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Viscoelastic emulsion improved the bioaccessibility and oral bioavailability of crystalline compound: A mechanistic study using *in vitro* and *in vivo* models

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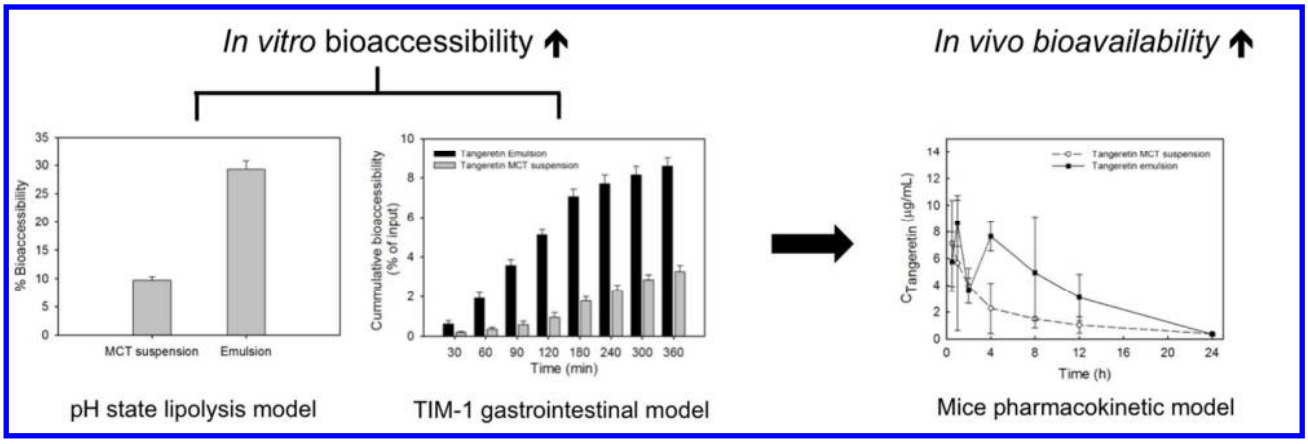

Abstract The oral bioavailability of hydrophobic compound is usually limited by the poor aqueous solubility in the gastrointestinal (GI) tract. Various oral formulations were developed to enhance the systemic concentration of such molecules. Moreover, compounds with high melting temperature that appear as insoluble crystals imposed great challenge to the development of oral vehicle. Polymethoxyflavone, an emerging category of bioactive compounds with potent therapeutic efficacies, were characterized as having a hydrophobic and highly crystalline chemical structure. To enhance the oral dosing efficiency of polymethoxyflavone, a viscoelastic emulsion system with a high static viscosity was developed and optimized using tangeretin, one of the most abundant polymethoxyflavones found in natural sources, as modeling compound. In the present study, different *in vitro* and *in vivo* models were used to mechanistically evaluate the effect of emulsification on oral bioavailability of tangeretin. *In vitro* lipolysis revealed that emulsified tangeretin was digested and became bioaccessible much faster than unprocessed tangeretin oil suspension. By simulating the entire human GI tract, TNO's gastrointestinal model (TIM-1) is a valuable tool to mechanistically study the effect of emulsification on the digestion events that lead to a better oral bioavailability of tangeretin. TIM-1 result indicated that tangeretin was absorbed in the upper GI tract. Thus, a higher oral bioavailability can be expected if the compound becomes bioaccessible in the intestinal lumen soon after dosing. *In vivo* pharmacokinetics analysis on mice again confirmed that the oral bioavailability of tangeretin increased 2.3 fold when incorporated in the viscoelastic emulsion than unformulated oil suspension. By using the combination of *in vitro* and *in vivo* models introduced in this work, the mechanism that underlie the effect of viscoelastic emulsion on the oral bioavailability of tangeretin was well elucidated.

Key words: tangeretin/ viscoelastic emulsion/ pharmacokinetic/ TIM-1/ *in vitro* lipolysis/ bioaccessibility/ bioavailability

1. Introduction

Tangeretin is one of the major polymethoxyflavones (PMFs) found in the peel of numerous citrus fruits [1, 2]. With its methoxy functional groups on the flavonoid backbone, tangeretin, similar to many other members in the polymethoxyflavone class, is a potential bioactive agent that is capable of reducing the risk of degenerative diseases. According to many of the previous published literatures, tangeretin is documented to have a wide array of biological functionalities including anti-inflammation [3], anti-tumorigenesis [4-8], neuroprotective effects [9], metabolic modulations [10-12], and protection against cardiovascular diseases [13-15]. In particular, due to its selective growth inhibition on the carcinoma cells [8, 16], 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 [17]. 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 unavailability 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 systemic concentration. Hydrophobic compounds such as tangeretin were found to be better absorbed when ingested with lipid [18-20], 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 [21]. The aqueous solubility and *in vitro* anti-cancer proliferation of tangeretin were significantly improved when processed into the VE formulation [21, 22].

