method described in our previously published paper (145) with minor modifications. In short, fed state lipolysis buffer was prepared with Tris maleate, NaCl, CaCl$_2$·H$_2$O, NaTDC, and phosphatidylcholine in concentrations of 50, 150, 5, 20, and 5 mM, respectively. Pancreatin was freshly prepared for each lipolysis study by mixing 1 g of pancreatin powder with 5 mL lipolysis buffer, centrifuged at 2000 rpm and stored on ice. To begin the lipolysis study, equivalent amounts of lipid samples (0.25 g of MCT suspension; 0.5 g of emulsion sample) and 1 mL of prepared pancreatin solution were added to 9 mL of lipolysis buffer. During the 2-hour lipolysis study, the temperature was maintained at 37 ± 1 °C and pH was maintained at 7.50 ± 0.02 by 0.25 N NaOH titration. The volume of NaOH added at each time point was recorded, and the total amount consumed was calculated for data analysis. Upon completion of the 2-hour lipolysis study, the whole lipolysis solutions were subject to ultracentrifugation (Type 60 Ti rotor, Beckman Coulter) for 1 hour at 50,000 rpm. After ultracentrifugation, the middle layer of supernatant was collected and stored at -80°C for later HPLC analysis. For HPLC analysis, 200 µL of lipolysis supernatant sample was filtered through a 0.22 µm filter and mixed well with 400 µL of DMSO.

\textit{Percent bioaccessibility calculation}

The concentration of PMFs (tangeretin or 5-demethyltangeretin) solubilized in the supernatant after the lipolysis study was determined using HPLC. The aqueous lipolysis supernatant simulated the small intestinal lumen where PMFs solubilized in this portion were most likely bioaccessible to intestinal cells. The percent bioaccessibility of PMFs
was calculated according to previously published literature (145) using the equation below:

\[
\% \text{ Bioaccessibility} = \frac{\text{Total mass of solubilized PMFs}}{\text{Total mass of PMF in original lipid samples}} \times 100\% \quad (\text{Eq. 4.1})
\]

The mass of solubilized PMFs was calculated using the concentration of PMFs per volume of supernatant (g/ml) multiplied by the total volume of lipolysis aqueous phase. The mass of PMFs in the original lipid sample was calculated from the concentration of PMFs in MCT suspension or the emulsion and mass of the lipid added.

**Determining the extent of lipolysis**

The extent of lipolysis, defined as the percentage of triglycerides digested by lipase, can be calculated using the amount of NaOH consumed during a set period of time. To better compare the digestion kinetics of MCT (unformulated) and emulsion, the extent of lipolysis was evaluated at the 30-minute time point since both the MCT suspension and the emulsion sample were 100% digested at the end of the 2-hour lipolysis experiment. The calculation for the extent of lipolysis assumed that two molecules of fatty acid were released from digestion of one triglyceride unit while consuming two molecules of NaOH. Since the lecithin molecule from the emulsion formulation also contributes to the total number of triglycerides present, the calculation of NaOH consumption for emulsion samples includes both compositional MCT (0.27 g) and lecithin (0.0075 g). Extent of lipolysis was calculated using the following equation with reference to the previously published paper (145):

\[
\text{Extent of lipolysis} = \frac{\text{Volume of NaOH} \times \text{Conc.of NaOH}}{2 \times \text{mol of triglyceride}} \times 100\% \quad (\text{Eq. 4.2})
\]
Volume of NaOH is obtained from the calculation of total NaOH volume added during lipolysis and is corrected by subtracting the NaOH volume added to blank lipolysis (no lipid added). The concentration of NaOH is 0.25 N in this experiment. A mole of triglyceride was estimated from the average molecular weight of triglyceride with Equation (3) using the saponification value of MCT and/or lecithin.

\[
Mw \text{ of triglyceride} = \frac{3 \times 1000 \times Mw \text{ KOH}}{SV}
\]  

(Eq. 4.3)

The molecular weight of KOH can be found in the literature as 56.1 g/mol. The saponification value used for MCT and lecithin are 334 and 190, respectively.

**HPLC analysis**

The UltiMate 3000 HPLC system (Dionex, CA, USA) consisted of a quaternary solvent delivery system and an auto sampler; a variable wavelength detector was connected to Supelco's RP-Amide column, 15 cm x 64.6 mm id, 3 μm, (Bellefonte, PA, USA). The detection of PMFs was performed using gradient elution composed of the mobile phase of water (solvent A) and ACN (solvent B). The optimized condition was modified from previous literature (147). A total of 22 minutes of elution gradient started from 40% of B, then linearly increased to 55% of B in 10 minutes, then linearly increased to 70% in 15 minutes, then linearly increased to 80% in 20 minutes, and then finally linearly reduced back to 40% at 21 minute and lasted for 1 minute until the end of analysis. The flow rate during HPLC analysis was kept constant at 1.0 ml/minute. Injection volume was 30 μL, and detection wavelength was 320 nm.

**Cell culture**

Human hepatic cancer cell line, HepG2 (American Type Culture Collection, HB-8065, Manassas, VA, USA) was grown and maintained in MEM supplemented with 10%
fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The incubation condition for cell culture was 95% relative humidity and 5% CO₂ at 37°C.

**In vitro anti-cancer activity evaluation**

Methyl thiazol tetrazolium bromide (MTT) assay was used to evaluate the *in vitro* anti-cancer activity of PMFs emulsions in comparison with DMSO-dissolved pure compounds. At the beginning of the test, HepG2 cells were seeded in a flat-bottom 96-well plate with cell density of 1 x 10⁴ cells/well, and then incubated for 24 hours. Subsequently, the seeded cells were treated with serum complete media containing various concentrations of PMFs (tangeretin or 5-demethyltangeretin) in either emulsion or DMSO. Negative control (untreated) and blank emulsion vehicles were also cultured along with PMF-treated cells, which were used as reference value for evaluation of *in vitro* anti-cancer activity. After 24 h of incubation, cell culture medium was replaced by 100 μL of MTT solution (0.5 mg/mL in RPMI 1640 medium). After incubation for 2 h at 37 °C, MTT solution was carefully aspired and 100 μL of DMSO was added to each well to dissolve the purple formazan crystals. Plates to which DMSO had been added were stored in dark conditions for 10 minutes and then subjected to light absorbance evaluation at 560 nm using an Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The viabilities of cancer cells were calculated relative to the untreated control value.

**Statistical analysis**

All experiments and analyses were performed in triplicate. Results were expressed as means ± SD. Using Sigmaplot 10.0 software, the student t-test was
performed to examine the mean difference between two groups where $p < 0.01$ (denoted as *) and $p < 0.001$ (denoted as **) were considered statistically significant.

**Results and discussion**

*Effect of emulsion on the digestion kinetic*

The therapeutic dosages of many bioactives that are beneficial to health are difficult to achieve as a result of low aqueous solubility. It has been stated in many previous studies that the oral bioavailabilities of lipophilic compounds could be greatly increased when consumed with lipids (26, 28, 30, 55, 148). Therefore, wide varieties of lipid-based delivery systems have gained popularity among researchers to boost the bioavailable concentrations and to improve the bioefficacies of such compounds. In order to efficiently determine the most suitable lipid-based option for each specific bioactive candidate, many choose to conduct *in vitro* screening of potential vehicle formulations before proceeding to *in vivo* animal studies, which are more costly and time-consuming. Hence, there is a need for simplified *in vitro* screening results in the emergence of an *in vitro* lipolysis model. The dynamic *in vitro* lipolysis model offers a good mockup of the *in vivo* lipid digestion process and achieved good *in vitro-in vivo* correlation (IVIVC) when predicting compound bioavailability (148-151).

In the present study, PMFs (tangeretin or 5-demethyltangeretin) were integrated into a lecithin-emulsified system. The change of digestion kinetics in an emulsion system was compared with crude MCT oil, which is also the base oil for the emulsion lipid dispersion core. As the result of lipid digestion, the pH of the lipolysis sample was lowered and required constant titration using NaOH to maintain the solution’s environment at pH 7.5, at which the enzyme exhibited the highest activity. Based on the
assumption that digestion of one triglyceride unit will consume two molecules of NaOH, the digestion process can be monitored by recording the time and volume of NaOH deposited to the lipolysis solution. Specifically, the faster the lipid digestion proceeded, the more frequently NaOH was required to be added. The majority of the lipid digestion in the emulsion sample occurred within the first 5 minutes of the lipolysis study, while a similar degree of lipid digestion in unformulated MCT lipid sample was reached only after 60 minutes (Figure 4.1 A). The reason for the faster digestion rate observed in the emulsion sample was due to the larger surface area at the lipid-water interface for contact of lipases that are only soluble in aqueous environment. Since this lipolysis study intends to account for the amount of time required for complete (100%) lipid digestion, the extent of lipolysis for emulsion and unformulated oil samples was compared at the 30-minute time point. The extent of lipolysis, defined as the percentage of lipid digested, exhibited a great difference between emulsion (>100%) and crude MCT sample (64.5%) (Figure 4.1 B). Since digested lipids are constantly adapted into mixed micelles during lipolysis, any nearby lipophilic compounds may as well be incorporated into the micelles available for intestinal absorption. Since intestinal activity is a dynamic process of digestion, absorption, and excretion, lipophilic compounds will have longer intestinal retention times and, thus, a higher chance to be absorbed if they are rapidly incorporated into the mixed micelle and become soluble in the intestinal lumen.
Figure 4.1 In vitro lipolysis. (A) digestion kinetic curve of blank emulsion (▽) and blank MCT oil (●). (B) Extent of lipolysis at 30 minute for blank emulsion and blank MCT oil.

**Effect of emulsion on total PMF bioaccessibility**

Oral bioavailability is the integration of a compound being bioaccessible to intestinal absorption, the amount that is actually transported across the intestinal lining, and the concentration that remains unchanged by the systemic metabolism. Accordingly, increasing bioaccessibility will contribute to the overall enhancement of bioavailability. To be bioaccessible for intestinal uptake, bioactive compounds must solubilize in the intestinal lumen, which is the medium for active and passive transport. Apart from compounds that naturally have good solubility in the aqueous intestinal lumen, lipophilic compounds become aqueous-soluble and bioaccessible after inclusion into mixed...
micelles. During the *in vitro* lipolysis study presented here, the lipid is simultaneously digested to form mixed micelle, which imitates the component of intestinal lumen where lipophilic compounds may be corporately absorbed. The percent of PMFs from the original emulsion or MCT suspension that becomes bioaccessible was determined after *in vitro* lipolysis digestion. As total PMF is defined as the sum of all PMF compounds present in the lipolysis solution, the percent of total PMF bioaccessibility improved for both tangeretin and 5-demethyltangeretin emulsions when compared with MCT suspensions (Figure 4.2 A and B). In comparison with the MCT suspension, tangeretin emulsion increased the percent of total PMF bioaccessibility from 23.0% to 30.7% and the concentration of total solubilized PMF from $1.75 \pm 0.39$ mg to $2.27 \pm 0.20$ mg (Table 4.1). With the more lipophilic structure that 5-demethyltangeretin possesses, the aqueous solubility of this compound is naturally very low and can be improved only slightly by an emulsion delivery vehicle. The percent of bioaccessibility of the total PMFs in 5-demethyltangeretin samples increased from 0.6% in MCT suspension to approximately 0.9% in emulsion, while the total PMF concentration rose from $4.74E^{-2} \pm 3.54E^{-3}$ mg to $10.3E^{-2} \pm 1.24E^{-2}$ mg (Table 4.1). The result from the percent bioaccessibility evaluation was consistent with previous studies reporting that faster lipid digestion kinetics will result in higher bioaccessibility of lipophilic compounds (52, 102).
Figure 4.2 (A) Percent bioaccessibilities of total PMFs MCT oil suspension and emulsion system containing tangeretin or 5-demethyltangeretin. (B) Enlarged picture of total PMFs in 5-demethyltangeretin MCT suspension and emulsion. Total PMFs is defined as the sum of tangeretin and 5-demethyltangeretin present in the digested lipolysis solutions.
Table 4.1 Solubilized tangeretin and 5-demethyl tangeretin concentrations in digested lipolysis solutions.

<table>
<thead>
<tr>
<th>Samples digested</th>
<th>Tangeretin (mg/mL)</th>
<th>5-demethyl tangeretin (mg/mL)</th>
<th>Total PMF (mg/mL)</th>
<th>Avg. NaOH consumption (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tangeretin MCT suspension</td>
<td>1.75±0.39</td>
<td>-</td>
<td>1.75±0.39</td>
<td>4.67±0.10</td>
</tr>
<tr>
<td>Tangeretin emulsion</td>
<td>2.27±0.20</td>
<td>-</td>
<td>2.27±0.20</td>
<td>5.57±0.10</td>
</tr>
<tr>
<td>5-demethyltangeretin MCT suspension</td>
<td>(3.60±0.33)×10^{-3}</td>
<td>(4.38±0.34)×10^{-2}</td>
<td>(4.74±0.35)×10^{-2}</td>
<td>4.62±0.41</td>
</tr>
<tr>
<td>5-demethyltangeretin emulsion</td>
<td>(5.80±0.39)×10^{-2}</td>
<td>(4.52±0.86)×10^{-2}</td>
<td>(10.30±1.24)×10^{-2}</td>
<td>5.52±0.21</td>
</tr>
</tbody>
</table>

Effect of lecithin-based emulsion on 5-demethyltangeretin transformations

HPLC standard curves for PMFs compounds were prepared using intact tangeretin or 5-demethyltangeretin compounds with average elution times of 10.7 ± 0.1 and 15.3 ± 0.1 minutes, respectively. Both PMF compounds had >98% purity (Figure 4.3 A and B) when intact compounds were sampled by the HPLC system. Furthermore, the undigested emulsion-processed samples were also examined by the HPLC system, and no changes in compound purity or composition were found. However, changes in PMF composition were observed in samples that contained 5-demethyltangeretin after lipolysis digestion. Meanwhile, the PMF present in lipolysis solutions after the digestion of tangeretin samples were exclusively tangeretin (Figure 4.3 E and F). The transformation of 5-demethyltangeretin into its permethoxylated counterpart was unprecedented and has not been reported in any previous literature. To further confirm the presence of tangeretin in lipolysis-digested 5-demethyltangeretin samples, a total of 6 replicates were conducted with both 5-demethyltangeretin emulsions and MCT suspensions on two separate sample
batches to avoid any inter- or intra-sample variation. Moreover, the digested 5-demethyltangeretin solutions were also subjected to LC-MS analysis and the chemical structures of both tangeretin and 5-demethyltangeretin were confirmed by comparing with standard compounds.
Figure 4.3 HPLC chromatography of (A) intact tangeretin standard, (B) intact 5-demethyltangeretin standard, (C) lipolysis-digested tangeretin MCT suspension sample, (D) lipolysis-digested tangeretin emulsion sample, (E) lipolysis-digested 5-demethyltangeretin MCT suspension sample, and (F) lipolysis-digested 5-demethyltangeretin emulsion sample. Non-filled arrows indicate tangeretin elution points and filled arrows indicate 5-demethyltangeretin elution points.
As discussed above, the total PMF bioaccessibilities were convincingly augmented by incorporation into an emulsion-based delivery system. However, when breaking down the compositional compounds of PMF present in 5-demethyltangeretin lipolysis samples, the amount of 5-demethyltangeretin essentially reduced from $4.38 \times 10^{-2} \pm 3.63 \times 10^{-3}$ mg in MCT suspension samples to $4.52 \times 10^{-2} \pm 8.59 \times 10^{-3}$ mg in emulsion samples (Table 4.1). Namely, the percentage of 5-demethyltangeretin in total suspended PMF reduced from 93.0% to 40.6% when digested in the form of emulsions (Fig. 4.4). Since the presence of tangeretin in undigested 5-demethyltangeretin MCT suspensions and emulsion samples was not observed, the appearance of tangeretin in digested samples could be conclusively attributed to transformation during the digestion processes. Since the mechanism underlying the transformation is not yet known, one proposed mechanism of such transformation was suggested as in Figure 4.5. As the process of lipolysis is executed in a basic environment (pH 7.5), the constant shock of a free hydroxyl group ($\text{OH}^-$) from the NaOH titration caused the de-hydrogenation of the phenolic group on the 5-demethyltangeretin. The negative charge on the resulting oxygen ($\text{O}^-$) at the 5-position then quickly attacked the labile methyl groups (-$\text{CH}_3^+$) provided by the choline head of lecithin.

Lecithin, phosphatidylcholine, is naturally present in various places within the biological system, such as the digestive tract, cell membrane, organs, etc. Due to the inclusion of a choline head group as part of its structure, lecithin is commonly regarded as a good dietary source of choline. Choline, as a structural labile methyl donor, is critical for cellular signaling, normal neural functions, hormone secretion, protein synthesis, and DNA methylations (82-88). Therefore, lecithin, as simple derivative of choline, is a
potential reservoir of labile groups. When lecithin was digested as part of an emulsion system, the release of a labile methyl group causes interaction with any compounds that could hypothetically be a methyl group receiver. In the lipolysis experiment, the concentration of the labile methyl group was much higher in samples containing lecithin-based emulsion than MCT oil suspension. While tangeretin was not affected by the presence of labile methyl groups, the phenolic group of 5-demethyltangeretin was much more reactive to methylation under basic environment, as proposed in the mechanism. The difference of tangeretin, in terms of percent of total PMFs, present in the digested 5-demethyltangeretin solutions was significantly ($p < 0.001$) different between MCT suspensions and emulsions. Since the chemical reaction is dependent upon the reactant concentrations, higher concentrations of labile methyl groups in the lipolysis solution may cause methyl substitution and result in the faster reaction rate.
Figure 4.4 The percent of tangeretin and 5-demethyltangeretin relative to total PMFs presented in lipolysis-digested 5-demethyltangeretin MCT suspensions and emulsions. (* $p < 0.01$, ** $p < 0.001$).

According to the findings of the lipolysis experiment, one can conclude that the emulsion-based delivery system is an effective means to enhance digestion kinetics, overall PMF bioaccessibility and, thus, bioavailability. Still, the selection of compositional material may require special consideration to account for possible interactions with the target compound. In our lipolysis experiment, the lecithin-based emulsion system was effective for augmenting the solubilized tangeretin concentration when compared with that of the MCT suspension. However, the sample emulsion
formulation did not work well when 5-demethyltangeretin was incorporated. Due to potential compound interactions with labile methyl groups, transformation of 5-demethyltangeretin was observed and resulted in the reduction of solubilized 5-demethyltangeretin in the final lipolysis solution.

