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14. ABSTRACT
The Center for Advanced Food Technology's (CAFT) Cooperative Research Program provided a unique research interface with representatives from the food industry, government and academia. The mission of the program was to develop new knowledge in food science and transfer the knowledge to the food industry resulting in more healthful and safer foods. The mission was fulfilled through funding of research projects funded by membership in the Center. Unfortunately the Cooperative Research Program was discontinued in August 2012. Past reports capture the output of the Center.

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Report Title

Improvement of the Quality, Health Promoting and Performance Enhancement Attributes of Combat Rations

ABSTRACT

The Center for Advanced Food Technology's (CAFT) Cooperative Research Program provided a unique research interface with representatives from the food industry, government and academia. The mission of the program was to develop new knowledge in food science and transfer the knowledge to the food industry resulting in more healthful and safer foods. The mission was fulfilled through funding of research projects funded by membership in the Center. Unfortunately the Cooperative Research Program was discontinued in August 2012. Past reports capture the output of the Center.
However two new short term projects were funded for Year 3 of the contract and this serves as the final report. The projects were: Development of a Center of Excellence for Food Safety Risk Analysis Decision Making Tools and Development of Novel Nutraceutical Nanodelivery Systems.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

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TOTAL:

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(b) Papers published in non-peer-reviewed journals (N/A for none)

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Number of Papers published in non peer-reviewed journals:

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Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Number of Manuscripts:

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Patents Submitted

Patents Awarded

Awards

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Names of Post Doctorates

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Total Number:

Names of Faculty Supported

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The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

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Names of Personnel receiving masters degrees

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Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

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Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment

Technology Transfer

Final Report

Improvement of the Quality, Health Promoting and Performance Enhancement Attributes of Combat Rations

Proposal Number: 57305LS

Sub-reports:

Development of Novel Nutraceuticals Nanodelivery Systems

Development of a Center of Excellence for Food Safety Risk

Analysis Decision Making Tools

Final Project Report

Development of Novel Nutraceuticals Nanodelivery Systems

Project Director: Professor Qingrong Huang

September 2014

Department of Food Science
Rutgers University
New Brunswick, New Jersey 08901

Research Objectives

The overall aim of this proposal is to develop novel, well-characterized nutraceutical nanodelivery systems which can meet the Army's needs. The common problem of these dietary flavonoids is their poor water solubility and the resulted poor oral bioavailability. Our central hypotheses of this proposal include: (1) nanodelivery systems will enhance the absorption of nutraceuticals in gastrointestinal (GI) tract, thus will have higher bioavailability than the corresponding free nutraceuticals; and (2) by optimizing the formulations and sizes of nanodelivery systems, safe functional food ingredients with improved bioavailability can be obtained. To test these hypotheses, we plan to explore some lipid-based nanodelivery systems recently developed in the PI's laboratory to engineer high-loading nutraceutical nanocapsules using food-grade wall materials, characterize their physicochemical parameters (i.e., rheological properties, droplet charges, sizes and size distributions, and shapes), study how physicochemical conditions and processing parameters affect their physical properties, as well as the distribution of nutraceuticals within the engineered nutraceutical nanodelivery systems, and investigate the absorption and toxicity of these nanostructured food ingredients using in vitro (i.e., cell lines) and in vivo animal (i.e., rats/mice) models. Here, to simplify and speed up the process to test our hypotheses, we have chosen a model compound (i.e., tangeretin) of PMFs for the development of delivery system and biological efficacy evaluation.

Tangeretin (4',5,6,7,8-pentamethoxyflavone) belongs to a sub-group of flavonoid called polymethoxyflavone and is majorly gathered from citrus peel. Tangeretin may function as a potential chemopreventive agent since it exhibits potent anti-inflammatory(*1, 2*), anti-proliferative(*3*), and anti-carcinogensis(*4, 5*) activities (Fig. 1-A). Previous studies has shown that tangeretin reduced inflammation related cyclooxygenase (COX-2) expression in human lung epithelial carcinoma cells(*1*) and induced G1 cell cycle arrest in breast and colon carcinoma cells(*6, 7*). However, when ingested orally, the bioavailability of tangeretin is low due to its low aqueous solubility arise from its lipophilic chemical structure. Consequently, the required tangeretin concentration for many intended therapeutic purposes is difficult to reach using oral delivery route. The bioavailability is directly related to amount of compound being accessible for intestinal absorption(*8*). Since the gastrointestinal (GI) tract is a constant flow of aqueous environment, the solubility of ingested compound becomes the critical factor determining the absorption rate and availability to the system circulation. Many approaches have been investigated aiming to augment oral bioavailability of active molecules through formulating various delivery systems such as chemical modification, emulsion, liposome, nanoparticle, micelle encapsulation, nanodispersion, and hydrogel, and so on. Among all, emulsion is one of the most common applied delivery methods for manufacturing or research purposes. The advantages of utilizing emulsion as delivery system includes wide application range, versatility, easy fabrication and processing, and convenient to be incorporated to food and supplemental products. However, the design of delivery vehicle for tangeretin is quite challenging due to its high melting temperature and rapid recrystallization behavior during processing. Therefore, the overall aim of this project was to utilize novel nanotechnology to design an optimized tangeretin delivery vehicle for augmenting bioavailability and bioefficacy in both *in vitro* and *in vivo* model.