To further elucidate the role of emulsion-based delivery systems and associated factors that contribute to the oral bioavailability, this work aims to mechanistically study the pre-absorption events using the *in vitro* lipolysis assay and the dynamic gastrointestinal simulating model (TIM-1). The *in vivo* pharmacokinetic of tangeretin was then assessed using an animal model. This work provides the systematic 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 formulated with lipidic carriers. Thus, the methodology used in this study can be used as good reference for future elucidation of mechanism that underlies the effect of delivery system on the oral bioavailability of bioactive ingredients.

2. Materials and Methods

2.1. 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.

2.2. 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 ^{o}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 valve 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 (50.4% MCT, 0.5% lecithin, 47% DI water, and 2.1% of tangeretin) were collected from each processing batch.

2.3. 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 (0.1g) into a 10 ml volumetric flask and filled with 95% ethanol.

2.4. In Vitro Lipolysis of PMFs in Emulsion or MCT Suspension

The *in vitro* lipolysis study was carried out using our method previously published [22]. 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, $CaCl_2 \cdot H_2O$, 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 micelle containing layer (usually the layer in between the undigested oil layer and solid precipitant) of the 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 [23] using the equation below:

$$\%\ Bioaccessibility = \frac{Total\ mass\ of\ solubilized\ PMFs}{Total\ mass\ of\ PMF\ in\ original\ lipid\ samples} \times 100\% \quad (1)$$

To determine the change in the digestion kinetics after emulsion processing, the extent of lipolysis at 30 min was compared between MCT suspension (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 [22]:

$$Extent\ of\ lipolysis = \frac{Volume\ of\ \ NaOH \times Conc.of\ NaOH}{2 \times mol\ of\ triglyceride} \times 100\% \quad (2)$$

2.5. 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 [24]. 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 of the fed state digestion fluids according to the previously published method [24].

To compare the bioaccessibility of tangeretin in 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.

2.6. 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).

2.7. 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 CO_2 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.

Peak concentration (C_{max}) and time to peak concentration (T_{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_{el}) was obtained from the terminal linear regression slope of logarithmic-transformed plasma concentration-time curves.

2.8. 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 [25]. 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.

2.9. 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 suspension and emulsion tangeretin. Statistical significance was concluded when $p < 0.05$.

3. Results

3.1. Characterization of tangeretin viscoelastic emulsion

In our previous study, the VE system was optimized for oral delivery of tangeretin [21]. 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, thus, the bioacessibility 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.

3.2. Comparison of in vitro lipolysis profiles between tangeretin MCT suspension and its 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 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 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 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.

3.3. 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 tangeretin samples were fed to the TIM-1 system, the portion that is considered bioaccessible for absorption was collected from the jejunum and ileum section with filtration that allow only solubilized compound to get through. On the other hand, the portion not is regard as not bioaccessible or simply not absorbed in the GI tract was also collected without further filtering from the efflux outlet. Since the TIM-1 system does not include the lower GI compartment (colon), the compound collected from efflux was considered lost from the unavailable for absorption. The sample collected from each section was analyzed by HPLC to determine the tangeretin concentration (Table 1).

The bioaccessibility of tangeretin determined by TIM-1 were expressed as the concentration of tangeretin recovered from both the jejunum and ileum compartments in relative to the original input weight. The bioaccessibility of tangeretin increased 2.6 fold when it was incorporated to the VE system than in the MS. The accumulated tangeretin bioaccessibility (jejunum and ileum) expressed as percent of input was then plotted as a function of time, as shown in Figure 3. According to the kinetic profile, the rate of tangeretin become bioaccessible was highest in the first 3 hours after dosing and gradually slowed down thereafter. To better understand the mechanism of tangeretin absorption, the kinetic of accumulative bioaccessibility was then individually analyzed for jejunum and ileum (figure 4A and 4B). This mechanistic examination suggested that the absorption of tangeretin majorly happened in the upper jejunum section and was continuously eliminated from the system through efflux. The result from the TIM-1 gastrointestinal study

indicated that faster dissolution rate in the intestinal lumen could be great beneficial for achieving higher oral bioavailability. Moreover, higher efflux recovery rate of tangeretin was also observed from feeding emulsified sample (figure 4C) suggesting that VE not only induced better bioaccessibility of tangertin but also faster GI clearance rate. This observation was again confirmed when total recovered tangeretin was 3.5 times higher when dosing with emulsified samples than with unformulated sample. More than 80% of the total recovered tangeretin from feeding VE was obtained within the first few hours, indicating that the emulsion-based delivery system could facilitate rapid absorption through enhancing the solubility and bioaccessibility of hydrophobic compounds.

3.4. 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, and pharmacokinetic parameters are summarized in Table 2.