Figure 4.5 Proposed mechanism by which 5-demethyltangeretin transformed to tangeretin during the in vitro lipolysis study.

Effect of 5-demethyltransformation on the in vitro anti-cancer activity

Although the chemical structures of tangeretin and 5-demethyltangeretin differ only in the substituted functional group at the 5-position, their physical properties and bioactivities deviate significantly. Whereas tangeretin was proven to inhibit cancer cell proliferation from G_{0}/G_{1} cell cycle arrest (81), 5-demethyltangeretin was found to exhibit
higher potency in anti-cancer activity through G2/M arrest while inducing cell apoptosis (133). To investigate the effect of emulsion formulation on the in vitro anti-cancer activities of the two PMF compounds, HepG2 cells were treated with serum complete medium containing PMFs (tangeretin or 5-demethyltangeretin) dissolved in DMSO or dispersed as emulsion. The potency of the anti-cancer activities was evaluated based on the relative cell viability of each treated cell to the untreated control. The result from the MTT study was consistent with previously reported literature that DMSO-dissolved tangeretin exhibits lower anti-cancer potency than 5-demethyltangeretin at all concentrations. However, the emulsion delivery system utilized in this study showed significant improvement ($p < 0.001$) of the tangeretin activities, with the largest difference being 26.7% at 25μM (Figure 4.6 A). With the impressive improvement seen in the case of tangeretin, the emulsion delivery system did not produce similar results when combined with 5-demethyltangeretin. The 5-demethyltangeretin emulsion-treated cell groups exhibited higher cell viability than groups that were treated with DMSO-dissolved 5-demethyltangeretin in a statistically significant manner (Figure 4.6 B). The lower bioactivity of 5-demethyltangeretin emulsion-treated groups may be explained by the findings from the lipolysis study. As discussed in previous sections, the excessive transformation of 5-demethyltangeretin to tangeretin in the presence of a labile methyl donor (lecithin in this case) may be the major reason that the average bioefficacy of the emulsion group was lowered, since tangeretin has a lower anti-cancer potency than 5-demethyltangeretin. Even though the mechanism underlying the transformation and effects on bioactivities has not yet been confirmed, the finding of such transformation
allows us to better comprehend the process of cancer inhibition in relation to surrounding biological compounds.
Figure 4.6 The plot of relative viabilities vs. PMFs concentrations. The equal concentration of PMFs, (A) tangeretin or (B) 5-demethyltangeretin, dissolved in DMSO or included as emulsion were added to HepG2 cell. Data was presented as mean ± SD, n = 6 in each of 3 separate replications. (* p < 0.01, ** p < 0.001).
Conclusion

In summary, the lipid-based delivery system has often been regarded as an efficient solution to enhance the bioavailability of lipophilic compounds. In fact, many investigators have formulated their delivery vehicles based on the hypothesis that improved solubility will simultaneously increase the absorption of such compounds. This hypothesis was proven valid in many pharmacokinetic studies showing that emulsion was indeed very effective to increase concentrations of encapsulated compounds in the systemic circulation. However, according to our results, the development of emulsion delivery vehicles may fail if interactions between formulation materials and target compounds are undesirable. In our study, we examined the effectiveness of lecithin-based emulsions to enhance the digestion kinetics, bioaccessibilities and bioactivities of two PMF compounds, tangeretin and 5-demethyltangeretin. Emulsions promoted faster lipid digestion kinetics, which result in higher total solubilized PMF concentrations. However, due to the presence of labile methyl donors (choline from lecithin), 5-demethyltangeretin was transformed to its permethoxylated counterpart, tangeretin, and, thus, lowered the solubilized 5-demethyltangeretin concentrations. The anti-cancer activities examined using MTT assay again address the decrease in compound bioactivities when affected by labile methyl donors. As a result, the potential interactions between compositional materials and target compounds may be worthy of careful consideration. The findings from our investigation may serve as a reference for the future development of delivery vehicles for many other compounds that may possess similar chemical properties. Since lecithin is a naturally occuring compound in the digestive tract, the in vivo transformation of 5-demethyltangeretin or other potential methyl receiver may also occur and require
further investigation. The consequent result from such transformations in biological systems may serve as a link to elucidate the mechanism that underlies the important anti-cancer efficacy of such compounds.
CHAPTER V. EFFECT OF VISCOELASTIC EMULSION SYSTEM ON THE IN VITRO AND IN VIVO ORAL BIOAVAILABILITY OF TANGERETIN

PROJECT TITLE: VISCOELASTIC EMULSION IMPROVED THE BIOACCESSIBILITY AND ORAL BIOAVAILABILITY OF TANGERETIN: IN VITRO AND IN VIVO EVALUATION

As of submission of this dissertation, the work in this chapter has been submitted in the title of “Viscoelastic emulsion improved the bioaccessibility and oral bioavailability of tangeretin: in vitro and in vivo evaluation” to Journal of Controlled Release for consideration of publication.

Abstract

Tangeretin (5,6,7,8,4′-pentamethoxyflavone), a polymethoxylated flavonone found predominantly in citrus fruit peels, has numerous bioactivities including anti-inflammation, anti-cancer and anti-obesity etc. Due to its hydrophobicity, tangeretin exhibits poor oral bioavailability. In the present study, an optimized viscoelastic emulsion system was formulated for tangeretin oral delivery, and different in vitro and in vivo models have been developed to investigate the effect of emulsification on the digestion and absorption of tangeretin. In the present study, the ability of emulsion-based delivery system to improve the oral bioavailability of tangeretin was examined with both in vitro and in vivo models. In vitro lipolysis (static model) revealed that emulsified tangeretin was digested considerably faster than tangeretin MCT suspension. TNO’s gastrointestinal model (TIM-1) (kinetic model) indicated that Jejunum was the main absorption site, as
evidenced by the consistently higher bioaccessibility than that of Ileum at all the time periods. In vitro lipolysis may overestimate the bioaccessible fraction as evidenced by the about 3.2-fold increase in bioaccessibility for emulsified tangeretin compared with about 2.6-fold increase for TIM-1 model. In vivo pharmacokinetics analysis on mice again confirmed that the oral bioavailability of tangeretin in the emulsion-based system was increased 2.3 fold with 23% increase in C\text{max} when compared with the unformulated oil suspension. Emulsified tangeretin is significantly more bioaccessible/bioavailable than its simple oil suspension, which may result in higher bioactivities and nutritional values to the consumers. TIM-1 is a better model to simulate the absorption and digestion of upper gastrointestinal tract, and correlate well with \textit{in vivo} bioavailability results.

\textbf{Introduction}

Tangeretin is one of the major polymethoxyflavones found in the peel of numerous citrus fruits (111, 152). With its methoxy functional groups on the flavonoid backbone, tangeretin, similar to many other members of the polymethoxyflavone class, is a potential bioactive capable of reducing the risk of dietary-related diseases. According to much previous research, tangeretin is documented to have a wide array of biological functionalities including anti-inflammation (75), anti-tumorigenesis (80, 81, 153-155), neuroprotective effects (156), metabolic modulations (157-159), and protection against cardiovascular diseases (160-162). In particular, due to its selective growth inhibition on the carcinoma cells (80, 163), many investigations have been performed addressing the ability of tangeretin to serve as an alternative to anti-cancer agents that universally cause toxic adverse effects to all cells.
The nature of tangeretin as a highly crystalline hydrophobic compound has led to its poor bioavailability when consumed orally. In one previous report, the plasma concentration of tangeretin was less than 0.49 μg/mL in rats fed at a dose level of 50 mg/kg (164). Due to its high melting point, tangeretin typically presents as crystals and is poorly soluble in most common dietary solvents, such as water and oil, at room temperature. Thus, the oral uses of tangeretin to date are limited by the availability of suitable formulations to increase its bioavailability.

As the oral bioavailability is closely dependent on the aqueous solubility, gut wall permeation, and metabolic stability, strategies that improve one or more of these factors could be applied to enhance the oral efficacy of compounds with problematic system concentration. Hydrophobic compounds such as tangeretin were found to be better absorbed when ingested with lipid (28, 30, 31), thus lipid-based formulations are popular among investigators when designing delivery systems targeting oral uses of such ingredients. In our previous study, a tangeretin-containing viscoelastic emulsion (VE) system was optimized to achieve higher formulation loading, good stability, and suitable particle size (113). The aqueous solubility and in vitro anti-cancer proliferation of tangeretin were significantly improved through proper emulsification using the VE formulation (113, 115).

To further elucidate the role of emulsion-based delivery systems and associated factors that contribute to oral bioavailability, this work aims to systematically study the pre-absorption events using the in vitro lipolysis assay and the dynamic gastrointestinal simulating model (TIM-1). The in vivo pharmacokinetics of tangeretin were then assessed using an animal model. This work provides the sole examination on the fate of tangeretin
when passed through the gastrointestinal (GI) tract and evaluates the possibility of using in vitro models to predict the in vivo oral bioavailability of hydrophobic compounds. Thus, the methodology used in this study can be used as reference for future selection of in vitro assays to evaluate the effectiveness of lipid-based oral formulations targeting improved compound oral bioavailability.

**Materials and methods**

**Materials**

Tangeretin of 98% purity was purchased from Quality Phytochemicals, LLC (NJ, USA). Rapeseed PC75 lecithin was gifted by American Lecithin Company (CT, USA). A Neobee Medium chain triglyceride sample was requested from Stepan Company (Northfield, IL, USA). Pancreatin of 8X USP specification and Tris maleate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium taurodeoxycholate (Na TDC) was purchased from CalBiochem (La Jolla, CA, USA). Pancrex V powder (lipase activity = 25,000 units/g, protease activity = 1,400 units/g, and amylase activity = 30,000 units/g) was purchased from Paines & Byrne, UK. Fresh pig bile was purchased from Farm to Pharm (NJ, USA). Rhizopus lipase (150,000 units/mg F-AP-15) was obtained from Amano Enzyme Inc. (Nagoya, Japan). Trypsin from bovine pancreas (7500 N-α-benzoyl-L-arginine ethyl ester (BAEE) units/mg, T9201) was purchased from Sigma Aldrich (add city, state, country). HPLC-grade acetonitrile (ACN) and HPLC-grade water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sterile filtered, cell culture compatible dimethyl sulfoxide (DMSO) (Sigma–Aldrich) was used as HPLC sample solvent. Other chemicals were of reagent grade and used without further purification. Milli-Q water was used throughout the experiment.
Preparation of tangeretin viscoelastic emulsion

Tangeretin VE was produced according to our recently published method [21]. In brief, an emulsion dispersed phase was prepared by adding tangeretin and emulsifier (lecithin) to the carrier oil comprised of 100% medium chain triglyceride (MCT) and maintained at 130 °C until completely solubilized and then cooled to 70 °C before the aqueous phase was added. The aqueous phase (100% double deionized water) was preheated to 70 °C to avoid rapid crystallization due to an abrupt temperature drop. Once the aqueous phase was added to the oil phase, the solution was maintained at 70°C and continuously stirred until a crude emulsion formed. To prevent blocking the narrow vale of the high-pressure homogenization instrument (EmulsiFlex-C6, AVESTIN Inc., Ottawa, Canada), the viscosity of the crude emulsion was first reduced by subjecting to high-speed homogenization (ULTRA–TURRAX T-25 basic, IKA Works Inc., Wilmington, NC, USA) at 24,000 rpm speed before undergoing pressure treatment at 500 bar and 55 °C. Finally, approximately 25-30 g of emulsion samples were collected from each processing batch.

Loading concentration analysis of tangeretin emulsion

The loaded tangeretin VE concentration was then determined using a microplate reader (Molecular Devices, Sunnyvale, CA) at 326 nm. A standard curve from 0.002 mg/ml to 0.125 mg/ml tangeretin in ethanol was constructed in triplicate. The loading capacity of tangeretin into the VEs were determined by dispensing a pre-measured VE sample (known weight) into a 10 ml volumetric flask and filled with 95% ethanol.
**In vitro lipolysis of PMFs in emulsion or MCT suspension**

The *in vitro* lipolysis study was carried out using our method previously published (115). To be consistent with the other bioavailability study in this work, a fasted-state buffer was selected for this part of the evaluation. In short, a fasted-state lipolysis buffer was prepared with Tris maleate, NaCl, CaCl$_2$·H$_2$O, NaTDC, and phosphatidylcholine in concentrations of 50, 150, 5, 5, and 1.25 mM, respectively. Pancreatin was freshly prepared for each study by mixing 1 g of pancreatin powder with 5 mL lipolysis buffer, centrifuging at 2000 rpm, and storing on ice. To begin the lipolysis study, an equivalent amount of samples and 1 mL of prepared pancreatin solution were added to 9 mL of fasted-state lipolysis buffer. During the 2-hour lipolysis study, the temperature was maintained at 37 ± 1°C and the pH was maintained at 7.50 ± 0.02 with 0.25 N NaOH titration. The volume of NaOH added at each time point was recorded for later analysis. Upon completion of the 2-hour lipolysis study, the resulting lipolysis solutions were subject to ultracentrifugation (Type 60 Ti rotor, Beckman Coulter) for 1 hr at 50,000 rpm. After ultracentrifugation, the middle layer of supernatant was collected and stored at -80°C for later HPLC analysis.

For HPLC analysis, 200 µL of lipolysis supernatant sample (0.22 µm filtered) was mixed with 400 µL of DMSO. The percent bioaccessibility of PMFs was calculated according to previously published literature (145) using the equation below:

$$\% \text{Bioaccessibility} = \frac{\text{Total mass of solubilized PMFs}}{\text{Total mass of PMF in original lipid samples}} \times 100 \quad \text{(Eq. 5.1)}$$

To determine the change in the digestion kinetics after emulsion processing, the extent of lipolysis at 30 min was compared between MS and VE samples. The extent of lipolysis, defined as the percentage of triglycerides digested by lipase, can be calculated
from the mols of NaOH consumed. The calculation for the extent of lipolysis assumed that two mols of fatty acid are released during digestion of one mol of triglyceride consuming two mols of NaOH. Since lecithin from the VE formulation may also contribute to the total number of fatty acids released, the calculation of NaOH consumption for the VE sample included both compositional MCT (0.27 g) and lecithin (0.0075 g). The extent of lipolysis was calculated using the following equation in reference to a previously published paper (115):

\[
\text{Extent of lipolysis} = \frac{\text{Volume of NaOH} \times \text{Conc. of NaOH}}{2 \times \text{mol of triglyceride}} \times 100\% 
\]

(Eq. 5.2)

**Gastrointestinal model**

The dynamic *in vitro* gastrointestinal model TIM-1 (TNO, Zeist, The Netherlands) was composed of four compartments that simulate the stomach, duodenum, jejunum, and ileum. It was used to study the pre-absorption events after ingestion. To mimic physiological states, the secretion of digestive juices and adjustment of pH conditions were controlled by computer programs according to physiological data described in previous literature (165). The half-life of gastric emptying was set at 70 min. Temperature during the digestion simulation was maintained at 37°C. For fasted state, secretion fluids were prepared at 5 times dilution from a previously published method (165) that utilized a fed-state digestion process.

To compare the bioaccessibility of tangeretin in MCT suspension (MS) and VE from digestion, the sample “meals” were “fed” into the stomach compartment and tested during 6-hour experiments. To determine the bioaccessible concentration of tangeretin, dialysates were collected at 30, 60, 90, 120, 180, 240, 300, and 360 min from jejunal and
ileal filtrate, which passed through semipermeable hollow capillary membranes (Spectrum Milikros modules M80S-300-01P) with pore size of 0.05μm. At the same time, efflux samples were obtained without filtration from outlet of the ileal compartment. Collected samples were stored on ice until subsequent HPLC analysis. The experiments were performed in duplicate and were analyzed in triplicate.

For HPLC analysis, 500 μL of sample was inoculated with an internal standard (nobiletin, 10 μg/mL) that was then extracted by mixing with 600 μL of ethyl acetate and centrifuge at 16000 g for 30 min at ambient temperature. After centrifuge, the 200 μL of supernatant was obtained and mixed with an equal amount of DMSO for use in HPLC analysis.

**Animals**

Female ICR mice aged seven weeks were purchased from Charles River Laboratories (NY, USA). Animals were randomly divided into control and experimental groups after 1 week of acclimation. All mice were maintained in a controlled atmosphere (25 ± 1 °C at 10% relative humidity) with 12 h light/12 h dark cycle. All animals were fed with Purina Laboratory Chow 5001 and ad libitum water (Ralston-Purina, Co., St. Louis, MO). The experimental protocol was approved by Rutgers University (no. 99-015).

**Pharmacokinetics study**

Mice used in the pharmacokinetics study were fasted overnight before administrating 100 mg/kg of tangeretin in MS or emulsion through oral gavage. At selected time intervals (0.5, 1, 2, 4, 8, 12, and 24 hr), blood samples were taken after the animals were sacrificed by CO₂ asphyxiation and whole blood samples were acquired
through cardiac puncture. Collected whole blood samples were immediately centrifuged at 5000 g for 15 min at 4°C. Plasma was collected and stored at −80 °C until later HPLC analysis. For HPLC analysis, a final concentration of 10 μg/mL of nobiletin was added to 200 μL of thawed plasma sample as an internal standard. The inoculated plasma was then extracted by combining with 600 μL of ethyl acetate and centrifuged at 16000 g for 30 min at ambient temperature. After centrifuge, the supernatant was collected in a separate container and then dried under nitrogen. The dried samples were redissolved in 100 μL of DMSO and were used for HPLC analysis.

$C_{\text{max}}$ and $T_{\text{max}}$ were recorded from the analysis of plasma concentration-time curves. The total areas-under-curve (AUC) of the time-concentration plot were calculated using the linear trapezoidal rule. The apparent elimination rate constant ($K_{\text{el}}$) was obtained from the terminal linear regression slope of logarithmic-transformed plasma concentration-time curves.

**HPLC Analysis**

The UltiMate 3000 HPLC system (Dionex, CA, USA) consisted of a quaternary solvent delivery system and an auto sampler; a variable wavelength detector was connected to Supelco's RP-Amide column, 15 cm x 64.6 mm id, 3 μm, (Bellefonte, PA, USA). The detection of PMFs was performed using a gradient elution of water (solvent A) and ACN (solvent B). The optimized condition was modified from previous literature (147). The total elution time was 22 min, where the mobile phase started from 40% ACN, then linearly increased to 55% of ACN over 10 min, then increased to 70% in 5 min, then to 80% in 5 min, and was then linearly reduced back to 40% at 21 min and held
isocratically for the final minute. The flow rate was held constant at 1.0 ml/min, injection volume was 30 μL, and detection wavelength was 320 nm.

Statistical analysis

All results were expressed as means ± standard deviation. One-way student t-tests were performed using Sigmaplot 10.0 software to examine the difference in oral bioavailability between unformulated and emulsion tangeretin. Statistical significance was concluded when $p < 0.05$.

Results

Characterization of tangeretin viscoelastic emulsion

In our previous study, the VE system was optimized for oral delivery of tangeretin (113). Sufficient loading capacity and storage stability make further in vivo bioavailability and bioefficacy evaluation possible. In this work, all tangeretin emulsion samples used for the studies were freshly prepared and characterized before use. To account for variations in the loading of tangeretin, each production batch was individually assessed for concentration in triplicate using a microplate reader. The tangeretin loading capacity of VE used in this work ranges from 2.3 – 2.5% (by HPLC), with an average droplet size of ~500 nm (by light scattering technique). The hydrophobic chemical structure of tangeretin is the major limiting factor to its oral absorption. Thus, methods that can improve the solubility and bioaccessibility of tangeretin in the aqueous environment could greatly improve its bioavailability. Viscoelasticity, for the tangeretin emulsion system, was characterized and exhibited good stability under normal temperature conditions. On the other hand, the fact that it can be
easily dispersed into aqueous environment makes it a well-qualified candidate for oral delivery.

**Comparison of in vitro lipolysis profiles between tangeretin MCT suspension and viscoelastic emulsion**

The oral bioavailability is positively related to the amount of ingested component that becomes accessible for intestinal uptake. The aqueous solubility and stability in the GI environment are important factors that determine the portion of the dietary component being absorbed through the gut wall. For hydrophobic compounds, low aqueous solubility and rapid elimination greatly limit its absorption via the oral route. Since hydrophobic ingredients usually have higher solubility in lipids, the presence of lipids during digestion has a positive impact on their oral bioavailability. As lipids are hydrolyzed by lipase and micellized with bile salts, the nearby hydrophobic compound may be incorporated into the hydrophobic micelle core and is then collectively absorbed through the intestinal lining. In other words, the greater the degree of micellization and the faster the rate of micelle formation in the intestinal lumen, the more likely the hydrophobic component can avoid rapid elimination and become bioaccessible. The *in vitro* lipolysis model is a very useful tool to study the impacts of oral formulation on the lipid digestion kinetics and the bioaccessibility of target compounds in the system.

During lipid digestion, fatty acids are continuously released, causing a decrease in pH. To maintain the optimum pH for enzymatic digestion, NaOH is constantly added into the digestion buffer. Thus, plotting the volume of NaOH added vs. time curve allows the monitoring of digestion kinetics. In this study, the titration kinetics of the emulsion sample proceeds at a much faster rate than the unformulated MS sample (Figure 5.1A).
Due to the greater surface area available for lipase digestion, the rate of mixed micelle formation from the digestion products is greater. According to the NaOH concentration-time curve, the majority of lipid digestion in the emulsion system occurred within 5 minutes from the onset of the study, whereas less than 10% is digested in unformulated MS. When comparing the extent of lipolysis at 30 min, all lipids in the emulsion system were fully digested, but only 29% was consumed in unformulated MS (Figure 5.1B). The change of lipid digestion kinetics compared between the unformulated and emulsified tangeretin increased the bioaccessibility from 9.7 to 29.3% of the original input concentration (Figure 5.2), respectively. Results from this work again confirmed that the rate and extent of lipid digestion indeed played an important role in the solubility and bioaccessibility of tangeretin.

**Figure 5.1** Comparison of *in vitro* lipolysis profiles of tangeretin viscoelastic emulsion and MCT suspension. (A) The lipid digestion kinetics expressed as the amount of NaOH added as a function of time. (B) The extent of lipid digestion after 30 minutes of *in vitro* lipolysis. Data in (B) are presented as mean ± standard deviation (n = 3). **p < 0.01**
**Figure 5.2** Comparison of tangeretin percent bioaccessibility relative to the original dose in the MCT suspension and viscoelastic emulsion. Data in (B) are presented as mean ± standard deviation (n = 3). **p < 0.01

*Comparison of in vitro gastrointestinal digestion between tangeretin MCT suspension and viscoelastic emulsion*

Besides lipid digestion, the level of orally-ingested tangeretin that becomes bioavailable may also be affected by other pre-absorption factors including temperature, pH, gastric emptying time, ionic strength, and enzymatic interactions. Thus, an *in vitro* system, the TIM-1, was utilized which simulates the digestion event in the upper GI tract to study the mechanism underlying the impact of oral formulation on changing the GI absorption rate. After the sample was ingested, tangeretin absorbed in the jejunum and
ileum was collected after filtration, allowing only digestate to pass through. The efflux concentration was regarded as the amount of tangeretin obtained unfiltered from the outlet of the duodenum section, since the TIM-1 system does not include the lower GI compartment (colon). The sample collected from each section was analyzed by HPLC to determine the tangeretin concentration (Table 5.1).

**Table 5.1 Bioaccessibility of tangeretin measured in TIM-1 system**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Tangeretin Input (mg)</th>
<th>Total recovery (mg)</th>
<th>Total recovery as of % input</th>
<th>Bioaccessibility as of % input</th>
<th>Efflux as of % input</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT suspension</td>
<td>2500</td>
<td>134.4±23.0</td>
<td>5.4±0.9</td>
<td>3.3±0.3</td>
<td>2.1±0.8</td>
</tr>
<tr>
<td>Emulsion</td>
<td>2400</td>
<td>255.2±28.7</td>
<td>19.1±1.24</td>
<td>8.6±0.4</td>
<td>10.5±1.2</td>
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</tbody>
</table>

The bioaccessibility, using the TIM-1, is defined as the concentration of tangeretin recovered from both the jejunum and ileum compartments compared with the original input. The bioaccessibility and the total recovered tangeretin was 2.6- and 3.5-fold higher when incorporated into the VE than in MS. However, when the emulsion sample was applied, a higher amount of tangeretin was also recovered from the efflux, indicating better solubility and a faster GI transit rate than MS. This observation implied that lower GI tract absorption of tangeretin in the emulsion-based system might well play a role in contributing to the overall bioavailability in living organisms. Cumulative bioaccessibility profiles of tangeretin from jejunum and ileum, along with the unabsorbed fraction were included in supplementary information (Figure 5.3). Here, only the accumulated tangeretin bioaccessibility (jejunum and ileum) expressed as percent input was plotted as a function of time, as shown in Figure 5.4. Within the first 3 hrs of the experiment, the rate of tangeretin bioaccessibility was much faster in VE than in MS.
More than 80% of the total recovered tangeretin from feeding VE was obtained within the first 3 hours, indicating that the emulsion-based delivery system could potentially facilitate rapid absorption through enhancing the solubility and bioaccessibility of hydrophobic compounds.
Figure 5.3 Cumulative bioaccessibility profiles of tangeretin from (A) jejunum and (B) ileum sections of the TIM-1 system expressed as percent of input concentration. Unabsorbed fraction was also collected during TIM-1 simulation and presented as (C) Efflux. The study was performed in duplicate and analyzed in triplicate.
Figure 5.4 Cumulative bioaccessibility profile of tangeretin in the TIM-1 system expressed as percent of input concentration. The study was performed in duplicate and analyzed in triplicate.
Comparison of oral bioavailability between tangeretin MCT suspension and viscoelastic emulsion

To directly investigate the effect of emulsion-based oral formulation on the oral bioavailability of tangeretin, a pharmacokinetic study was conducted using mice fed with either tangeretin VE or MS through gavage ingestion. Apart from the *in vitro* digestion studies discussed in earlier sections, an *in vivo* pharmacokinetic study measures the available system concentration of ingested compounds, which takes into account all physiological factors including absorption, membrane permeation, and metabolism. Single oral administration of tangeretin (100 mg/kg) in either VE or MS to mice resulted in distinctive pharmacokinetic profiles between oral formulations. The curve showing plasma concentration of tangeretin against time is given in Figure 5.5, and pharmacokinetic parameters are summarized in Table 5.2.

**Table 5.2 Pharmacokinetic parameters of tangeretin after oral administration**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Tangeretin dose (mg/kg)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (hr)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (μg/mL)</th>
<th>AUC&lt;sub&gt;0-24&lt;/sub&gt; (μg/mL*hr)</th>
<th>K&lt;sub&gt;e1&lt;/sub&gt; (hr&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Relative Bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT suspension</td>
<td>100</td>
<td>0.5</td>
<td>7.1±3.2</td>
<td>35.5</td>
<td>0.126</td>
<td></td>
</tr>
<tr>
<td>Emulsion</td>
<td>100</td>
<td>1</td>
<td>8.7±1.7</td>
<td>83.0</td>
<td>0.153</td>
<td>2.3</td>
</tr>
</tbody>
</table>
Figure 5.5 Profile of plasma concentration of tangeretin as a function of time after oral administration in form of viscoelastic emulsion (solid line) or MCT suspension (dashed line). Data are presented as mean ± standard deviation (n = 3 or 4).

Administration of VE resulted in a delayed $T_{\text{max}}$ at 1 hour after oral administration, while $T_{\text{max}}$ for mice fed with MS appeared rapidly after 30 minutes. The more viscous characteristic of VE the formulation could potentially extend the gastric retention time and delay the time to peak concentration. Interestingly, in mice fed with VE, a second peak concentration was observed at 4 hrs, indicating the release of tangeretin from viscous formulation. During 4 to 12 hrs, the plasma concentration of tangeretin was significantly higher in those groups fed with VE than MS. Together with the observation of delayed release of tangeretin and higher plasma concentration after the
second peak (at 4 hour), one can postulate that the VE could be used for controlled-release applications, maintaining adequate drug levels and reducing the need for frequent dosing. Despite the delayed $T_{\text{max}}$ value, the VE formulation gave a $C_{\text{max}}$ value of $8.7 \pm 1.7 \mu g/mL$, which was 23% higher than the MS formulation. Moreover, the relative bioavailability of tangeretin in VE was 2.3 times greater than the bioavailability of MS, suggesting that the $\text{AUC}_{0-24}$ value for VE and MS were 83.0 and 35.5 $\mu g/mL*hr$, respectively. At 24 hrs, the level of tangeretin plasma concentration for both VE and MS decreased to 0.37 $\mu g/mL$ with $K_{\text{el}}$ at 0.126 and 0.153 per hr, respectively.

From the analysis of pharmacokinetic profiles, VE is an effective application to improve the oral bioavailability of tangeretin where the mechanism may reside in enhancing aqueous solubility, extending the gastric retention time, and/or modifying the compound release kinetics. However, as the extracted plasma samples were analyzed by HPLC, one metabolite was consistently found in all plasma samples withdrawn from mice fed with tangeretin aglycone. In one representative HPLC chromatography (Figure 5.6), three distinctive peaks were seen at 8.813, 10.94, and 15.067 min, which correspond to the presence of nobiletin (internal standard), tangeretin (aglycone), and 5-demethyltangeretin (metabolite), respectively. To better analyze the metabolic kinetics, the concentration of 5-demethyltangeretin calculated at each time point was plotted in Figure 5.7. The concentration-time profile of 5-demethyltangeretin was similar regardless of either oral formulation ingested. Even though not statistically significant, the concentration of 5-demethyltangeretin, however, was always higher in mice fed with MS than VE. This observation could be attributed to the fact that tangeretin is an inhibitory agent to the metabolic enzyme (157, 158). Thus, when a higher concentration of
tangeretin reaches the metabolic facility, more of the enzyme-catalyzed metabolic activities were inhibited. This phenomenon again confirmed the efficacy of the VE system to enhance the plasma concentration of tangeretin through higher absorption rates, from which a larger portion of orally-ingested tangeretin could reach the metabolic sites and exit unchanged.

Figure 5.6 HPLC elution profile for plasma samples of mice fed with tangeretin. Data presented were selected at 12-hr time point for a clear indication of metabolite appearance. Three elution peaks correspond to nobeletin (internal standard, 8.81 minute), tangeretin (aglycone, 10.94 minute), 5-demethyltangeretin (metabolite, 15.07 minute)
Figure 5.7 Plasma concentration of 5-demethyltangeretin as a functional of time profile after oral administration of tangeretin in viscoelastic emulsion (empty circles) or MCT suspension (solid circles). Data are presented as mean ± standard deviation (n = 3 or 4).
Discussion

In this study, VE was used as a carrier system for oral delivery of hydrophobic crystalline tangeretin. Rapeseed lecithin (PC 75) has a generally recognized as safe (GRAS) status and was selected as the only emulsifier, which, in this case, stabilized the emulsion system and provided it a viscoelastic characteristic. The oral bioavailability provides a fundamental explanation of the mechanism that links the improved physical and chemical compound properties of the VE system to the better biological efficacy. The oral bioavailability of the ingested compound is the sum of three major parameters: bioaccessibility, membrane permeability, and metabolic stability (112). Here, we used two in vitro digestion studies as well as in vivo pharmacokinetic analysis to examine the effect of the VE system on the bioavailability of tangeretin.

The in vitro lipolysis model is an effective tool to examine the bioaccessibility of hydrophobic components (119, 145). Using this model, the VE system demonstrated that lipid digestion is more efficient when a larger surface area is available for lipase activity. Moreover, higher formation rates of mixed micelles resulting from the accelerated lipid breakdown increased tangeretin bioaccessibility by 3.2-fold. However, due to the fact that this method used a closed compartment, analysis excludes the effect of other physiological factors, and some have argued that this model could result in the overestimation of bioavailability (150, 166). Therefore, we further examined the pre-absorption events using the TIM-1 in vitro gastrointestinal simulating system that mimics the digestion process in the upper GI tract to provide an advanced estimation of bioaccessibility. Interestingly, the TIM-1 system estimated a 2.6-fold enhancement in bioaccessibility when it was fed with VE vs. MS. This observation indicates that other
biological factors, besides lipase, could influence the amount of tangeretin that become available for absorption. That is, the TIM-1 system, which includes more physiological factors during absorption, may provide a more accurate estimate of oral bioavailability than the lipolysis model.

Even though both in vitro systems gave consistent predictions of bioavailability, it is still not possible for the in vitro models to address all of the physiological influences that together contribute to the overall bioavailability. For example, in the TIM-1 system the gastric emptying rate is pre-determined regardless of the food ingested while, in reality, the gastric retention time would be variable when different dietary matrices are encountered. Moreover, the in vitro digestion systems, in general, rule out the absorption and metabolic mechanisms that are important factors to oral bioavailability. The in vivo pharmacokinetic study in mice was conducted to provide a realistic assessment on the effect of oral formulation with regard to the system availability of tangeretin. Even though the level of tangeretin in the plasma was still low (since peak plasma concentrations were $7.1 \pm 3.2$ and $8.7 \pm 1.7$ μg/mL from feeding MS and VE formulations, respectively), the oral bioavailability of tangeretin in those mice fed with VE was 2.3 times of that in mice fed with MS. The data from the pharmacokinetic study implied that oral bioavailability of tangeretin could be improved by oral formulations that enhance its solubility. Moreover, the bioavailability of tangeretin is not only affected by pre-absorption events, but also other metabolic activities following absorption. The decrease in the appearance of 5-demethyltangeretin in the VE-fed mice suggested that metabolic activities could be downregulated when larger amounts of tangeretin reach the metabolic facilities.
When assimilating all of the presented analysis, despite their limitations, the \textit{in vitro} methods used in this work were useful in providing prediction of \textit{in vivo} oral bioavailability from different oral formulations. According to the data collected, solubility and metabolic conversion are the two main hurdles for tangeretin bioavailability. Using VE as the oral delivery vehicle for tangeretin, improved its bioaccessibility in intestinal lumen, which resulted in higher oral bioavailability. Even though the oral bioavailability analysis of tangeretin is still limited, the pharmacokinetic values obtained in this work were higher than in the work of Manthey et al., in which 50 mg/kg of tangeretin in corn oil was fed to SD rats (164). The difference could be attributed to the lower solubility of tangeretin in corn oil (102) and physiological variability between animal species.

\textbf{Conclusion}

In summary, VE developed for the oral delivery of tangeretin proved effective in enhancing the oral bioavailability of tangeretin by improving the aqueous solubility, and promoting rapid digestion, increasing the absorption of tangeretin, and resulting in higher metabolic stability and, thus, better oral bioavailability. The \textit{in vitro} digestion models used in this work demonstrated a positive correlation in predicting the bioavailability in living organisms and should be used when screening the efficacy of delivery systems before proceeding to \textit{in vivo} assessment, which could subject to great individual variability. Emulsion-based delivery systems were demonstrated to be an efficient strategy to overcome the poor bioavailability of tangeretin and may also be used for other hydrophobic ingredients with similar chemical properties. However, more research is needed on the related toxicity that accompanies the benefits that oral delivery achieves.
With more careful safety evaluation, the advantage of using an emulsion-based delivery system may then be applied to consumer products to extend the range of available health-promoting benefits.
CHAPTER VI. EFFECT OF VISCOELASTIC EMULSION SYSTEM ON THE IN VITRO AND IN VIVO ANTICANCER EFFICACY OF TANGERETIN

PROJECT TITLE: IN VITRO AND IN VIVO ANTI-CANCER ACTIVITY OF TANGERETIN AGAINST COLORECTAL CANCER WAS ENHANCED BY EMULSION-BASED DELIVERY SYSTEM

As of submission of this dissertation, the work in this chapter has been submitted in the title of “In vitro and in vivo anti-cancer activity of Tangeretin against colorectal cancer was enhanced by emulsion-based delivery system” to the Molecular Nutrition & Food Research for consideration of publication.