Administration of VE resulted in a delayed T_{max} at 1 hour after oral administration, while T_{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[26, 27]. 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_{max} value, the VE formulation gave a C_{max} value of 8.7 ± 1.7 μg/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 AUC_{0-24} value for VE and MS were 83.0 and 35.5 μg/mL*hr, respectively. At 24 hrs, the level of tangeretin plasma concentration for both VE and MS decreased to 0.37 μg/mL with Kel 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 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 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 CYP450 [10, 11]. 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.

4. Discussion

In this study, VE was used as a carrier system for oral delivery of hydrophobic crystalline compound, 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 [28]. Here, we used two *in vitro* digestion studies as well as *in vivo* pharmacokinetic analysis to examine the mechanism that underlie the effect of VE system on the bioavailability of tangeretin.

The *in vitro* lipolysis model is an effective tool to examine the bioaccessibility of hydrophobic components [23, 29]. 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 [30, 31]. 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 ± 3.2 and 8.7 ± 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 down regulated when larger amounts of tangeretin reach the metabolic facilities.

When assimilating all of the presented analysis, despite their limitations, the *in vitro* methods used in this work were useful in providing prediction of *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 [17]. The difference could be attributed to the lower solubility of tangeretin in corn oil [32] and physiological variability between animal species.

5. 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 *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 *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. It should be noted that even though viscoelastic emulsion (VE) based formulations showed improved oral bioavailability and bioactivities than the inferior suspension formulations, the VE formulations still receive less

attention due to their limited practical interest in pharmaceutical industry. More research is needed on the development of spray-dryable formulations that can be reconstituted into emulsions when water is added. In addition, 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.

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References

1. Li, S. M.; Lo, C. Y.; Ho, C. T. Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (Citrus sinensis) peel. *J. Agric. Food Chem.* **2006,** *54*, 4176-4185.
2. Manthey, J. A.; Grohmann, K. Phenols in citrus peel byproducts. Concentrations of hydrogycinnamates and polymethoxylated flavones in citrus peel molasses. *J. Agric. Food Chem.* **2001,** *49*, 3268-3273.
3. Chen, K. H.; Weng, M. S.; Lin, J. K. Tangeretin suppresses IL-1 beta-induced cyclooxygenase (COX)-2 expression through inhibition of p38 MAPK, JNK, and AKT activation in human lung carcinoma cells. *Biochem. Pharmacol.* **2007,** *73*, 215-227.
4. Hirano, T.; Abe, K.; Gotoh, M.; Oka, K. Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. *Br. J. Cancer* **1995,** *72*, 1380-8.
5. Pan, M. H.; Chen, W. J.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. Tangeretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinases 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells. *Carcinogenesis* **2002,** *23*, 1677-84.
6. Rooprai, H. K.; Kandanearatchi, A.; Maidment, S. L.; Christidou, M.; Trillo-Pazos, G.; Dexter, D. T.; Rucklidge, G. J.; Widmer, W.; Pilkington, G. J. Evaluation of the effects of swainsonine, captopril, tangeretin and nobiletin on the biological behaviour of brain tumour cells in vitro. *Neuropathol. Appl. Neurobiol.* **2001,** *27*, 29-39.
7. Arafa el, S. A.; Zhu, Q.; Barakat, B. M.; Wani, G.; Zhao, Q.; El-Mahdy, M. A.; Wani, A. A. Tangeretin sensitizes cisplatin-resistant human ovarian cancer cells through downregulation of phosphoinositide 3-kinase/Akt signaling pathway. *Cancer Res.* **2009,** *69*, 8910-7.
8. Morley, K. L.; Ferguson, P. J.; Koropatnick, J. Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. *Cancer Lett.* **2007,** *251*, 168-78.
9. Datla, K. P.; Christidou, M.; Widmer, W. W.; Rooprai, H. K.; Dexter, D. T. Tissue distribution and neuroprotective effects of citrus flavonoid tangeretin in a rat model of Parkinson's disease. *Neuroreport* **2001,** *12*, 3871-5.
10. CanivencLavier, M. C.; Bentejac, M.; Miller, M. L.; Leclerc, J.; Siess, M. H.; Latruffe, N.; Suschetet, M. Differential effects of nonhydroxylated flavonoids as inducers of cytochrome P450 1A and 2B isozymes in rat liver. *Toxicol. Appl. Pharm.* **1996,** *136*, 348-353.