Abstract

The oral bioavailability and efficacy of citrus polymethoxyflavone, tangeretin, was attenuated by its poor aqueous solubility. An emulsion-based delivery system was utilized to enhance the anti-tumoregenesis activity of tangeretin. The in vitro anti-proliferative activity of tangeretin was first evaluated using MTT test on colonic carcinoma cell lines, HCT116 and HT29. The utilization of emulsion system significantly improved the efficacy of tangeretin at dosing concentrations at 12.5 and 25μM. The effectiveness of emulsion system to enhance the in vivo oral efficacy of tangeretin against colorectal cancer development was also evaluated by AOM/DSS-induced colitis related colon tumoregensis model. The tumor incidence, multiplicity, and pathological signs of colorectal adenoma were significantly reduced when tangeretin emulsion was applied. The regulation on tumoregenesis related protein expression was more effective in mice
treated with tangeretin emulsion than unformulated suspension. Moreover, the ability of tangeretin to modulate the lipid metabolism efficiency was also enhanced by emulsion-based delivery system. Our finding indicated that emulsion-based delivery system was an effective means to increase the oral efficacy of tangeretin. This study is the first successful demonstration of the effect of delivery system on the oral efficacy of nutraceutical using long-term animal model.

Introduction

Colorectal cancer (CRC), a collective name for colon cancer and rectal cancer, is initiated when polyps develop in the small intestinal inner surface lining and progressively expands towards the center cavity. According to statistics published by the American Cancer Society, CRC is the third most common cancer in both sexes and accounts for 9% of all cancer deaths in the United States. In 2013, 102,480 cases of colon cancer and 40,340 cases of rectal cancer, as well as 50,830 deaths from CRC, are projected. Epidemiological studies and research have indicated that the risk of developing CRC is closely related to age, genetic heredity, dietary habits, physical activity level and other environmental pro-carcinogens that can cause inflammation and oxidation stress (167). When internal homeostasis is disturbed, CRC develops in three stages that include initial gene mutation in normal cells, promotion of the mutated gene, and progression of uncontrolled cell proliferation, differentiation and disrupted apoptosis (168). Moreover, inflammatory bowel diseases (IBD) including Crohn’s disease and ulcerative colitis (UC) are a major cause of aberrant chronic inflammation, which significantly increase the risk of CRC development (71, 169-171). In this sense, many bioactive compounds with anti-inflammatory activity are being regarded as potential anti-cancer agents for inhibiting
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CRC (96).

Tangeretin (4’,5,6,7,8-pentamethoxyflavone) belongs to a sub-group of flavonoids called polymethoxyflavones and is mainly sourced from citrus peel. Tangeretin may function as a potential chemopreventive agent since it exhibits potent anti-inflammatory (74, 75), anti-proliferative (76), and anti-carcinogenic (77, 78) activities (Figure 6.1 A). Previous studies has shown that tangeretin reduced inflammation-related cyclooxygenase (COX-2) expression in human lung epithelial carcinoma cells (75) and induced G1 cell cycle arrest in breast and colon carcinoma cells (80, 81). However, when ingested orally, the bioavailability of tangeretin is low due to its low aqueous solubility arising from its lipophilic chemical structure. Consequently, the required tangeretin concentration for many intended therapeutic purposes is difficult to reach using an oral delivery route.

The bioavailability of a compound is directly related to the amount of compound that is accessible for intestinal absorption (44). Since the gastrointestinal (GI) tract is a constantly-flowing aqueous environment, the solubility of an ingested compound becomes the critical factor in determining the absorption rate and availability of ingested compounds to the system circulation. To increase the solubility of lipophilic compounds in the aqueous environment, emulsion is one of the most commonly applied delivery methods for manufacturing or research purposes. The advantages of utilizing emulsion as a delivery system include a wide application range, versatility, easy fabrication and processing, and convenience to be incorporated to food and supplemental products. From our previous investigation, a stable emulsion-based delivery system containing high
loading of tangeretin (>2.5%) was successfully developed to enhance solubility and bioaccessibility (113, 115). The effect of the emulsion system on the anti-cancer activity of tangeretin against HepG2 human hepatic carcinoma cells was significantly improved by more than 20% at a concentration of 25 μM (115).

In earlier literature, many have demonstrated great improvement in the pharmacokinetic properties (66, 119, 138, 139, 141-144, 146, 172) and bioactivity (24, 52) of compounds using an emulsion-based delivery system. However, due to constraints on the loading of most delivery systems’ encapsulating lipophilic compounds, most researchers have found it difficult to conduct long-term in vivo anti-cancer studies at concentration levels that will produce meaningful therapeutic results. Therefore, ours may be the first orally-delivered long-term cancer inhibition study on emulsion systems containing bioactive food ingredients. In the present study, we aim to examine the effect of an emulsion-based delivery system on tangeretin to prevent azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced colon tumorigenesis in mice. Preliminary screening of the anti-proliferation potency of tangeretin in an emulsion-based system was compared with unformulated samples using HCT116 and HT29 human colonic carcinoma cell lines before proceeding to an in vivo animal study. Since the AOM/DSS model was designed to mimic colitis-related human CRC development (173), we will be evaluating the amount of expression on biomarkers related to inflammation activity and tumor proliferation. Evaluation of key biomarkers is an efficient means to quantitatively compare the in vivo bioactivity of tangeretin in unformulated oil suspension and emulsion-based systems. The related safety and toxicity issue was also assessed through monitoring the changes in body weight, organ weights, and survival rates of mice at the
time of sacrifice.

Materials and methods

Materials

Tangeretin of 98% purity was purchased from Quality Phytochemicals, LLC (Edison, NJ, USA). PC75 rapeseed lecithin containing 75% phosphatidylcholine was a gift from American Lecithin Company (Oxford, CT, USA). Neobee 1053 medium-chain triacylglycerol (MCT) was a gift from Stepan Company (Northfield, IL, USA). Sterile filtered, cell culture compatible dimethyl sulfoxide (Sigma–Aldrich, MO, USA) was used for HPLC sample solvent. Minimum essential medium (MEM) was purchased from HyClone Laboratories, Inc. (Logan, UT, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Penicillin and streptomycin were purchased from Invitrogen, (Carlsbad, CA, USA). AOM was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against COX-2 were purchased from BD Transduction Laboratories (Lexington, KY, USA). Polyclonal antibodies for β-catenin, PCNA, and VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals were of reagent grade and used without further purification. Milli-Q water was used throughout the experiment.

Preparation of tangeretin emulsion

Tangeretin emulsion was prepared according to our recently published method (113). The designed formulation contained 2.1% tangeretin in 30 g of processed emulsion, which was composed of 50.4% medium-chain triglyceride (MCT), 1.5% lecithin, and 46% double deionized (DI) water. To describe the processing procedure briefly, the
preparation of oil phase was performed by fully dissolving tangeretin and lecithin in MCT at 130 °C. The aqueous phase, DI water, was preheated to 70 °C for preventing rapid tangeretin re-crystallization due to sudden temperature drop. When the aqueous phase was to be added to the oil phase, the temperature was reduced to 70 °C and continued to be mixed by magnetic stirring until a single-phase crude emulsion was formed. After removal of the magnetic stir bar, the crude emulsion was then subjected to high-speed homogenization (ULTRA–TURRAX T-25 Basic, IKA Works Inc., Wilmington, NC, USA) to reduce the viscosity for easier processing of high-pressure homogenization (EmulsiFlex-C6, AVESTIN Inc., Ottawa, Canada). Throughout the high-pressure homogenization process, 500 bar pressure was applied while the temperature was maintained at 55 °C. In each processing batch, approximately 25-30 g of sample was able to be collected.

Particle size and loading concentration analysis of tangeretin emulsion

Particle size of the tangeretin emulsion was determined using dynamic light scattering (DLS)-based photon correlation spectroscopy (PCS), which includes a BIC 90 Plus particle size analyzer equipped with a Brookhaven BI-9000AT digital correlator (Brookhaven Instrument Corporation, New York, NY, USA). To rule out multiple scattering effects, the emulsion samples were diluted 5000 times by DI water in 1-cm path length cuvettes. The applied light source was a solid-state laser set at 658 nm and 30mW power. The resulting signal was detected by a high-sensitivity avalanche photodiode detector and measured at 90° fixed scattering angle while temperature maintained at 25 ± 1°C. Particle size measurements were conducted in triplicate (n = 3).
The actual loadings of the finished emulsion samples were measured by a microplate reader (Molecular Devices, Sunnyvale, CA) at the absorption wavelength of 234 nm. A tangeretin concentration standard curve was constructed using a concentration range from 0.002 mg/ml to 0.125 mg/ml. Loading of tangeretin in emulsion was determined by measuring the weight of the emulsion sample into a 10 ml volumetric flask and filling the flask to 10 ml with 95% ethanol.

Cell culture

Human colorectal carcinoma cell lines, HCT 116 and HT29 (American Type Culture Collection, HB-8065, Manassas, VA, USA) were grown and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin. The incubation condition for cell culture was 95% relative humidity and 5% CO₂ at 37 °C.

MTT cell proliferation assay and growth morphology observation

Methyl thiazol tetrazolium bromide (MTT) assay was used to quantify cell proliferation of HCT116 and HT29 after treated with emulsion or DMSO-dissolved tangeretin. Briefly, cells suspended in serum complete medium were seeded into a flat-bottom 96-well clear plate at density of 1 x 10⁴ cells/well. After 24 hours of incubation, the culture media were carefully aspirated and replaced by a medium containing emulsion or DMSO-dissolved tangeretin. Separate sets of negative control (untreated) and blank emulsion vehicles were also cultured for reference value and background subtraction. After 24 hours, the medium containing tangeretin in the form of emulsion or dissolved in DMSO were removed and then 100 µL of MTT solution (0.5 mg/mL in RPMI 1640
medium) was added to each well. MTT solution caused the cell to crystallize within 2 hours of incubation, and cells were removed from the plates by aspiration. Finally, formed crystals were dissolved in 100 µL of DMSO before absorbance was read at 560 nm using Microplate Reader (Molecular Devices, Sunnyvale, CA). The relative proliferation values were calculated against the control value. Experiments were conducted in triplicate with 6 repeats in each investigation for avoiding inter- and intra-experimental error. Cells were also observed using an inverted microscope (Leica Microsystems) with 200 times magnification.

**Animals**

Male ICR mice aged 6 weeks were purchased from BioLASCO Experimental Animal Center (Taiwan Co., Ltd). Animals were randomly divided into control and experimental groups after 1 week of acclimation. All mice were maintained at the National Kaohsiung Marine University animal facility in a controlled atmosphere (25 ± 1 °C at 50% relative humidity) with 12 h light/12 h dark cycle. All animals were able to liberally access water and food that was replenished every day. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU).

**AOM/DSS-induced colorectal cancer model**

In the present study, an AOM/DSS-induced colorectal cancer model was conducted in conjunction with a preventive feeding pattern, which contained 1 week of pre-induction treatment and 10 weeks of post-induction treatment (Figure 6.1). A total of 45 male ICR mice were randomly separated into 1 negative control, 2 positive control and 2 experimental groups (Figure 6.1 B). For a total of 11 weeks, all mice were gavage
fed either with blank MCT oil, blank emulsion, tangeretin MCT suspension or tangeretin emulsion (tangeretin concentration = 100mg/kg body weight). During week 0, all mice were fed with designated feeding samples and did not receive any carcinogenic chemical treatment. Starting from week 1, all groups (n = 36) besides the negative control received a single intraperitoneal injection (ip) of AOM (20mg/kg body weight) for induction of colorectal cancer development. After 1 week of post-AOM period, 2% DSS was added to the drinking water for groups that received the AOM ip injection for 7 days and then switched back to normal drinking water. At the end of 11 weeks, all mice were sacrificed by CO₂ asphyxiation and subjected to necropsy inspection. The terminal body weight, liver, spleen, and kidney were weighted and recorded. Upon necroscopy, colons from mice were carefully removed and flushed with PBS. After weight and length were precisely measured and recorded, the colons were cut longitudinally and feces removed. The incidences of tumor development from each group were calculated and tumor multiplicities were also determined by microscopic examination. Representative colons from each group were fixed in 10% buffered formalin for 1 week and then used for histopathological analysis. Colon mucosa from un-fixed colons were scraped off from colon film and stored at -80 °C for immunochemical evaluation.
Figure 6.1 (A) Structure of tangeretin and (B) Experimental design for *in vivo* animal study.

*Immunoblotting*

For immunoblot analysis, the tissues scraped off from the colon mucosa and 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 1% NP-40, and 10 mg/mL leupeptin) were homogenized by Polytron tissue homogenizer for 15 seconds. After homogenization, tissue mixtures were then lysed on ice for 30 minutes with vortex at intervals of 5 minutes. The lysed tissue samples were then centrifuged at 10000 g for 30 minutes at 4 °C. After centrifugation, the supernatant containing solubilized protein was collected and used for immunoblot analysis. Fifty micrograms of protein from each sample were
mixed with buffer (0.3 M pH 6.8 Tris-HCl, 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate, 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue) in a ratio of 1:5 and then boiled at 100 °C for 5 minutes. After boiling, the mixtures were subjected to electrophoresis on SDS-polyacrylamide gels under 100 mA constant current. After electrophoresis, the resolved proteins on the gel were electrotransferred onto the immobile membrane (45 μm PVDF; Millipore Corp., Bedford, MA, USA) using transfer buffer (25 mM pH 8.9 Tris-HCl, 192 mM glycine, and 20% methanol). The membranes were then blocked with blocking solution consisting of 20 mM Tris-HCl, pH 7.4, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide. After blocking, the membranes were incubated with specific antibodies of COX-2 monoclonal antibodies and anti-VEGF at 1:1000 dilutions by blocking solution. β-actin antibody was also probed to evaluate the consistency of the loaded protein concentration.

Immunohistochemical staining

The colon mucosa samples that were fixed in 10% buffered formalin were cut into 3 mm sections, deparaffined, rehydrated, and treated with 0.3% hydrogen peroxide (H₂O₂) for 15 minutes to block endogenous peroxidase. To unmask epitopes, sections were pressure-cooked four times at 7 minutes each in 10 mM citrate buffer with pH 6.0 (Immuno DNA retriever with citrate, BIO SB, Inc., Santa Barbara, CA, USA). After pressure treatment, tissue sections were incubated with primary antibodies to PCNA and β-catenin (1:100 dilution in PBS) for 1 hour. Immunoreactivity was determined by respective incubation with biotin-labeled secondary antibody and streptavidin-biotin peroxidase for 30 minutes each. The positive signal was detected, with the substrate, 3,3’-Diaminobenzidine tetrahydrochloride (0.05%, DAB), presenting as brown color
under a light microscope. The procedure for analyzing the stained tissue was reported in previously published literature (174). In particular, the positive expression for PCNA and β-catenin was evaluated by the degree of nuclear and cytoplasmic staining on the sampled tissue sections. The percentage of positive cells and the staining intensity was multiplied to give the immunoreactive score (IRS).

**Clinical chemistry**

At the end of 11 weeks, blood samples were collected from mice from all groups of mice immediately after CO₂ anesthetization through cardio puncture. Collected blood was mixed with heparin and centrifuged at 3000 g for 15 minutes at 4° C. After centrifugation, serum was collected and stored at -80 °C before subjection to clinical chemistry analysis. Serum triglycerides (TG) and total cholesterol (TCHO) were evaluated using commercially available kits (BioVision Inc., Mountain View, CA, USA) and Fuji DRI-CHEM40000 (FUJIFILM, Tokyo Japan).

**Statistical analysis**

Cell line studies and biochemistry analysis for this animal study were performed in at least triplicate. Results were expressed as means ± standard deviation. One-way student t-tests were performed using Sigmaplot 10.0 software to examining the bioactivity difference between unformulated and emulsion tangeretin. Statistical significance was concluded when $p < 0.05$. 
Results and discussion

Emulsion particle size and loading analysis

In our previous study, an emulsion-based system was successfully formulated as a delivery vehicle for tangeretin. An identical tangeretin emulsion formulation composed of 2.1% tangeretin, 50.4% MCT, 1.5% lecithin, and 46% DI water was used for the present study. However, due to the constraint of experimental location, the local high-pressure homogenization machinery and processing parameters were slightly different than in the original experiment. To control the formulation quality and to confirm compound loading, the metrics of emulsion stability, particle size and precise loading were evaluated for each production batch throughout the study. For each processing batch, approximately 30 g of tangeretin emulsion was deposited into a clean glass bottle with cap and stored in a dark at room temperature until used (Figure 6.2 A). Daily observation of the stored emulsion samples was conducted, and the storage stability was good, with no signs of phase separation and precipitation before consumption by later experiments. The morphology of the undiluted tangeretin emulsion sample was further observed using an optical microscope with 500x magnification (Figure 6.2 B). As shown in the picture, the tangeretin emulsion system is a heterogeneous system, with part of the compounds either encapsulated in` emulsion droplets or homogeneously entrapped in the viscous emulsion systems. According to our previous investigations, the size of the emulsion droplets plays a critical role in the bioavailability and efficacy of the included compound (24, 138); accordingly, the sizes of the emulsion droplets and entrapped tangeretin crystals were precisely measured. With the image processing software (Image J, nih.gov) and correct reference scale, tangeretin crystals present in the system were measured, on
average, as $9.33 \pm 1.3 \, \mu m$. The mean hydrodynamic diameter of the tangeretin emulsion was evaluated using the DLS method at room temperature. The DLS autocorrelation function curve and single stretched exponential fitting of diluted tangeretin emulsion (1:5000 in DI water) is shown in Figure 6.2 B. The mean hydrodynamic diameter of the emulsion droplets was determined as $482.3 \pm 1.8 \, \text{nm}$. The particle size determined using the DLS method included the surrounding hydrated layer, which may in turn give a systematically higher value than measurement using the dried method. The polydispersity of each sample was measured as $0.127 \pm 0.007$, indicating a narrow size deviation among the tangeretin emulsion samples. Moreover, the particle sizes of each production batch were relatively constant, with a standard deviation value of $\pm 50 \, \text{nm}$.
tangeretin emulsion at 500x magnification. (C) The DLS autocorrelation function curve and single stretched exponential fitting of diluted tangeretin emulsion (1:5000 dilution by DI water). (D) The loading of tangeretin samples (black triangles) was measured using a standard curve constructed at 234 nm absorbance.