11. Obermeier, M. T.; White, R. E.; Yang, C. S. Effects of bioflavonoids on hepatic P450 activities. *Xenobiotica* **1995,** *25*, 575-84.
12. Sundaram, R.; Shanthi, P.; Sachdanandam, P. Effect of tangeretin, a polymethoxylated flavone on glucose metabolism in streptozotocin-induced diabetic rats. *Phytomedicine* **2014,** *21***,** 793-799.
13. Seo, J.; Lee, H. S.; Ryoo, S.; Seo, J. H.; Min, B. S.; Lee, J. H. Tangeretin, a citrus flavonoid, inhibits PGDF-BB-induced proliferation and migration of aortic smooth muscle cells by blocking AKT activation. *Eur. J. Pharmacol.* **2011,** *673*, 56-64.
14. Kurowska, E. M.; Manthey, J. A.; Casaschi, A.; Theriault, A. G. Modulation of HepG2 cell net apolipoprotein B secretion by the citrus polymethoxyflavone, tangeretin. *Lipids* **2004,** *39*,143-51.
15. Guthrie, N.; Kurowska, E. M.; Manthey, J. A.; Horowitz, R. M.; Horowitz, S. Compositions and methods of treating, reducing and preventing cardiovascular diseases and disorders with polymethoxyflavones. **2006**, US patent no. 6987125.
16. Gupta, S.; Afaq, F.; Mukhtar, H. Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. *Biochem. Biophys. Res. Commun.* **2001,** *287*, 914-20.
17. Manthey, J. A.; Cesar, T. B.; Jackson, E.; Mertens-Talcott, S. Pharmacokinetic study of nobiletin and tangeretin in rat serum by high-performance liquid chromatography - electrospray ionization - mass spectrometry. *J. Agric. Food Chem.* **2010,** *59*, 145-151.
18. Chakraborty, S.; Shukla, D.; Mishra, B.; Singh, S. Lipid - An emerging platform for oral delivery of drugs with poor bioavailability. *Eur. J. Pharm. Biopharm.* **2009,** *73*, 1-15.
19. Humberstone, A. J.; Charman, W. N. Lipid-based vehicles for the oral delivery of poorly water soluble drugs. *Adv. Drug Deliver. Rev.* **1997,** *25*, 103-128.
20. Porter, C. J.; Trevaskis, N. L.; Charman, W. N. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. *Nat. Rev. Drug Discov.* **2007,** *6*, 231-48.
21. Ting, Y. W.; Xia, Q. Y.; Li, S. M.; Ho, C. T.; Huang, Q. R. Design of high-loading and high-stability viscoelastic emulsions for polymethoxyflavones. *Food Res. Int.* **2013,** *54*, 633-640.
22. Ting, Y. W.; Li, C. C.; Pan, M. H.; Ho, C. T.; Huang, Q. R. Effect of a labile methyl donor on the transformation of 5-demethyltangeretin and the related implication on bioactivity. *J. Agric. Food Chem.* **2013,** *61*, 8090-8097.
23. Yu, H. L.; Shi, K.; Liu, D.; Huang, Q. R. Development of a food-grade organogel with high bioaccessibility and loading of curcuminoids. *Food Chem.* **2012,** *131*, 48-54.
24. Anson, N. M.; Selinheimo, E.; Havenaar, R.; Aura, A. M.; Mattila, I.; Lehtinen, P.; Bast, A.; Poutanen, K.; Haenen, G. R. M. M. Bioprocessing of wheat bran improves in vitro bioaccessibility and colonic metabolism of phenolic compounds. *J. Agric. Food Chem.* **2009,** *57*, 6148-6155.
25. Wang, Z.; Li, S.; Ferguson, S.; Goodnow, R.; Ho, C. T. Validated reversed phase LC method for quantitative analysis of polymethoxyflavones in citrus peel extracts. *J. Sep. Sci.* **2008,** *31*, 30-7.
26. Deshpande, A. A.; Rhodes, C. T.; Shah, N. H.; Malick, A. W. Controlled-release drug delivery systems for prolonged gastric residence: An overview. *Drug Dev. Ind. Pharm.* **1996,** *22*, (6), 531-539.
27. Golding, M.; Wooster, T. J. The influence of emulsion structure and stability on lipid digestion. *Curr. Opin. Colloid Interface Sci.* **2010,** *15*, (1-2), 90-101.
28. Ting, Y. W.; Jiang, Y. K.; Ho, C. T.; Huang, Q. R. Common delivery systems for enhancing in vivo bioavailability and biological efficacy of nutraceuticals. *J. Funct. Foods* **2014,** *7***,** 112-118.

29. Yu, H.; Huang, Q. Improving the Oral Bioavailability of Curcumin Using Novel Organogel-Based Nanoemulsions. *J. Agric. Food Chem.* **2012,** *60*, 5373-5379.
30. Brissette, L.; Noël, S.-P. An in vitro model to study lipolysis of rat hepatic very low density lipoprotein using cardiac lipoprotein lipase. *Can. J. Biochem. Cell B.* **1984,** *62*, 1076-1080.
31. Dahan, A.; Hoffman, A. Use of a dynamic in vitro lipolysis model to rationalize oral formulation development for poor water soluble drugs: correlation with in vivo data and the relationship to intra-enterocyte processes in rats. *Pharm. Res.* **2006,** *23*, 2165-2174.
32. Li, Y.; Zheng, J.; Xiao, H.; McClements, D. J. Nanoemulsion-based delivery systems for poorly water-soluble bioactive compounds: Influence of formulation parameters on polymethoxyflavone crystallization. *Food Hydrocoll.* **2012,** *27*, 517-528.