As a result of the presence of lipid in the emulsion-based delivery system, frequent sample analysis may increase the chance of machine clotting and dysfunctions when high pressure liquid chromatography (HPLC) system was utilized. Therefore, tangeretin loadings in emulsion samples were evaluated by an automatic microplate reader using the ultraviolet (UV) absorbance method. The precision of this method was redundantly verified by HPLC analysis when an identical value was obtained from the
tangeretin control. As shown in Figure 6.2C, the quantifications of tangeretin concentration were calculated using a concentration standard curve with $R^2 = 0.9994$. Due to the lack of a suitable temperature control system, the produced emulsion batches may have contained different amounts of tangeretin, which ranged from 35 mg/mL to 55 mg/mL. To accurately calculate the dosing concentration, the tangeretin loading in each emulsion sample was precisely measured in triplicate immediately before being used for experimental investigations (represented as solid triangles in Figure 6.2D).

**In vitro proliferation evaluations and morphology observations**

The mechanisms that bioactive compounds have been shown to use to prevent and constrain the development of cancer include anti-inflammation, anti-proliferation, apoptosis promotion, and anti-metastasis. As a potential anti-cancer agent, tangeretin has been documented to inhibit cell proliferation through inducing G$_0$/G$_1$ cell cycle arrest (80, 81). Due to its lipophilic nature, higher dosing concentrations are generally required for tangeretin to produce a meaningful anti-proliferation effect in cancer cells. Therefore, to investigate the effectiveness of an emulsion-based delivery system to improve the bioidentification of tangeretin, an in vitro MTT essay was performed as efficient assessment before executing the subsequent in vivo animal study, which is typically much more complex and time-consuming. In this study, two colorectal carcinoma cell lines, HCT116 and HT29, seeded in 96-well plates with a density of $1 \times 10^4$ cells/well were treated with serum complete medium containing tangeretin either dissolved in DMSO or dispersed by an emulsion delivery system. The potency of the anti-proliferative effect of tangeretin was determined by the relative viability of treated cell wells to the untreated controls. After 24-hour incubation with tangeretin (DMSO dissolved or as emulsion), both cell
lines exhibited dose-dependent inhibition of cell proliferation in all treatment concentrations (3.125, 6.25, 12.5, and 25 μM) as shown in Figure 6.3 A and B. Cells treated with tangeretin emulsion showed statistically significant reductions ($p < 0.05$) in cell proliferation when compared with cells dissolved in DMSO. Moreover, the improvement in anti-proliferative activities by the emulsion-based delivery system was higher in the HT29 cell line than in the HCT116 cell line. The HT29 cell line, in comparison with the HCT116 cell line, is considered to be a more malignant type of colorectal carcinoma cells that contain a mutated $p53$ tumor suppressor gene, which is responsible for many anti-cancer functions, such as regulating normal DNA repair, cell cycle, normal cell apoptosis, and autophagy (175-177). Higher proliferative reduction rates promoted by the tangeretin emulsion in the HT29 cell line than in the HCT116 cell line indicates that the emulsion system may be an effective method to enhance the anti-cancer efficacy of tangeretin.
Figure 6.3 Relative cell viability of (A) HCT116 and (B) HT29 from MTT essay.

Microscopic observation of cell morphologies: HCT116 (C) untreated control, (D) blank emulsion treated, (E) treated with DMSO solubilized tangeretin, and (F) tangeretin emulsion. HT29 (G) untreated control, (H) blank emulsion treated, (I) treated with DMSO solubilized tangeretin, and (J) tangeretin emulsion. * $P < 0.05$

To obtain a direct observation of cell conditions, cells treated with tangeretin in both systems (solubilized in DMSO or incorporated in emulsion) were examined using an optical microscope under 100x magnification. The effectiveness of tangeretin to inhibit cell growth and proliferation has been discussed in terms of the observed cell size and density relative to the untreated control. While cells treated with blank emulsion (Figures 6.3 D and H) show no difference in cell density to the untreated controls (Figures 6.3 C
and G), the cells treated by tangeretin in both systems showed various degrees of growth inhibition. A similar trend was seen when the tangeretin emulsion presented the lowest cell density in both colorectal cancer cell lines (Figure 6.3 F and J). Moreover, the cell sizes were also smaller in tangeretin emulsion-treated groups than in groups treated with DMSO-solubilized tangeretin. The microscope examinations allowed the simple and direct realization that the emulsion-based delivery system augmented the efficacy of tangeretin for reducing cancer cell growth and proliferation. Together with the results from MTT essay and microscopic observation, emulsion-based systems have been proven to be an effective and efficient means to augment the \textit{in vitro} anti-proliferative cancer-related properties of tangeretin.

\textit{General observation of in vivo AOM/DSS-induced mice colorectal cancer model}

As discussed in the previous section, the emulsion-based delivery system was an effective means to enhance the \textit{in vitro} anti-proliferative activity of tangeretin. The effect of emulsion processing on the \textit{in vivo} oral bioefficacy of tangeretin was also evaluated using a colitis-related mouse colorectal cancer model. Among the experimentally-induced CRC models, the AOM/DSS model mimics the accelerated development of CRC induced by an inflammatory stimulant. After a single injection of AOM, a one-week exposure to the potent inflammatory agent DSS was proven to rapidly induce the progression of aberrant crypt foci to adenoma and then to carcinoma in as short a period as 8 weeks (173). In the present study, the experimental period lasting for 11 weeks was determined to allow consequential tumor development. During the 11-week study, the mean body weight of mice from all groups increased steadily, with no observable phenotypic characteristic of toxicity at any point during the experiment (Figure 6.4 A). By the end of
the experiment, all groups of mice presented approximately 80% body weight increase since the beginning of the experiment. After CO$_2$ asphyxiation, cautiously collected organs (liver, kidney, spleen) from all groups of mice were examined and showed no alteration in appearance and morphology, indicating no or low level of toxicity after 11 weeks of study. While the average weight of kidney and spleen showed no significant difference among all groups, the average liver weight of mice fed with tangeretin emulsion was lower than other groups due to the lower average body weight (Figure 6.4 B). Since the lower average body weight from the beginning of the experiment was observed for mice fed with tangeretin emulsion, the difference in liver weight was not caused by toxicity, as the relative liver weight (g/100 g body weight) showed no significant difference among all experimental groups. Overall, the dosing level of 100mg/kg produced no conspicuous sign of toxicity during and at the end of the animal study.
Figure 6.4 (A) Weekly body weight growth curve and (B) Weight of liver, kidney, and spleen at the end of the experiment.
Pathological findings

The progression of colitis-related CRC could be assessed by several characteristic large bowel symptoms. On this basis, the degree to which an emulsion-based delivery system improved the protective efficacy of tangeretin in the treatment of AOM/DSS-induced colorectal tumor development was evaluated. In the AOM/DSS-induced colitis CRC model, the increased weight and shortened length of colon are the most common pathological features identified as the disease proceeds. Mice colons from all experimental groups were carefully excised and measured, since DSS-stimulated colon inflammation results in reduced colon length. When compared with the non-treated control, AOM/DSS-induced mice showed a significant reduction in colon length ($p < 0.05$). The oral administration of tangeretin in the form of MCT oil suspension (TO) or emulsion (TE) resulted in longer colon lengths than groups fed with blank MCT and emulsion (Table 6.1). Due to uncontrollable cell proliferation, the process of developing colorectal tumors directly increased the cell density, mucosal thickness and, thus, the colon weight. The calculation of the colon weight-to-length (W/L) ratio was then established based on the rationale that heavier and shorter colons are viewed as a sign of a greater malignant gradient of CRC. While a significant increase of the colon W/L ratio was seen in positive controls (Group 2 and 3, $p < 0.01$), the change in mucosal morphology was alleviated in groups treated by TO and TE. The reduction in W/L ratio was statistically significant ($p < 0.05$) in TE-treated groups when compared with positive control groups fed with empty emulsion. In contrast, only mild improvement was seen in the mice fed with TO, relative to the blank MCT positive control mice.
As mentioned above, anomalous cell proliferation caused mucosal thickening and eventually resulted in the occurrence of colorectal adenomas. To efficiently study and compare the anti-tumorigenic efficacy of both tangeretin formulations, the incidence and multiplicity of colon adenomas is correspondingly summarized in Table 6.1. Single ip injection of AOM followed by a one-week exposure of drinking water containing 2% DSS resulted in 82% and 92% of large bowel adenoma incidence in the groups fed with blank MCT and emulsion, respectively. In contrast to the mice that did not receive any preventive treatment, mice that were treated by either TO or TE decreased the frequency of colorectal adenomas to 73% and 64%, respectively. Besides looking at the incidence of large bowel tumors, the number of adenomas presenting on each mouse colon was also an important parameter in determining the effectiveness of a bioactive compound to prevent or regulate CRC development. In this study, mice that were orally administered with TE decreased 37% of adenoma multiplicity when compared with the group that

Table 6.1 Body weight, colon weight and length, adenoma incidence, and multiplicity of mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight (g)</th>
<th>Colon Weight (g)</th>
<th>Length (cm)</th>
<th>W/L ratio</th>
<th>Incidence</th>
<th>Multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>42.85 ± 5.20</td>
<td>0.15 ± 0.03</td>
<td>7.18 ± 0.75</td>
<td>0.02 ± 0.01</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>AOM/DSS/MCT</td>
<td>40.13 ± 3.18</td>
<td>0.24 ± 0.13</td>
<td>6.15 ± 0.63</td>
<td>0.04 ± 0.02*</td>
<td>82%</td>
<td>6.22 ± 6.16</td>
</tr>
<tr>
<td>AOM/DSS/Blank emulsion</td>
<td>43.76 ± 4.54</td>
<td>0.27 ± 0.08</td>
<td>6.50 ± 1.45</td>
<td>0.04 ± 0.01*</td>
<td>90%</td>
<td>7.25 ± 3.92</td>
</tr>
<tr>
<td>AOM/DSS/Tangeretin MCT Suspension</td>
<td>42.33 ± 4.97</td>
<td>0.24 ± 0.08</td>
<td>6.70 ± 0.98</td>
<td>0.04 ± 0.02*</td>
<td>73%</td>
<td>5.80 ± 3.36</td>
</tr>
<tr>
<td>AOM/DSS/Tangeretin Emulsion</td>
<td>36.69 ± 3.91</td>
<td>0.24 ± 0.06</td>
<td>6.90 ± 1.20</td>
<td>0.03 ± 0.01*</td>
<td>64%</td>
<td>4.60 ± 3.27</td>
</tr>
</tbody>
</table>
consumed only blank emulsion sample (4.6 ± 3.27 vs. 7.25 ± 3.92, p < 0.01). While the tangeretin emulsion showed the lowest incidence of adenoma per mice, TO only insignificantly decreased the multiplicity by 7% relative to PO (5.8 ± 3.36 vs. 6.22 ± 6.16).

In Figure 6.5 A, the macroscopic pictures of representative colons from each group are presented for direct gross morphology observation. According to earlier discussion, a healthy mice colon should be reasonably long and lean, as represented by the picture from the untreated negative control (Figure 6.5 A). However, in the picture of positive controls (AOM/DSS treated with oral administration of blank MCT or emulsion), the large bowel became brittle and appeared as short and flappy, as the incidence of abnormal colonic mucosal inflammation and proliferation arose (Figure 6.5 B and C). The change in colon morphology was in agreement with the previously reported pathological signs of the AOM/DSS murine model (173). The colonic adenomas observed were distributed unevenly along the colon, with higher frequency in the lower rectal portion. The advancement of colonic disease was indicated when colonic adenomas appeared as large clusters resulting from higher tumor density. In comparison with MCT suspension (Figure 6.5 D), preventive treatment with tangeretin emulsion (Figure 6.5 E) for a total of 11 weeks more effectively alleviated the symptomatic morphological changes and suppressed the number of adenomas. Moreover, the histological examination of colonic adenoma showed serious disruption of the mucosal architecture, as the goblet cell structure in the normal intestinal mucosa (Figure 6.5 F) had generally disappeared in the AOM/DSS-treated positive control groups (Figures 6.5 G and H). The mucosal structure retained its architecture to various degrees when tangeretin was consumed
orally in the form of MCT suspension or emulsion. The goblet cell structures were better preserved by tangeretin incorporated in the emulsion-based delivery system than in unformulated MCT suspension. Overall, tangeretin dietary treatment in the AOM/DSS model was proven effective to prevent the colitis-related colon adenoma progression and to alleviate the related symptoms. Nevertheless, the utilization of an emulsion-based delivery system more significantly improved the dose efficiency and therapeutic efficacy of tangeretin, as evidenced by the experimental results.
Figure 6.5 Photographic observation of mice colon from AOM/DSS-induced tumorigenesis study: (A) negative control: MCT; (B) positive control: AOM/DSS/MCT; (C) positive control: AOM/DSS/Empty emulsion; (D) AOM/DSS/Tangeretin MCT suspension; (E) AOM/DSS/Tangeretin emulsion. The blue circles represent the occurrence of large group of tumor while the arrows indicate single tumor incidence. Histological analysis of large bowl morphology by H&E staining: (F) negative control: MCT; (G) Positive control: AOM/DSS/MCT; (H) Positive control: AOM/DSS/Empty emulsion; (I) AOM/DSS/Tangeretin MCT suspension; (J) AOM/DSS/Tangeretin emulsion.
Effect of emulsion-based delivery system on the efficacy of tangeretin to inhibit colitis-related colon tumorigenesis

The combination of AOM/DSS administration mimicked human colitis-related colorectal carcinogenesis by stimulating a state of chronic inflammation (173). Therefore, the suppression of large bowel inflammation by bioactives is then considered a potential preventive strategy to inhibit the formation of AOM-induced colorectal lesions (178, 179). A cytoplasmic protein, COX-2, is the common biomarker used for the evaluation of inflammatory stress of many in vivo models. While normal epithelial cells show negative expression, anomalous COX-2 expression plays a critical role in the development of the colitis-associated CRC. In this study, the effectiveness of tangeretin to regulate the expression of the COX-2 pro-inflammatory cytokine was evaluated using immunoblotting methods. After challenged by AOM/DSS, the expression of COX-2 markedly increased in positive control groups fed with blank MCT and emulsion, as denoted by the relative band intensity scores of 4.5 and 3.7, respectively. While TO did not show any suppression effect, COX-2 expression was successfully lowered in the mice treated by TE (Figure 6.6). The efficacy of tangeretin to regulate the in vivo COX-2 production was notably enhanced by the emulsion delivery system due to enhanced dose efficiency resulting from better solubility, release profile, intestinal uptake, and transportability. A similar phenomenon was seen when we further examined the expression of protein related to the pathogenesis of tumor growth and metastasis. VEGF is the common cell signaling protein that mediates the formation of new blood vessels (angiogenesis) in the endothelial cells and becomes excessively expressed during tumorigenesis (180, 181). In earlier literature, the inhibition of VEGF is an effective
approach to suppress pathological angiogenesis and associated tumor growth (180, 182).
Since VEGF expression is positively related to tumor formation, the efficacy of tangeretin to reduce tumor growth was assessed by immunodetection of VEGF levels in colonic epithelial cells collected from all groups of mice studied. According to the immunoblotting result, the level of VEGF expression was decreased appreciably in the group treated with TE when compared to the emulsion-treated positive control. However, the group treated by TO presented the highest level of VEGF expression, with no evidence of inhibition activity when compared to the positive controls (Figure 6.6). That is, the unformulated tangeretin suspended in MCT was not capable of producing significant \textit{in vivo} colorectal anti-tumorigenesis activity in the AOM/DSS murine model.

\textbf{Figure 6.6} The result from immunoblotting. Tangeretin emulsion treatment effectively decreased the expression of COX-2 inflammation cytokine and VEGF.

Since the occurrence of tumors is the result of unregulated cell growth, the expression of proliferative related protein can serve as important indicator to estimate the progression of tumorigenesis. Under normal conditions, PCNA is present in the cell nucleus of proliferating cells situated in various parts of organ tissues. When incidences of abnormal
cell growth occur, PCNA is extensively expressed and variably distributed in the tumor cells ranging from <1% to >20% (183). In this study, the degree of PCNA elevation in the AOM/DSS-triggered CRC model was histologically examined after antigen staining. The PCNA positive cell was determined when the nucleus was stained and detected as brown-colored. As shown in Figure 6.7, the proportion of browned nucleus was significantly elevated in positive controls (Figures 6.7 B and C) when compared with untreated negative controls (Figure 6.7 A). As discussed earlier, tangeretin was reported to serve as an anti-proliferative agent through arresting cells at the G1 phase. The results from our study were in agreement with the previous finding, since the administrations of tangeretin considerably lessened the appearance of PCNA in the colonic epithelial cells (Figures 6.7 D and E). Moreover, the IRS for TE treated group was significantly lowered when compared with the positive controls ($p < 0.05$), indicating the reduced proliferative activity within the cells (Figure 6.7 K).
Figure 6.7 Microscopic picture of immunohistological study. Mice treated by tangeretin MCT suspension and emulsion decrease the expression of PCNA (A-E) and β-catenin (F-J). ***p < 0.001, **p < 0.01, *p < 0.05

In the present study, the downstream tumorigenesis-related protein expression was found to be variably regulated by treatment with tangeretin in both administrated oral formulations. To further elucidate the mechanism that underlies the efficacy of tangeretin in preventing colitis-related colorectal cancer, the existence in the nucleus of β-catenin, the well-known regulator protein for inflammation-related protein expression (184), was studied by the IHC method. Being part of the Wnt signaling pathway, β-catenin proteins are involved in the progression of cell cycles that include cell growth, proliferation, and differentiation. β-catenin, discovered as cadherin-binding protein, exists on the cell membrane as part of the cytoskeletal structure under normal cell conditions
However, abnormal cell phosphorylation caused by chronic inflammation induces the release of β-catenin into the cytoplasm and the nucleus. Once β-catenin migrates into the nucleus, it tends to bind to the Tcf DNA binding protein and to cause the transcription of downstream carcinogenic gene expression. As the positive link between the overexpression of β-catenin to CRC formation had been widely studied in previous research, the modulation of the β-catenin-mediated signaling pathway then became a major target for CRC prevention (184-188). Here, the groups treated with AOM/DSS (Figures 6 G and H) significantly boosted the nuclear concentration of β-catenin, indicating higher transcription activities of downstream tumorigenesis genes. After dietary administration of TO and TE, the relocation of β-catenin into the nucleus was prevented and a lower overall concentration (interpreted as the intensity of brown pigment) was observed. Once again, the tangeretin emulsion resulted in a significant reduction of β-catenin IRS when compared with the positive controls ($p < 0.05$). To sum up, the result from the immunochemistry study clearly indicated that the utilization of the emulsion-based delivery system significantly increased the efficacy of tangeretin to inhibit the development of colitis-related tumorigenesis.