Table 1. Bioaccessibility of tangeretin measured by TIM-1 system.

Formulation	Tangeretin Input (mg)	Total recovery (mg)	Total recovery as of % input	Bioaccessibility as of % input	Efflux as of % input
MCT suspension	2500	134.4±23.0	5.4±0.9	3.3±0.3	2.1±0.8
Emulsion	2400	255.2±28.7*	19.1±1.2*	8.6±0.4*	10.5±1.2*

* $p < 0.05$

Table 2. Pharmacokinetic parameters of tangeretin after oral administration.

Formulation	Tangeretin dose (mg/kg)	T_{max} (hr)	C_{max} (µg/mL)	AUC_{0-24} (µg/mL*hr)	K_{el} (hr-1)	Relative Bioavailability
MCT suspension	100	0.5	7.1±3.2	35.5	0.126	
Emulsion	100	1	8.7±1.7	83.0	0.153	2.3

Figure Captions

Figure 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 min of *in vitro* lipolysis. Data in (B) are presented as mean ± standard deviation (n = 3). ** $p < 0.01$

Figure 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). ** $p < 0.01$

Figure 3. 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.

Figure 4. 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. 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).

Figure 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 nobiletin (internal standard, 8.81min), tangeretin (aglycone, 10.94 min), 5-demethyltangeretin (metabolite, 15.07 min).

Figure 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).

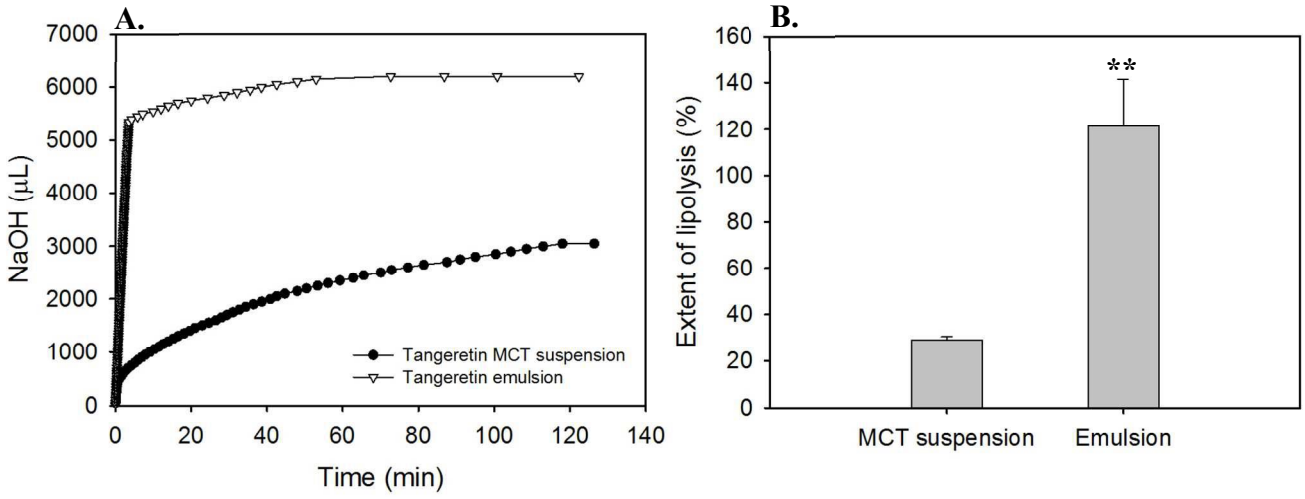

A.
NaOH (μL)
Time (min)
Tangeretin MCT suspension
Tangeretin emulsion
B.
Extent of lipolysis (%)
**
MCT suspension
Emulsion