**Clinical chemistry**

In this study, both the MCT suspension and the emulsion system used for the oral administration of tangeretin were primarily composed of lipid. The association of lipid metabolic syndrome to colorectal carcinogenesis has been well investigated (189-192). From earlier discussion, the mouse group fed with TE exhibited the lowest average weight among all experimental groups throughout the study. To reveal whether the ability of tangeretin to prevent colonic tumorigenesis also correlated with the regulation of
serum lipid metabolism, the serum TG and TCHO was analyzed (Figure 6.8). While the dietary administration of blank emulsion produced a drastically higher serum TG level than blank MCT oil \( (p < 0.05) \), mice treated with TE but not TO showed significantly lowered serum TG level than the positive controls \( (P < 0.05) \). While no statistically significant increase in the serum TCHO level was detected in all groups, the TE-treated group exhibited a noteworthy drop \( (p < 0.05) \) when compared with the positive control. The result from blood lipid analysis correlated well with the previous immunochemical analysis, in which TE treatment significantly enhanced the in vivo efficacy of tangeretin. Moreover, the interesting decrease in the serum TG and TCHO levels after tangeretin oral application suggested that the blood lipid regulation ability of tangeretin might be worthwhile for future investigation.
**Figure 6.8** Clinical chemistry analysis on serum TG and TCHO levels of experimental mice from the AOM/DSS-induced colon tumorigenesis study. Tangeretin emulsion significantly decreased both TG and TCHO serum levels, compared with tangeretin MCT suspension. *p < 0.05

**Conclusion**

The results from this study clearly indicate that the emulsion-based delivery system was an effective application method to enhance both the *in vitro* and *in vivo* efficacy of tangeretin to inhibit the growth of colon cancer. Resulting from its lipophilic nature, the insufficient oral bioavailability of tangeretin leads to the low *in vitro* and *in vivo* correlation in terms of its biological efficacy. With this limitation in mind, the
emulsion system served as a platform to increase the efficacy correlation between the \textit{in vitro} and \textit{in vivo} investigations. Depending on the design of different emulsion systems, the strategy that each system must utilize to enhance the efficacy of bioactive compounds varies considerably. In this study, the applied emulsion system was designed to improve the efficacy of tangeretin by improving the aqueous solubility, gastric retention time, intestinal uptake, and metabolic stability. As shown in the present study, the unformulated tangeretin showed an insignificant dose-dependent reduction in HT29 and HCT116 colon cancer cell lines. After the incorporation of the emulsion delivery system, the dose efficiencies of tangeretin were significantly improved at higher application concentrations. Moreover, the \textit{in vivo} anti-tumorigenic efficacy of tangeretin was also significantly enhanced when an orally-administered emulsion formulation was applied.

Because chronic inflammation has been reported to be one of the leading causes of CRC, the AOM/DSS-induced colitis-related colon carcinogenesis model was developed as a pre-clinical animal study for therapeutic evaluation. The regulation of β-catenin expression and translocation is an established pathway to inhibit the transcription of downstream inflammation and proliferation-related gene expression. Despite many \textit{in vitro} investigations reporting the anti-cancer efficacy of tangeretin, the oral ingestion of intact tangeretin suspended in MCT showed low or no inhibition of colonic tumor development. On the other hand, significant reduction in the pathological symptoms was observed when tangeretin was orally applied as part of the emulsion delivery system. In emulsion-treated mice groups, the increased β-catenin regulation resulted in lower expression of the COX-2 inflammation cytokine and proliferation-related protein expression. In summary, this study clearly indicated that the applied emulsion-based
delivery system was an effective approach to increase the bioefficacy of tangeretin. Moreover, to the best of our knowledge, this is the first long-term *in vivo* evaluation of the effect of an oral delivery system on the efficacy of nutraceuticals. The result from this study showed that these delivery systems are indeed worthwhile investigations that will allow the efficient oral application of bioactive compounds.
CHAPTER VII. EFFECT OF VISCOELASTIC EMULSION SYSTEM ON ORAL TOXICITY OF TANGERETIN

PROJECT TITLE: INVESTIGATION OF TOXICITY OF EMULSIFIED TANGERETIN USING IN VIVO MICE MODELS

As of submission of this dissertation, the work in this chapter has been submitted in the title of “Investigation of toxicity of emulsified tangeretin using in vivo mice models” to the Journal of Functional Food for consideration of publication.

Abstract

The acute toxicity of tangeretin and the toxicity response of emulsified tangeretin over 28 days have been investigated using mice model. The hepatic side effects in response to tangeretin administration were identified for both cases. When 1, 2, and 3 g/kg of tangeretin were ingested, the alteration in hepatic cell morphology and related value of clinical chemistry increased dose dependently when responding to higher metabolic stress. In the sub-acute study, the effect of emulsification was found negligible but the repeated daily low dose application of tangeretin exhibited hormesis dose response trend, in which 50 mg/kg of tangeretin created more physiochemical alteration to mice than 100 mg/kg. The hormesis effect was observed in both male and female mice. Our results indicated that it is necessary to further evaluate the toxicity response of tangeretin in wider dosing range. Overall, the effect of tangeretin was sub-lethal to mice and generated more of the adaption response than toxicity effect.
Introduction

As significant effort has been dedicated to promoting better human health, numerous active compounds derived from dietary sources have been identified as potential agents to prevent or treat human diseases\(^{(193, 194)}\). However, the actual therapeutic effectiveness of these bioactive ingredients is often reduced by their unfavorable interactions with the physiological environment after they are consumed. That is, depending on the chemical structures, different limiting factors, such as pH instability, rapid degradation, low aqueous solubility, poor membrane permeability, and extensive metabolism by the human body, have been identified to prevent bioactives from being efficiently utilized by the biological system. In this sense, much research is now devoted to formulating various delivery systems for overcoming the potential biological challenges that each active compound may encounter. Various processing technologies and ingredients have been used to produce tailored delivery vehicles of various sizes, chemical and physical properties that could effectively change the biological fate of targeted active ingredients\(^{(112)}\).

Delivery systems are designed to enhance the therapeutic efficacy of targeted bioactives by modifying the physiochemical properties including stability, solubility, and pharmacokinetic profile. Thus, proper assessment and identification of the potential health risks from using different delivery methods is the most important prerequisite before any formulation can be released to the general consumer. Compared with invasive parenteral injections that usually produce immediate effects, bioactive ingredients that are consumed through an oral route pass through the entire digestive and metabolic mechanisms, reaches the target site much later, and requires a higher ingested dose since
most of the active ingredient could be lost during the transition process. Therefore, it is particularly critical to evaluate the effectiveness and potential toxicity of oral formulations over a longer application time course.

Tangeretin, categorized as being one of the polymethoxyflavones isolated from citrus peel, is recognized for its broad health benefits including anti-inflammation (75), anti-proliferation (81), anti-carcinogenesis (195), neuro-protection (156), and inhibition of cardiovascular diseases (161) and diabetes (159). However, despite its health-promoting properties, the application of tangeretin as a potential oral therapeutic agent is greatly attenuated due to its low oral bioavailability. The hydrophobic chemical structure of tangeretin results in its poor solubility in the aqueous environment of the gastrointestinal tract and reduces the absorption of tangeretin by intestinal enterocytes. Thus, in our previously published research, an optimized novel viscoelastic emulsion (VE) system composed solely of generally recognized as safe (GRAS) ingredients was formulated for the oral delivery of tangeretin (113, 196). It is necessary to evaluate the conceivable changes in toxicity and side effects that could be imposed by the delivery system. Therefore, to assess potential change and accumulation of toxicity produced by utilizing the VE system, a 28-day sub-acute oral toxicity study was conducted using both male and female ICR mice. In addition, to better define the safety profile of tangeretin, the present investigation also included a 14-day acute toxicity study at dose up to 3 g/kg of tangeretin as medium-chain triglyceride (MCT) suspension. To the best of our knowledge, this is the first report on the toxicity of tangeretin as well as the first assessment of sub-acute safety on the emulsion delivery system for dietary bioactives.
Materials and Methods

Materials

Tangeretin of 98% purity was acquired from Quality Phytochemicals, LLC (Edison, NJ, USA). Rapeseed PC75 lecithin was provided by American Lecithin Company (CT, USA). Neobee medium chain triglyceride sample was provided by Stepan Company (Northfield, IL, USA). Hematoxylin and eosin stain were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of tangeretin viscoelastic emulsion

Emulsified tangeretin was produced using the method described in our previously published paper (113). To describe the process briefly, the disperse phase of VE was prepared by placing tangeretin and emulsifier (lecithin) into the carrier oil (100% MCT) that was pre-heated to 130°C. Until all materials were completely solubilized, the disperse phase was immediately cooled to 70°C and the pre-heated aqueous phase (100% double deionized water at 70°C) was added. Subsequently, the mixture was continuously stirred on a heated plate (70°C) until a crude emulsion was formed. To avoid blockage of the high-pressure homogenization device (EmulsiFlex-C6, AVESTIN Inc., Ottawa, Canada), high-speed homogenization (ULTRA−TURRAX T-25 basic, IKA Works Inc., Wilmington, NC, USA) was first applied to the crude emulsion at a speed of 24,000 rpm, which resulted in the reduction of emulsion viscosity. Finally, approximately 25-30 g of stable VE samples were gathered from each processing batch after undergoing pressure treatment at 500 bar under elevated temperature (55°C).
**Loading concentration analysis of tangeretin emulsion**

The loaded emulsified tangeretin concentration was then determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 326 nm. A standard curve from 0.002mg/ml to 0.125 mg/ml tangeretin in ethanol was constructed in triplicate. The loading capacity of tangeretin into the VEs was determined by dispensing a pre-measured VE sample (known weight) into a 10 ml volumetric flask that was subsequently filled with 95% ethanol.

**Experimental animals**

The experiment was conducted using healthy male and female ICR mice acquired from the BioLASCO Experimental Animal Center (Taipei, Taiwan). The mice were 5 to 6 weeks old, with an average weight between 20 to 25 g. All mice were housed at the animal facility of National Kaohsiung Marine University under controlled atmosphere (25 ± 1 °C at 50% relative humidity) with 12 h light/12 h dark cycle. All animals had free access to water and food that was refilled every day. The experimental procedures were authorized by the Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU)

**Oral acute toxicity assay**

After 1 week of acclimation, mice were randomly divided into control and experimental groups receiving different treatment doses. The animals were divided into five groups containing 5 male and 5 female mice. The oral acute toxicity of tangeretin was conducted with procedures according to the Organization for Economic Co-operation and Development (OECD) Guidelines 423. All animals were fasted for 12 hours before dose administration. Taking into account that oil might affect the physiological condition
of the mice, 2 out of 5 groups of mice were used as control groups, in which one of the
group was fed with 200 μL of DI water and the other with 200 μL of MCT. Three dose
levels of tangeretin at 1 g/kg, 2 g/kg, 3 g/kg was administered to mice at equal feed
volume (200 μL). Due to limitations on the loading of VE, tangeretin tested for acute
toxicity was only administered to mice as suspension in MCT. Food was returned to the
animal approximately 2 hours after treatment. Following administration, mice were
closely observed every 30 minutes during the first 6 hours, and, subsequently, checked
daily for 14 days, for signs of toxicity, recovery from toxic effect and incidence of
mortality. Body weights of mice were measured every two days until sacrifice at day 14.

*Oral sub-acute toxicity*

To study sub-acute toxicity, 140 mice were separated into 7 groups, with each
group containing 10 mice of each sex. The oral sub-acute toxicity test was adapted from
the OECD Guidelines 407. Before first dose administration, mice were fasted for 12
hours but were free to drink water if needed. The control group received 0.1 mL of clean
water. To determine the effect of the dosing vehicle itself on the animals, two extra
groups of mice were used as second controls and received either blank MCT or VE
vehicle. In the experimental groups, mice were administered 50 mg/kg or 100 mg/kg of
tangeretin either as MCT suspension or VE. Each mouse from all groups were gavage fed
daily for 28 days and weighed weekly. Food was provided to mice 2 hours after dosing.
Mice were closely observed for the first 3 hours after the very first dose administration
and daily thereafter. Mice were visually observed several times each day for signs of
toxicity and side effects.
Clinical test parameters

At the end of the study, blood samples were collected via cardio puncture after sacrifice using CO₂. To prepare the blood sample for clinical testing, blood samples were mixed with 10 μL of sodium heparin (5000 I.U./ml) and centrifuged for 10 minutes at 5,000 g and 4°C. After centrifuge, the clear plasma sample was carefully withdrawn and stored at –80°C until further analysis. Glutamic-oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), triacylglycerol (TG), total cholesterol (TCHO), and high-density lipoprotein (HDL) were analyzed by an enzyme method using Fuji DRI-CHEM4000 (FUJIFILM) and Fuji DRI-CHEM slides (GOT/AST-P3, GPT/ALT-P3, TG-P3, TCHO-P3, and HDL-P3 FUJIFILM). For the clinical test, 10 μL of plasma was used in each analysis.

Necropsy

Upon sacrifice, the vital organs from each group of the mice were collected. Liver, heart, spleen, lung and kidney were obtained, weighed, photographed, and examined for signs of toxicity. The data (expressed as % body weight) obtained were compared between control groups and experimental groups.

Histopathological analysis

Organs (liver, heart, spleen, lung and kidney) obtained from the mice were fixed in 10% neutral buffered formalin (pH 7.4) overnight and then enclosed in paraffin. Paraffin-encased tissues were sectioned into 4 μm thick slices, fixed onto treated microscope slides, stained with hematoxylin and eosin (H&E), and closed with mounted gel and cover glass. H&E stained tissue slides were then examined and photographed.
using an optical microscope (Leica, DM 1000, Germany). Photos of the specimen were then sent to a pathologist for histopathological analysis.

**Statistical analysis**

Results were expressed as means ± standard deviation (S.D.) for the number of repeated trials indicated for each experiment. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test. Statistical significance was concluded when $p < 0.05$.

**Results**

*Oral acute toxicity of tangeretin*

To date, information on the toxicology profile for bioactives in the polymethoxyflavone family has been still limited. Thus, to better define the potential toxicity effects, an oral acute toxicity study intended to determine the safe dose of tangeretin to be used as a dietary supplement was executed on healthy ICR mice. Due to the fact that tangeretin is a highly crystalline compound at room temperature, the dose higher than 3 g/kg was not able to be homogeneously suspended into the dosing medium (200 μL MCT). Thus, the threeselected dosages of 1, 2, and 3 g/kg of tangeretin were used in our oral acute toxicity assessment.

*Clinical observation and mortality*

Following oral administration, the mice, regardless of which dose was administered, showed a decrease in mobility in the first two hours. Shortly after dosing, groups that received 2 and 3 g/kg of tangeretin showed bristling of body hair, which later disappeared after 4 and 6 hours, respectively. With these exceptions, no other signs of
toxicity or behavior abnormality was observed during the 14-day post-treatment phase. During the observation period, there was no apparent difference in the behavior of food and water consumption between the control and experimental groups. All animals in this experiment survived until the termination of experiment with a normal weight gain pattern, indicating that the limit of dosage that may result in mortality was higher than 3 g/kg. Thus, an LD$_{50}$ value was not estimable in this case, since zero incidence of death was observed.

**Gross observation and analysis of organ and body weight**

Representative gross appearances for all groups of mice are shown in Figure 7.1. When compared with the non-treated controls, macroscopic examination did not suggest any morphological changes in systemic organs between groups of male and female mice. However, a slightly brown-pigmented liver was observed in mice (both sexes) that received 3 g/kg of tangeretin. When expressed in relative values of body weights, no obvious differences in any of the vital organs and were found among all 5 groups of male mice (Table 7.1). In females, the group that received tangeretin at the 3 g/kg level exhibited potential systemic toxicity, showing a consistently lower relative weight of all organs, of which the heart and kidney were significantly lower than in the un-treated control group ($^a p < 0.05$). In particular, a consistently smaller kidney size implied that renal toxicity could occur in higher-dose female mice groups (2 and 3 g/kg). Thus, from gross observation and weight analysis, female mice appeared to be more sensitive than male mice to high-dose acute toxicity.
Figure 7.1 Gross observation of systemic organs (heart, liver, spleen, lung and kidney) from the acute toxicity study, including the untreated control, control treated with blank MCT, 1, 2, 3 g/kg of tangeretin treated mice on the 14th day post-treatment.
Table 7.1 Relative organ weight expressed as percentage of body weight in mice treated with tangeretin.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Blank MCT</th>
<th>1000mg/kg</th>
<th>2000mg/kg</th>
<th>3000mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart (%)</td>
<td>0.53 ± 0.07</td>
<td>0.51 ± 0.09</td>
<td>0.51 ± 0.04</td>
<td>0.52 ± 0.06</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>Liver (%)</td>
<td>5.93 ± 0.67</td>
<td>5.88 ± 0.57</td>
<td>5.94 ± 0.27</td>
<td>6.01 ± 0.36</td>
<td>6.01 ± 1.06</td>
</tr>
<tr>
<td>Spleen (%)</td>
<td>0.36 ± 0.11</td>
<td>0.40 ± 0.07</td>
<td>0.36 ± 0.04</td>
<td>0.36 ± 0.01</td>
<td>0.32 ± 0.05</td>
</tr>
<tr>
<td>Lung (%)</td>
<td>0.81 ± 0.14</td>
<td>0.77 ± 0.10</td>
<td>0.73 ± 0.14</td>
<td>0.70 ± 0.10</td>
<td>0.67 ± 0.08</td>
</tr>
<tr>
<td>Kidney (%)</td>
<td>2.07 ± 0.19</td>
<td>2.01 ± 0.29</td>
<td>2.02 ± 0.21</td>
<td>2.05 ± 0.24</td>
<td>2.02 ± 0.38</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>31.94 ± 9.81</td>
<td>37.44 ± 2.81</td>
<td>37.79 ± 2.72</td>
<td>38.81 ± 2.29</td>
<td>35.46 ± 3.34</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart (%)</td>
<td>0.56 ± 0.04</td>
<td>0.55 ± 0.04</td>
<td>0.52 ± 0.02</td>
<td>0.55 ± 0.06</td>
<td>0.45 ± 0.10ab</td>
</tr>
<tr>
<td>Liver (%)</td>
<td>6.25 ± 0.59</td>
<td>5.85 ± 1.25</td>
<td>5.88 ± 0.65</td>
<td>5.62 ± 0.55</td>
<td>5.70 ± 1.19</td>
</tr>
<tr>
<td>Spleen (%)</td>
<td>0.48 ± 0.07</td>
<td>0.48 ± 0.08</td>
<td>0.47 ± 0.15</td>
<td>0.51 ± 0.12</td>
<td>0.45 ± 0.13</td>
</tr>
<tr>
<td>Lung (%)</td>
<td>0.83 ± 0.10</td>
<td>0.74 ± 0.02</td>
<td>0.77 ± 0.13</td>
<td>0.82 ± 0.08</td>
<td>0.72 ± 0.10</td>
</tr>
<tr>
<td>Kidney (%)</td>
<td>1.61 ± 0.10</td>
<td>1.58 ± 0.21</td>
<td>1.54 ± 0.20</td>
<td>1.41 ± 0.0a</td>
<td>1.41 ± 0.09a</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>25.88 ± 6.05</td>
<td>27.96 ± 3.37</td>
<td>28.70 ± 1.60</td>
<td>25.95 ± 1.52</td>
<td>29.60 ± 2.29</td>
</tr>
</tbody>
</table>

a Significantly different from control (untreated) \( p < 0.05 \)
b Significantly different from control treated with blank MCT \( p < 0.05 \)
n = 5

Histopathology examination

After examining the organs of male and female mice from the control and experimental groups, an alteration in liver structure was observed in all experimental mice groups (Figure 7.2 and 7.3). In male mice, hydropic swelling of hepatic cell was observed for all 3 doses. While mice groups treated with 1 and 2 g/kg of tangeretin maintained a well-defined cell structure, the group that received 3 g/kg of tangeretin showed irregular hepatic cell structure and vacuolated appearance. In female mice, irregular and vacuolated cell shape appeared for all doses. At 2 g/kg, loss of membrane integrity was perceived in conjunction with appreciable spotty necrosis. Moreover, the
complete loss of the regular cell lining structure due to cytoplasmic swelling at the periportal region was observed in microscopic observation of the group that received dose at 3 g/kg. Besides liver, no unfavorable change was perceived in the histological examination when experimental groups were compared with the untreated control group (Figures 7.4 and 7.5). In summary, single oral administration of high-dose tangeretin resulted in liver abnormality, which presented as various degrees of pathological lesions with increased dose-dependent severity. However, even though the kidney was found to be significantly lower in relative weight, this organ did not present perceivable organelle abnormality.
Figure 7.2 Histological observation of the liver from male mice used in the acute toxicity study, including (A) untreated control, (B) control treated with blank MCT, (C) 1 g/kg, (D) 2 g/kg, and (E) 3 g/kg of tangeretin-treated mice on the 14th day post-treatment. The histological morphology of liver was examined using H&E staining and microscopy (200x magnification). Bar = 100 μm.
Figure 7.3 Histological observation of the liver from female mice used in the acute toxicity study, including (A) untreated control, (B) control treated with blank MCT, (C) 1 g/kg, (D) 2 g/kg, and (E) 3 g/kg of tangeretin-treated mice on the 14th day post-treatment. The histological morphology of liver was examined using H&E staining and microscopy (200× magnification). Bar = 100 μm.
**Figure 7.4** Histological observation of the systemic organs (heart, liver, spleen, lung, and kidney) from male mice used in the acute toxicity study, including (A) untreated control, (B) control treated with blank MCT, (C) 1 g/kg, (D) 2 g/kg, and (E) 3 g/kg of tangeretin-treated mice on the 14\textsuperscript{th} day post-treatment. The histological morphology of liver was examined using H&E staining and microscopy (200× magnification). Bar = 100 μm.
Figure 7.5 Histological observation of the systemic organs (heart, liver, spleen, lung, and kidney) from female mice used in the acute toxicity study, including (A) untreated control, (B) control treated with blank MCT, (C) 1 g/kg, (D) 2 g/kg, and (E) 3 g/kg of tangeretin-treated mice on the 14th day post-treatment. The histological morphology of liver was examined using H&E staining and microscopy (200× magnification). Bar = 100 μm.
Clinical biochemistry and serum lipid profile analysis

The effects of high-dose tangeretin (1, 2, and 3 g/kg body weight) on the clinical biochemistry and lipid profile of male and female mice are presented in Table 7.2. While GOT measured from both male and female mice were not meaningfully different between the control and experimental groups, GPT, on the other hand, presented a significant change in male mice fed with tangeretin of 1 g/kg ($^a p < 0.05$), 2 and 3 g/kg ($^A p < 0.01$). Analyzing the serum lipid profile, measurements of TG, TCHO, and HDL from the experimental group were not significantly different among the control groups that did not consume tangeretin. However, a significant increase in the serum TG level was observed in male mice fed with tangeretin at a level of 1 g/kg ($^a p < 0.05$), 2 and 3 g/kg ($^A p < 0.01$). The increase in male serum TG level can most likely be attributed to the ingestion of tangeretin, since the serum TG levels in tangeretin-treated groups (2 and 3 g/kg) were also significantly higher than in the control group fed with blank MCT oil ($^B p < 0.01$), which had a similar TG level as the untreated control group. While the elevation of GPT and TG is a pathological indicator of non-alcoholic fatty liver (197, 198), this result suggested that males, compared with females, are more exposed to the potential risk of non-alcoholic fatty liver development after high-dose tangeretin ingestion.
Table 7.2 Change in the clinical chemistry and lipid profile in mice treated with tangeretin.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Blank MCT</th>
<th>1000mg/kg</th>
<th>2000mg/kg</th>
<th>3000mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GOT (IU/L)</td>
<td>76.2 ± 25.3</td>
<td>109.6 ± 95.0</td>
<td>99.2 ± 83.9</td>
<td>87.6 ± 21.5</td>
<td>113.0 ± 41.8</td>
</tr>
<tr>
<td>GPT (IU/L)</td>
<td>23.2 ± 5.2</td>
<td>32.4 ± 7.5</td>
<td>35.0 ± 7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.8 ± 8.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>41.1 ± 8.3&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>185.8 ± 43.5</td>
<td>189.4 ± 35.2</td>
<td>281.0 ± 45.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>330.2 ± 129.7&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>325.0 ± 61.7&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>TCHO (mg/dL)</td>
<td>187.6 ± 29.0</td>
<td>180.2 ± 30.6</td>
<td>166.2 ± 33.7</td>
<td>170.8 ± 23.5</td>
<td>182.0 ± 19.0</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>144.0 ± 28.6</td>
<td>154.0 ± 38.1</td>
<td>163.4 ± 55.0</td>
<td>161.0 ± 24.8</td>
<td>161.0 ± 23.6</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GOT (IU/L)</td>
<td>81.0 ± 18.4</td>
<td>74.7 ± 5.1</td>
<td>80.3 ± 25.1</td>
<td>86.0 ± 6.6</td>
<td>85.7 ± 35.2</td>
</tr>
<tr>
<td>GPT (IU/L)</td>
<td>30.5 ± 14.0</td>
<td>29.7 ± 5.0</td>
<td>33.3 ± 12.6</td>
<td>29.0 ± 5.3</td>
<td>33.5 ± 12.1</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>366.8 ± 69.5</td>
<td>312.6 ± 42.8</td>
<td>316.0 ± 90.2</td>
<td>260.6 ± 73.8</td>
<td>336.2 ± 78.4</td>
</tr>
<tr>
<td>TCHO (mg/dL)</td>
<td>111.0 ± 25.4</td>
<td>110.8 ± 21.5</td>
<td>117.4 ± 30.2</td>
<td>107.0 ± 29.6</td>
<td>119.3 ± 13.6</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>85.6 ± 17.5</td>
<td>80.4 ± 19.2</td>
<td>87.8 ± 27.8</td>
<td>60.2 ± 32.5</td>
<td>90.6 ± 17.3</td>
</tr>
</tbody>
</table>

<sup>A</sup> Significantly different from control (untreated) *p* < 0.01
<sup>a</sup> Significantly different from control (untreated) *p* < 0.05
<sup>B</sup> Significantly different from control treated with blank MCT *p* < 0.01
<sup>b</sup> Significantly different from control treated with blank MCT *p* < 0.05
n = 5

*Oral sub-acute toxicity of tangeretin ingested as MCT suspension or VE emulsion*

Being a hydrophobic crystalline compound, orally-consumed tangeretin is, in general, poorly absorbed into the systemic circulation and, thus, the potential therapeutic uses are attenuated due to insufficient concentration being accessible at target site of action. Therefore, the use of tangeretin as a dietary supplement is not effective without the incorporation of an oral formulation designed to enhance its bioavailability and resulting efficacy. The optimized emulsified tangeretin was a well-designed delivery system for this purpose. With the use of the VE system, the systemic availability and
anti-cancer efficacy was significantly improved in our animal model when compared with the unformulated MCT suspension. However, while VE increased the dosing efficacy of tangeretin, the enhanced oral bioavailability could also result in a change of the toxicity effect that each dosage had originally posed for the organisms. For instance, 25μg of emulsified tangeretin significantly reduced the relative viability of the hepatic and colonic carcinoma cells, which were not sensitive to 25μg DMSO-solubilized tangeretin (115). Therefore, it is necessary to evaluate the change in the tangeretin toxicity level imposed by using VE as an oral delivery system. Due to the limitation in the loading capacity of VE, only two dosages of 50 and 100 mg/kg were selected, with repeated ingestion daily for 28 days to permit (195) the examination of any side effects, toxic accumulation, and pathological indications.

*Clinical observation and mortality*

Following first ingestion of the designated doses, there was no behavior abnormality or signs of toxicity observed at any time point. However, besides the untreated control group, soft stool was observed from the first 2 days of dose application, potentially due to the mice being unaccustomed to the higher lipid consumption originating from the dosing vehicles. On the third day of application, the soft stool phenomenon diminished, and mice from all groups presented in the normal healthy condition for the remaining testing period. Regardless of the application dose, no other evidence of pathological symptoms was identified after exposure to repeated daily ingestion for 28 days, and all mice survived until the test was terminated. Thus, the dosing levels used in the sub-acute toxicity study were not capable of inducing perceptible toxic effects or mortality on the tested animal population.
**Gross observation and analysis of organ and body weight**

The gross morphology of representative systemic organs from all groups of mice used in this part of the study is presented in Figure 7.6. While no morphological change could be identified in all other organs, livers from the tangeretin-treated groups (50 and 100 mg/kg in MCT suspension or VE) presented a yellow-tinted discoloration. This observation indicates that daily consumption of tangeretin at doses higher than 50 mg/kg could potentially impose a stress to the normal liver anatomy. When looking at the relative weight of systemic organs (Table 7.3), the 28-day sub-acute ingestion of tangeretin did not create a change in heart sizes in either sex, regardless of the dosage level or oral formulations. Even though our observations about the gross morphology of the liver seem problematic, this organ had a relative weight not significantly different among the untreated control and experimental groups in male mice. In contrast, the relative liver weights of female mice increased in all tangeretin-treated groups and were more significant in groups fed with 50 mg/kg in the form of either MCT suspension or VE ($^a p < 0.05$). In addition, as an indication of potential inflammation, larger spleens were observed in male mice that were fed with 50 mg/kg of emulsified tangeretin and 100 mg/kg of tangeretin MCT suspension. Among male mice, significantly heavier relative lung weights than the untreated control group were measured in mice fed with tangeretin in the form of MCT suspension at the 100 mg/kg dosage level ($^a p < 0.01$) and in the form of VE for both 50 and 100 mg/kg doses ($^a p < 0.05$). For the kidney, male mice that received 50 mg/kg tangeretin in both types of oral formulations showed significantly smaller relative weight ($^a p < 0.05$). To summarize, our results from the
analysis of relative organ and body weights showed that males and females responded quite differently to the application of tangeretin dosages as well as to the dosing vehicle used.
Figure 7.6 Gross observation of systemic organs (heart, liver, spleen, lung and kidney) from the sub-acute study, including (A) untreated control, (B) control treated with blank MCT, (C) 50 mg/kg of tangeretin in MCT suspension, (D) 100 mg/kg of tangeretin in MCT suspension, (E) control treated with blank VE, (F) 50 mg/kg of tangeretin in VE, and (G) 100 mg/kg of tangeretin in MCT suspension.
Table 7.3 Relative organ weight expressed as a percentage of body weight in mice treated with tangeretin in MCT suspension or VE.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Blank MCT</th>
<th>Blank emulsion</th>
<th>Tangeretin in MCT suspension</th>
<th>Tangeretin Emulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50mg/kg</td>
<td>100mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50mg/kg</td>
<td>100mg/kg</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart (%)</td>
<td>0.48 ± 0.04</td>
<td>0.48 ± 0.06</td>
<td>0.47 ± 0.04</td>
<td>0.48 ± 0.06</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>Liver (%)</td>
<td>5.61 ± 0.70</td>
<td>5.62 ± 1.25</td>
<td>5.56 ± 0.49</td>
<td>6.04 ± 0.38</td>
<td>5.32 ± 0.41</td>
</tr>
<tr>
<td>Spleen (%)</td>
<td>0.30 ± 0.05</td>
<td>0.32 ± 0.05</td>
<td>0.33 ± 0.05</td>
<td>0.34 ± 0.04</td>
<td>0.37 ± 0.06*a</td>
</tr>
<tr>
<td>Lung (%)</td>
<td>0.58 ± 0.08</td>
<td>0.58 ± 0.07</td>
<td>0.58 ± 0.06</td>
<td>0.64 ± 0.04</td>
<td>0.67 ± 0.09A</td>
</tr>
<tr>
<td>Kidney (%)</td>
<td>2.21 ±0.16</td>
<td>2.12 ± 0.46</td>
<td>2.04 ± 0.33</td>
<td>1.95 ± 0.21*a</td>
<td>1.98 ± 0.22</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>39.79 ± 3.31</td>
<td>37.74 ± 2.51</td>
<td>37.96 ± 3.00</td>
<td>37.22 ± 3.31*a</td>
<td>37.69 ± 2.25</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart (%)</td>
<td>0.52 ± 0.07</td>
<td>0.49 ± 0.08</td>
<td>0.49 ± 0.09</td>
<td>0.49 ± 0.04</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>Liver (%)</td>
<td>4.95 ± 0.80</td>
<td>5.07 ± 0.68</td>
<td>4.84 ± 0.45</td>
<td>5.77 ± 0.52Ab</td>
<td>5.21 ± 0.48</td>
</tr>
<tr>
<td>Spleen (%)</td>
<td>0.42 ± 0.13</td>
<td>0.43 ± 0.10</td>
<td>0.44 ± 0.06</td>
<td>0.44 ± 0.10</td>
<td>0.43 ± 0.09</td>
</tr>
<tr>
<td>Lung (%)</td>
<td>0.77 ± 0.10</td>
<td>0.78 ± 0.20</td>
<td>0.76 ± 0.03</td>
<td>0.70 ± 0.07</td>
<td>0.79 ± 0.08</td>
</tr>
<tr>
<td>Kidney (%)</td>
<td>1.47 ± 0.14</td>
<td>1.50 ± 0.18</td>
<td>1.49 ± 0.16</td>
<td>1.41 ± 0.13</td>
<td>1.51 ± 0.13</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>29.59 ± 2.52</td>
<td>28.93 ± 3.11</td>
<td>28.93 ± 2.37</td>
<td>29.44 ± 1.88</td>
<td>29.52 ± 1.97</td>
</tr>
</tbody>
</table>

A Significantly different from control (untreated) p < 0.01
a Significantly different from control (untreated) p < 0.05
B Significantly different from control treated with blank MCT p < 0.01
b Significantly different from control treated with blank MCT p < 0.05
C Significantly different from control treated with blank emulsion p < 0.01
c Significantly different from control treated with blank emulsion p < 0.05
n = 10
Histopathology examination

The microscopic analysis of systemic organs in the mouse, including the heart, liver, spleen, lung, and kidney, revealed that the liver was the only affected organ after repeated treatments with tangeretin, with various degrees of cell disruption observed in regard to different dosage levels and types of oral formulations (Figures 7.9 and 7.10). While groups treated with blank MCT oil and VE showed no change in hepatic cell morphology, tangeretin-treated groups resulted in an irregular increase of cytoplasmic volume and vacuolated-appearance (Figures 7.7 and 7.8). The highest severity was observed in male and female mice fed with 50 mg/kg of tangeretin in MCT oil, followed by 100 mg/kg in MCT oil, and 50 and 100 mg/kg of emulsified tangeretin, respectively. That is, regardless of gender, the observable pathological indication was less severe in emulsified tangeretin treated groups than in MCT suspension. Densely-packed homogeneous cytoplasm in the perivenous region was observed in male mice treated with 50 mg/kg of emulsified tangeretin and in female mice treated with 50 mg/kg of tangeretin MCT suspension. Necrosis was also observed in female mice treated with 50 mg/kg of tangeretin in both oral formulations. The histological findings indicate that tangeretin is capable of causing metabolic stress to the liver, which can be well correlated with the larger relative liver weights measured. From the pathological analysis, female mice showed more sensitivity to the side effects of sub-acute ingested tangeretin imposed on the liver than male mice.
Figure 7.7 Histological observation of the liver from male mice used in the subacute toxicity study, including (A) untreated control, (B) control treated with blank MCT, (C) 50 mg/kg of tangeretin in MCT suspension, (D) 100 mg/kg of tangeretin in MCT suspension, (E) control treated with blank VE, (F) 50 mg/kg of tangeretin in VE, and (G) 100 mg/kg of tangeretin in MCT suspension. The histological morphology of liver was examined using H&E staining and microscopy (200× magnification). Bar = 100 μm.
Figure 7.8 Histological observation of the liver from female mice used in the subacute toxicity study, including (A) untreated control, (B) control treated with blank MCT, (C) 50 mg/kg of tangeretin in MCT suspension, (D) 100 mg/kg of tangeretin in MCT suspension, (E) control treated with blank VE, (F) 50 mg/kg of tangeretin in VE, and (G) 100 mg/kg of tangeretin in MCT suspension. The histological morphology of liver was examined using H&E staining and microscopy (200× magnification). Bar = 100 μm.
**Figure 7.9** Histological observation of the systemic organs (heart, liver, spleen, lung, and kidney) from male mice used in the subacute toxicity study, including (A) untreated control, (B) control treated with blank MCT, (C) 50 mg/kg of tangeretin in MCT suspension, (D) 100 mg/kg of tangeretin in MCT suspension, (E) control treated with blank VE, (F) 50 mg/kg of tangeretin in VE, and (G) 100 mg/kg of tangeretin in MCT suspension. The histological morphology of liver was examined using H&E staining and microscopy (200× magnification). Bar = 100 μm.
Figure 7.10 Histological observation of the systemic organs (heart, liver, spleen, lung, and kidney) from female mice used in the subacute toxicity study, including (A) untreated control, (B) control treated with blank MCT, (C) 50 mg/kg of tangeretin in MCT suspension, (D) 100 mg/kg of tangeretin in MCT suspension, (E) control treated with blank VE, (F) 50 mg/kg of tangeretin in VE, and (G) 100 mg/kg of tangeretin in MCT suspension. The histological morphology of liver was examined using H&E staining and microscopy (200× magnification). Bar = 100 μm.
Clinical biochemistry and serum lipid profile analysis