Figure 1

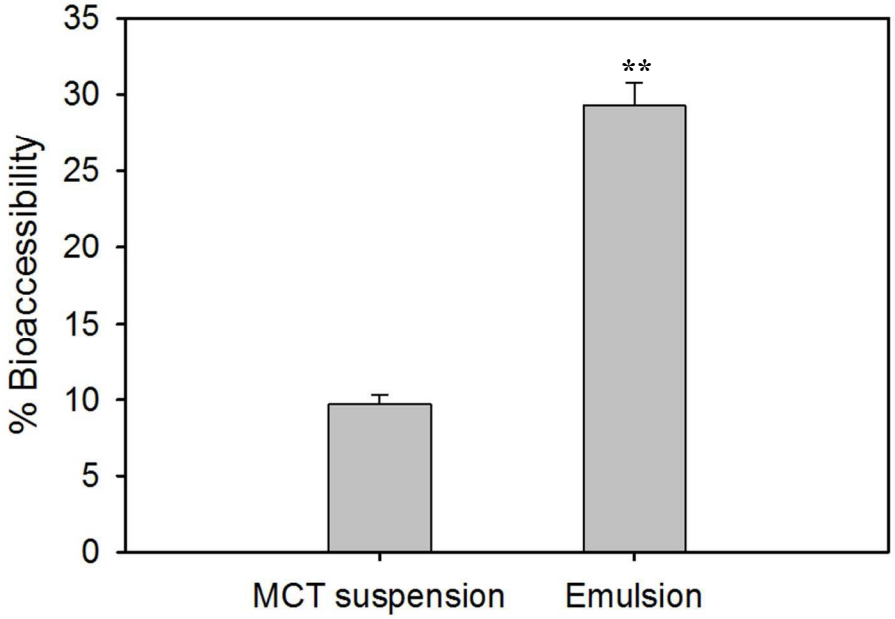

35
30
25
20
15
10
5
0
% Bioaccessibility
**
MCT suspension
Emulsion

Figure 2

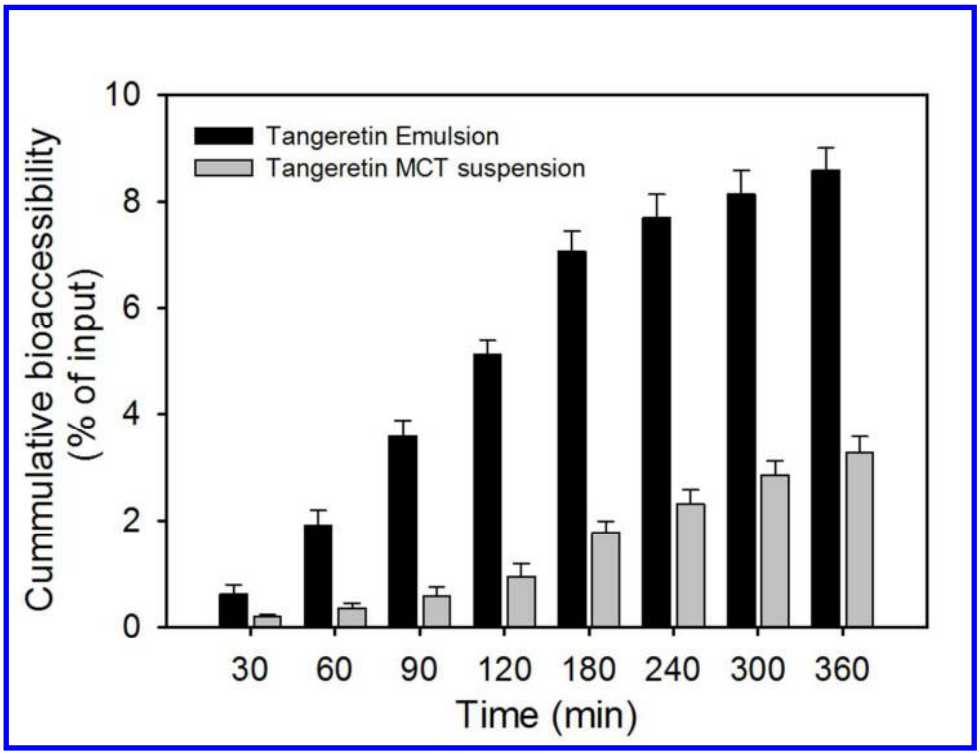
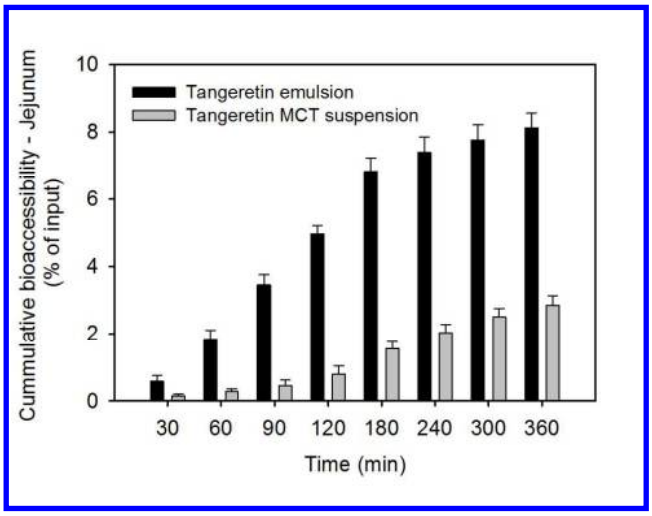
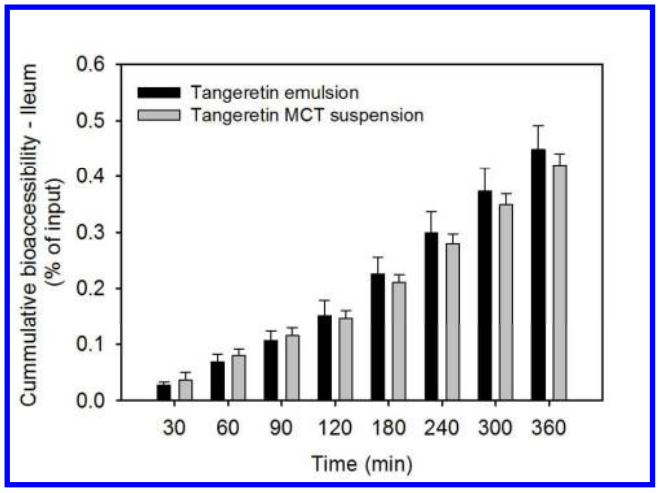