The result from our clinical biochemistry analysis of the 28-day sub-acute toxicity study is summarized in Table 7.4. Repeated sub-acute ingestion of tangeretin in either MCT suspension or VE emulsion at 50 and 100 mg/kg did not result in changes of the hepatic enzyme profile in experimental male mice. However, in female mice, significantly increased GPT values were measured in mice dosed with 50 mg/kg of tangeretin of both oral formulations ($^\Lambda p < 0.01$, $^B p < 0.01$, and $^C p < 0.01$). In female mice, serum lipid profile was found to be more responsive to the dosage level than to different oral formulations, since the result showed significantly amplified TG and TCHO concentrations in mice treated with tangeretin at the dosing level of 50 mg/kg ($^\Lambda p < 0.01$ for TG and $^a p < 0.05$ for TCHO), regardless of which formulation was used. Among serum lipid parameters in male mice, TCHO showed no significant change in most experimental groups. The male group treated with 100 mg/kg of tangeretin as MCT suspension had significantly lower serum TG ($^a p < 0.05$). Compare to the untreated control group, the HDL values decreased significantly in male groups fed with tangeretin of 50 mg/kg in VE and 100 mg/kg in MCT suspension ($^\Lambda p < 0.01$). However, HDL values were significantly higher in female mice treated with 100 mg/kg of tangeretin as MCT suspension. To summarize, differences in gender and types of oral formulations produced large variations in serum biochemistry values.
Table 7.4 Change in the clinical chemistry and lipid profile in mice treated with tangeretin in MCT suspension or VE.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Blank MCT</th>
<th>Blank Emulsion</th>
<th>Tangeretin in MCT suspension</th>
<th>Tangeretin Emulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50mg/kg</td>
<td>100mg/kg</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GOT (IU/L)</td>
<td>95.8 ± 70.1</td>
<td>119.7 ± 67.6</td>
<td>119.3 ± 70.6</td>
<td>125.1 ± 60.0</td>
<td>90.7 ± 42.9</td>
</tr>
<tr>
<td>GPT (IU/L)</td>
<td>28.3 ± 7.3</td>
<td>30.9 ± 12.5</td>
<td>30.9 ± 6.8</td>
<td>29.9 ± 6.5</td>
<td>26.3 ± 12.9</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>254.6 ± 80.3</td>
<td>250.3 ± 136.9</td>
<td>262.1 ± 73.5</td>
<td>210.6 ± 38.1</td>
<td>184.8 ± 33.4a</td>
</tr>
<tr>
<td>TCHO (mg/dL)</td>
<td>159.7 ± 29.6</td>
<td>154.8 ± 34.1</td>
<td>194.3 ± 46.3</td>
<td>170.0 ± 56.3</td>
<td>128.3 ± 15.3</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>144.3 ± 22.6</td>
<td>133.5 ± 38.8</td>
<td>176.0 ± 28.1a</td>
<td>139.0 ± 33.7</td>
<td>91.5 ± 18.6A</td>
</tr>
<tr>
<td><strong>Females</strong></td>
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<tr>
<td>GOT (IU/L)</td>
<td>114.3 ± 79.3</td>
<td>116.2 ± 20.0</td>
<td>123.8 ± 49.4</td>
<td>151.7 ± 40.2</td>
<td>125.3 ± 81.5</td>
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<tr>
<td>GPT (IU/L)</td>
<td>20.3 ± 7.2</td>
<td>23.6 ± 3.6</td>
<td>20.5 ± 9.0</td>
<td>63.3 ± 10.3AB</td>
<td>23.8 ± 10.6</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>173.2 ± 49.6</td>
<td>202.9 ± 41.8</td>
<td>173.6 ± 18.8</td>
<td>271.3 ± 37.2AB</td>
<td>213.2 ± 41.5</td>
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<tr>
<td>TCHO (mg/dL)</td>
<td>85.1 ± 36.3</td>
<td>105.0 ± 16.3</td>
<td>90.6 ± 20.9</td>
<td>110.3 ± 19.2a</td>
<td>111.4 ± 31.8</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>53.5 ± 26.5</td>
<td>64.0 ± 13.3</td>
<td>51.5 ± 13.6</td>
<td>56.0 ± 16.5</td>
<td>75.0 ± 25.6a</td>
</tr>
</tbody>
</table>

A Significantly different from control (untreated) p < 0.01
a Significantly different from control (untreated) p < 0.05
B Significantly different from control treated with blank MCT p < 0.01
b Significantly different from control treated with blank MCT p < 0.05
C Significantly different from control treated with blank emulsion p < 0.01
c Significantly different from control treated with blank emulsion p < 0.05
n = 10
Discussion

As numerous health-promoting bioactive compounds have been identified from a variety of dietary sources, the increasing demand for these functional ingredients has prompted the need for evaluation of not only the efficacy of these compounds, but also of related safety issues. Recently, a group of citrus-derived bioactives, polymethoxyflavones (PMFs), has drawn much attention in the field of functional food due to their reported wide-ranging biological functionalities. Thus, the introduction of these compounds as a dietary supplement to potential consumers will require much more extensive evaluations for their safety profile, which has not yet been evaluated.

Tangeretin, as one of the most abundant PMFs, is still far from being used as oral supplement because of its poor bioavailability, insufficient efficacy, and lack of safety characterization. The result from the previous work indicated that the inclusion of oral formulation could be an oral dosing solution for hydrophobic compounds with similar chemical properties to tangeretin. To extend those findings, the present study looked more closely into the safety profile of tangeretin itself and the potential alteration in the level of adverse effects that may be caused by the oral delivery formulation.

In our acute toxicity study, oral administration of tangeretin in doses from 1 to 3 g/kg did not induce mortality in male and female mice. The effect of ingesting large doses of tangeretin was sub-lethal and adaptable, since the physical abnormality observed in mice that received higher doses disappeared a few hours after dosing. However, further analysis on the gross morphology and histopathology of systemic organs found liver abnormality for all doses. This side effect was further confirmed by the clinical chemistry and lipid profile analysis, from which significant changes in the serum GPT and TG
levels were measured in male mice. However, the effect of tangeretin overdose on the liver and its functionality could be attributed more to the adaptation response than to toxicity. In previously published literature, tangeretin was found to be a substrate of the liver microsomal enzyme, cytochrome P450 (CYP450), which is extensively demethylated (199-201). Moreover, tangeretin and its resulting metabolites were also found to be potent inhibitors of CYP450(158, 202, 203), which is the major hepatic enzyme for metabolizing xenobiotics and other endogenous substrates (42, 204, 205), such as steroids.

When a larger dose of tangeretin was ingested and reached the liver through the portal system, the increased demand for metabolism by CYP450 prompted the liver to increase its capacity for the purpose of responding to this higher stress level (157). As a result, organ enlargement and hepatocellular hypertrophy then appeared proportional to the level of severity of metabolic stress. The observation that greater relative liver weight was measured in male mice with higher serum GOT, GPT, and TG concentrations again correlated with the pathological diagnosis. Moreover, CYP450 is a sex-dependent hepatic enzyme, which shows greater expression in female mice (206). In agreement with this sexual dimorphism finding, female mice presented no significant change in the clinical biochemistry evaluation and lipid profile, suggesting better adaptation to the overdosing shock, which can still be detected by looking at the morphological and structural changes in the hepatocytes.

While our acute toxicity study concluded that tangeretin could impose a metabolic stress to the liver, our sub-acute toxicity study evaluated the adverse effects that may occur over 28 days with repeated lower doses of 50 and 100 mg/kg. To elucidate the effect of oral formulation on the toxicity reaction, tangeretin was administered to the
mice after incorporation to the VE and was compared with the unformulated MCT suspension. The result showed that the VE system itself was safe for oral administration to mice, since it does not generate significant adverse changes in physiological status. However, the fact that the VE system enhanced the oral absorption of tangeretin 2.3-fold could involve a change in toxicity response when an identical concentration is ingested.

Instead of a linear dose-dependent reaction, the sub-acute treatment with tangeretin over 28 days showed a hormesis dose-response effect, in which a lower dose induced more hepatic side effects than the higher dose. The term, hormesis, was generally used to described the situation that a lower dose induces while a higher dose inhibits the pathological symptom (207). Compared to the linear dose-responsive curve, bioactive compounds that exhibit hormesis-related behavior require more plannings for safety evaluations, since most toxicity studies mainly focus on the side effects from higher dosages. That is, bioactives that exert no adverse effect at high doses may still be toxic at lower doses. This phenomenon has driven the establishment of lowest observed adverse effect levels (LOAELs), in addition to the traditional no observed adverse effect levels (NOAEL) for regulatory requirements (208).

According to the results obtained from the sub-acute study, mice treated with 50 mg/kg of tangeretin exhibited more severe pathological symptoms than the higher dosage from the same oral formulations. In clinical chemistry analysis, 50 mg/kg of tangeretin administered either as MCT suspension or VE formulation to female mice induced significant changes in the serum GPT and TG levels, indicating the impairment of lipid metabolism. In addition, vacuolated cytoplasm and irregular cell arrangement due to lipid accumulation in the hepatocytes was shown, with descending pathological severity in
order of 50 mg/kg MCT suspension, 100 mg/kg MCT suspension, 50 mg/kg VE, and 100 mg/kg VE. The effect of the dosing vehicle on pathological results could be excluded since the control groups fed with blank MCT or VE showed no significant adverse effects. Thus, to explain the difference in the pathological development among different treatments, the concentration that was absorbed by enterocytes and reached the liver should be the main point for consideration.

Since unformulated tangeretin in the MCT suspension is poorly absorbed, ingesting higher dosages of tangeretin in this type of oral formulation may induce only a minimal increase in the system concentration. Therefore, the tangeretin concentration that might reach the liver from ingesting oral treatments used in this study was expected to be the highest from 100 mg/kg VE, followed by 50 mg/kg VE, 100 mg/kg MCT suspension, and, last, 50 mg/kg MCT suspension. When comparing this approximation to the pathological severity order from the histological analysis, one can perfectly explain it with the hormesis phenomenon, in which lower doses induce hepatic stress, while higher doses may protect the liver from injury. In the sub-acute study, the occurrence of hormesis was consistent among different genders. However, a study using a wider dosing range is required before a definite safety profile of tangeretin can be concluded.

Even though two studies cannot be compared equally, a U-shaped dose-response phenomenon could still be predicted according to the results from both the acute and sub-acute studies. The U-shaped curve denotes a biphasic dose-response behavior that is in agreement with the theory of hormesis (208, 209). That is, the bottom of the U-shaped curve represents the dose showing the lowest adverse effect, and the curve increases or decreases as dosing concentrations raise the potential toxicity. Again, to determine the
actual safety profile of tangeretin, a more complete range of dosages, from high to low doses, should be used for the toxicity evaluation.

**Conclusion**

The administration of high-dose tangeretin to mice in acute toxicity induced sub-lethal liver side effects in a positive dose-dependent relationship. However, the adverse effects in the liver produced by over-dosing were both adaptable and reversible. In our sub-acute study, the interference of tangeretin with the hepatic metabolic activity was further confirmed by observed changes in clinical chemistry and serum lipid profile. Using VE as an oral delivery system increased the bioavailable concentration of tangeretin, but did not create additional side effects in the mice. The injury to the hepatocytes by repeated dosing was highly dependent on the concentration of tangeretin that reached the liver and exhibited a hormesis-related dose-response phenomenon. However, the hormesis phenomenon was unexpected and will need to be confirmed by further investigation using a wider dosing range. The lessons learned from the present investigation are that safety evaluations need to be conducted using a wider range of dose levels, and that the establishment of both NOAELs and LOAELs is of equal importance to high-dosage toxicity investigations, to ensure the safety of using bioactive ingredients.
SUMMARY AND FUTURE WORK

As many bioactive compounds have been isolated from dietary sources, much research has been dedicated to discover its potential biological functionalities of these compounds, which may ultimately be used for promoting better health in humans. Many of the bioactives found in natural sources are intended to be used to prevent the development of chronic disease. Thus, for long-term intake, most people prefer to consume these bioactive compounds through oral ingestion rather than other delivery routes.

However, most of these bioactives generally have poor oral bioavailability, due to the limitations imposed by their specific chemical structure. To improve oral dosing efficacy, many types of oral formulations and delivery systems have been developed using a variety of technologies. However, while much research has emphasized the development of novel technologies that could be used to orally deliver bioactives, very few of these studies have tested those delivery systems in actual biological settings to realistically evaluate the effectiveness of these approaches to improve the systemic bioavailability, which subsequently may lead to improved therapeutic efficacies.

The effectiveness of many oral delivery systems to enhance the bioavailability and efficacy of targeted compounds in real biological settings had been unknown. Therefore, this dissertation intended to eliminate all of the complexity of oral delivery systems and to use the most basic emulsion-based vehicle to systematically evaluate the potential efficacy of oral formulations to enhance the physiochemical properties of dietary bioactives. In this work, an emulsion-based system was utilized for the oral delivery of tangeretin. As a newly emerging bioactive, tangeretin is a highly crystalline hydrophobic compound that exhibits low solubility in either water or oil at room
temperature. Thus, a viscoelastic property was included in the optimized emulsion system for higher loading capacity. The loading of tangeretin in the viscoelastic emulsion system at levels higher than 2.5% was satisfactory for subsequent animal studies. This optimized formulation was then proven by the animal models as a very effective method to enhance the oral bioavailability and anti-cancer efficacy of tangeretin. However, the toxicity of tangeretin could increase due to the better dosing efficacy provided by utilization of the emulsion-based delivery system.

To summarize the results from this work, we may conclude that the emulsion-based delivery system was effective to enhance the dosing efficacy of tangeretin, but may require a separate toxicity evaluation for determining the safe level of usage. Overall, this work can serve as a good reference for future investigations that aim to systematically evaluate the interaction of delivery formulations with biological systems.

Meanwhile, there is definitely much future research that could be performed to extend the current study. First of all, toxicity evaluation of a wider dosing range should be conducted to determine the safety profile of tangeretin and the effect of this oral formulation, before application of this technique should be considered for consumer-ready products. Once the safety issue is fully addressed, employment of an emulsion-based delivery method for dietary uses of functional bioactives could be a convenient and cost-effective solution for disease prevention. A second area of research that could extend this work is the incorporation of other novel processing methods, such as nanotechnology and surface modification, for further improvement of the efficacy of such a system, in order to increase its biological functionalities. Another way to expand on this work is to apply this method to other bioactive compounds, which may have similar
properties and limitations. By doing so, this method could be generalized for future compound delivery. In conclusion, there is much more potential to further investigate the work performed in this dissertation. With the effort of future studies, more efficient and safer oral delivery systems could be developed for the improvement of human health.
REFERENCES


**APPENDIX: LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AOM</td>
<td>Azoxymethane</td>
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<tr>
<td>COX-2</td>
<td>Cyclooxygenase - 2</td>
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<tr>
<td>CRC</td>
<td>Colorectal Cancer</td>
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<tr>
<td>CYP</td>
<td>Cytochrome Protein</td>
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<tr>
<td>DI</td>
<td>Deionized Water</td>
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<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
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<tr>
<td>DSS</td>
<td>Dextran Sodium Sulphate</td>
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<tr>
<td>EGCG</td>
<td>(−)-Epigallocatechin Gallate</td>
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<tr>
<td>FA</td>
<td>Fatty Acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food Drug Administration</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GOT</td>
<td>Glutamic-Oxaloacetic Transaminase</td>
</tr>
<tr>
<td>GPT</td>
<td>Glutamic Pyruvic Transaminase</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally Recognized As Safe</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<tr>
<td>HDL</td>
<td>High-Density Lipoprotein</td>
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<tr>
<td>HMF</td>
<td>Heptamethoxyflavone</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>High Pressure Valve Homogenizer</td>
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<td>HSH</td>
<td>High Speed Homogenizer</td>
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<td>IBD</td>
<td>Inflammatory Bowel Diseases</td>
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<td>IL-6</td>
<td>Interleukin - 6</td>
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<tr>
<td>LOAEL</td>
<td>Lowest Observed Adverse Effect Level</td>
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<tr>
<td>MCT</td>
<td>Medium Chain Triglyceride</td>
</tr>
<tr>
<td>MDD</td>
<td>Mean Droplet Diameter</td>
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<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
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<tr>
<td>MG</td>
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<tr>
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<td>Medium Chain Triglyceride Suspension</td>
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<tr>
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<td>3-(4,5-Dimethylthiazol-2-Yl)-2,5-</td>
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<tr>
<td></td>
<td>Diphenyltetrazolium Bromide</td>
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<tr>
<td>Na TDC</td>
<td>Sodium Taurodeoxycholate</td>
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<td>NOAEL</td>
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</tr>
<tr>
<td>O/W</td>
<td>Oil in Water</td>
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<tr>
<td>OECD</td>
<td>Organization For Economic Co-Operation and</td>
</tr>
<tr>
<td></td>
<td>Development</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
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<td>Photon Correlation Spectroscopy</td>
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<td>Polymethoxyflavone</td>
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<td>S.D.</td>
<td>Standard Deviation</td>
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<td>Description</td>
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<tr>
<td>SEDDS</td>
<td>Self-Emulsifying Drug Delivery Systems</td>
</tr>
<tr>
<td>SLN</td>
<td>Solid Lipid Nanoparticle</td>
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<tr>
<td>ST</td>
<td>Sulfotransferase</td>
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<tr>
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<td>Total Cholesterol</td>
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<tr>
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