Cummulative bioaccessibility
(% of input)
Tangeretin Emulsion
Tangeretin MCT suspension
0
2
4
6
8
10
30
60
90
120
180
240
300
360
Time (min)

Figure 3

A


Cummulative bioaccessibility - Jejunum (% of input)
Tangeretin emulsion
Tangeretin MCT suspension
0
2
4
6
8
10
30 60 90 120 180 240 300 360
Time (min)

B


Cummulative bioaccessibility - Ileum (% of input)
Tangeretin emulsion
Tangeretin MCT suspension
0.0
0.1
0.2
0.3
0.4
0.5
0.6
30 60 90 120 180 240 300 360
Time (min)

C

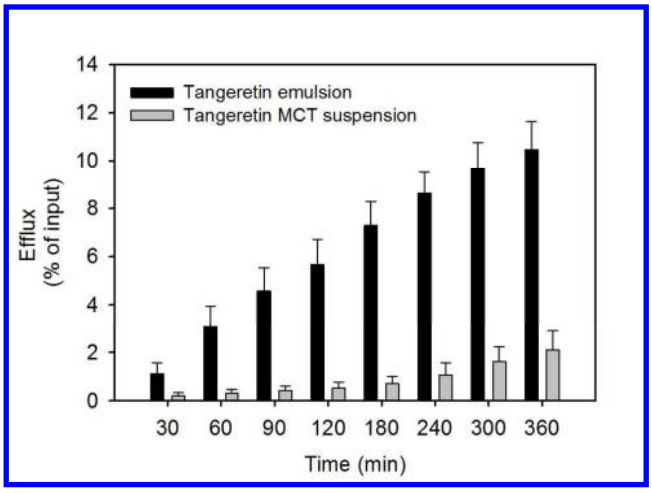

Efflux (% of input)
Tangeretin emulsion
Tangeretin MCT suspension
0
2
4
6
8
10
12
14
30 60 90 120 180 240 300 360
Time (min)

Figure 4

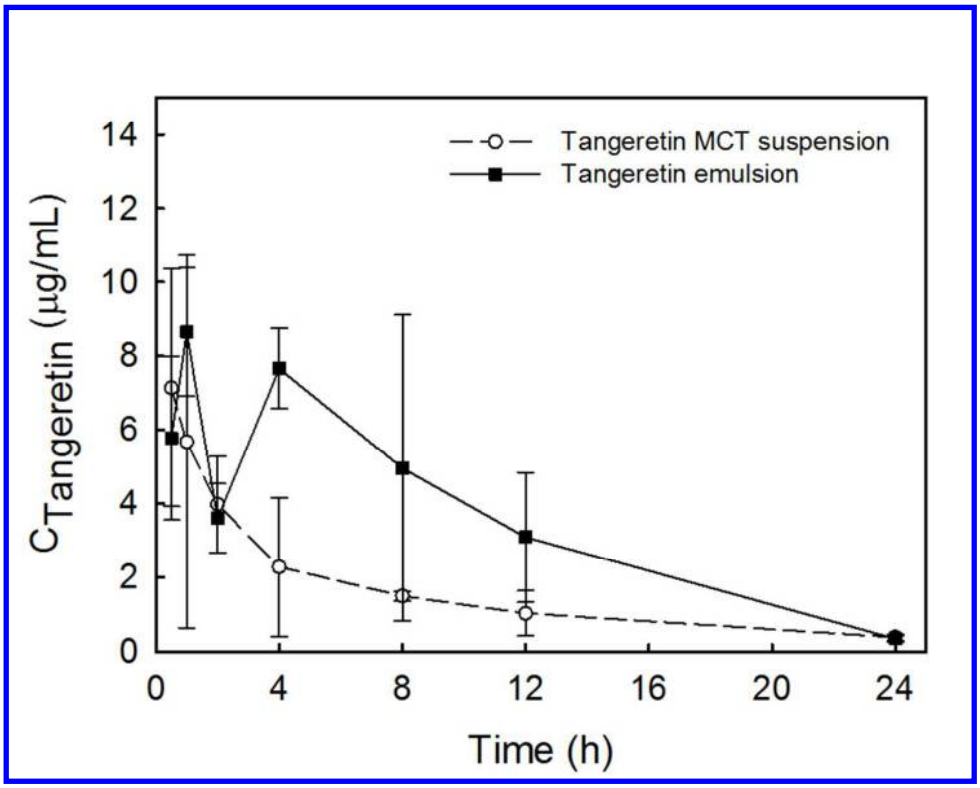

Tangeretin MCT suspension
Tangeretin emulsion
$C_{Tangeretin}$ (μg/mL)
Time (h)
0
2
4
6
8
10
12
14
0
4
8
12
16
20
24

Figure 5

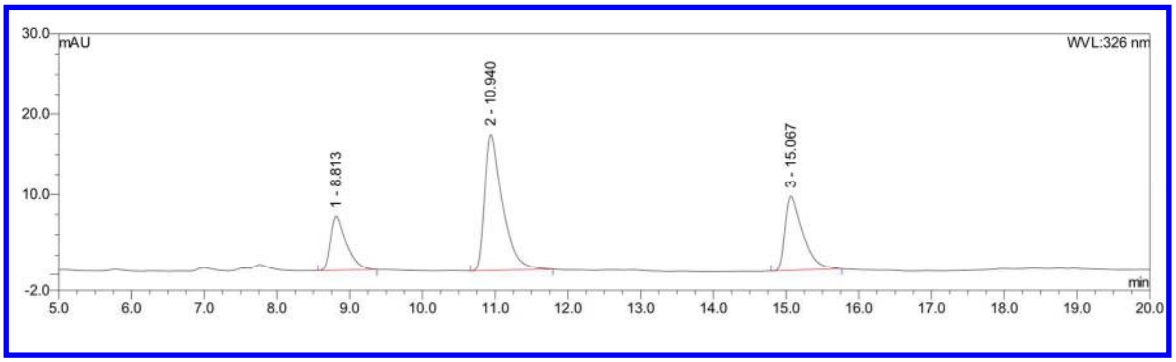


Figure 6

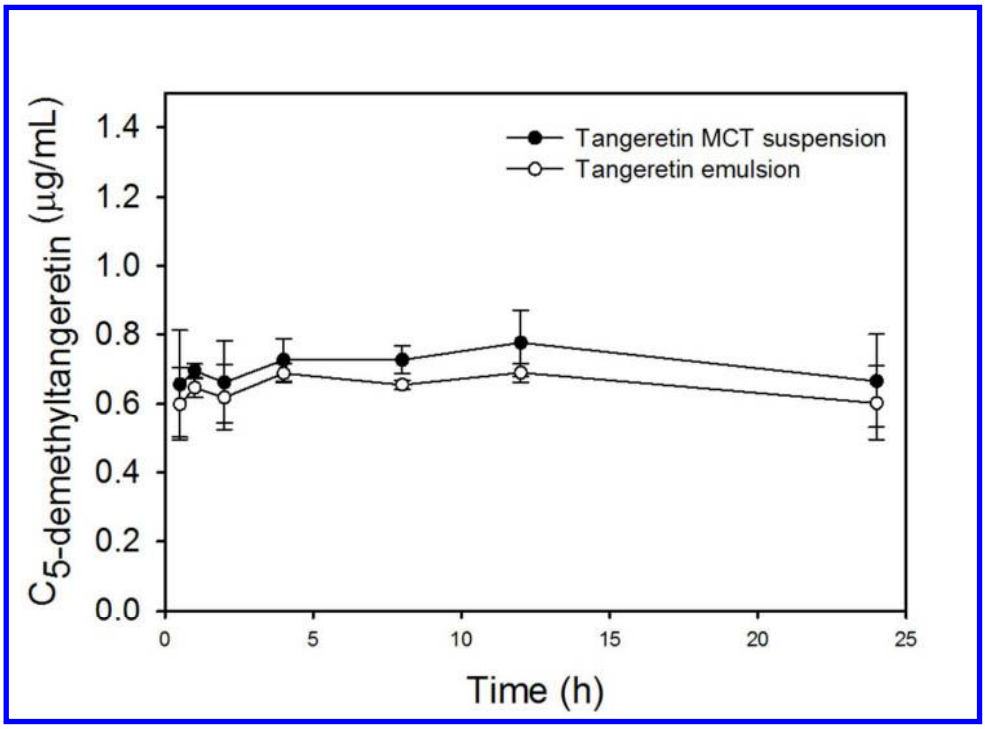

Tangeretin MCT suspension
Tangeretin emulsion
C5-demethyltangeretin (μg/mL)
Time (h)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
5
10
15
20
25

Figure 7