Objective 1. To engineer nanoemulsions containing high loadings (>1%) of polyphenols (i.e., curcumin, quercetine, nobiletin, and 5-demethyl nobiletin etc.) using innovative "green processing" technique without the use of organic solvents, and characterize detailed droplet characteristics which include droplet sizes and size distributions, viscosities, crystal formation, digestion, and droplet charges using a combination of dynamic light scattering (DLS), rheology, optical microscopy, lipolysis, and zeta potential measurements.

Objective 2. To determine the bioavailability of nanoemulsions-encapsulated polyphenols using in vitro human Caco-2 cell monolayer model and in vivo animal model.

Objective 3. To evaluate the potential toxicity of nanoemulsions-encapsulated polyphenols using in vivo animal model.

Materials and Methods

Materials

Tangeretin of 98% purity was purchased from Quality Phytochimical LLC (Edison, NJ, USA). PC75 rapeseed lecithin containing 75% phosphatidylcholine was a gift from American Lecithin Company (Oxford, CT). Neobee 1053 medium chain triacylglycerol (MCT) was a gift from Stepan Company (Northfield, IL). Sterile filtered, cell culture compatible dimethyl sulfoxide (Sigma–Aldrich) was used for HPLC sample solvent. Minimum essential medium (MEM) was purchased from HyClone Laboratories, Inc. (Logan, Utah). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Penicillin and streptomycin were purchased from Invitrogen, (Carlsbad, CA). AOM was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies against COX-2 were purchased from BD Transduction Laboratories (Lexington, KY). Polyclomal antibodies for β-catenin, PCNA, and VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 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 recent published method(*9*). The designed formulation contained 2.1% tangeretin in 30 g of processed emulsion, which composed of 50.4% medium chain triglyceride (MCT), 1.5% lecithin, and 46% double deionized (DI) water. To describe the processing procedure in short, the preparation of oil phase was done by fully dissolve 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 aqueous phase was to be added to oil phase, the temperature was reduced to 70°C and continue to mixed by magnetic stirring until a one phase crude emulsion was formed. After removal of maganetic stir bar, the crude

emulsion were then subjected to high-speed homogenization (ULTRA–TURRAX T-25 basic, IKA Works Inc., Willmington, USA) to reduce the viscosity for easier processing of high-pressure homogenization (EmulsiFlex-C6, AVESTIN Inc., Ottawa, Canada). Through out the high-pressure homogenization process, 500 bar pressure was applied while the temperature was kept at 55°C. In each processing batch, approximately 25-30 g of sample can be collected.

In vitro Lipolysis of PMFs in Emulsion or MCT Suspension

To better mimic the digestion activity in human small intestine, *in vitro* lipolysis study was carried out using the method described in our previously published paper (*10*) with minor modification. In short, fed state lipolysis buffer was prepared with Tris maleate, NaCl, $CaCl_2 \cdot H_2O$, NaTDC, and phosphatidylcholine in concentration of 50, 150, 5, 20, and 5 mM respectively. Pancreatin was freshly prepared for each lipolysis study by mixing 1g of pancreatin powder with 5 mL lipolysis buffer, centrifuged at 2000 rpm and stored on ice. To begin lipolysis study, equivalent amount 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 9mL of lipolysis buffer. During the 2-hour lipolysis study, the temperature was kept at 37 ± 1°C and pH is 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 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, middle layer of supernatant was collected and stored at -80°C for later HPLC analysis. For HPLC analysis, 200 μL of lipolysis supernatant sample were filtered through 0.22 μm filter and mixed well with 400 μL of DMSO.

In vivo pharmacokinetic study

Female CD-1 mice (22− 26 g) were used in the pharmacokinetics analysis of Tangeretin (Rutgers University protocol no.: 99−015). After fast overnight, mice were administrated with tangeretin emulsion or tangeretin MCT suspension at the dose of 100 mg/kg by oral gavage. At different time intervals (0.5, 1, 2, 4, 8, 12, and 24 h), blood samples were withdrawn by cardiac puncture. After centrifugation at 5000 ×g for 15 min at ambient temperature, plasma was removed and stored at −80 °C until HPLC analysis. The areas-under-the curve (AUC) for the plot of the plasma concentration over time was calculated using linear trapezoidal method. C_{max} and T_{max} were directly obtained from the curves.

Cell Culture

Human colorectal carcinoma cell lines, HCT 116 and HT29 (American Type Culture Collection, HB-8065, Manassas, VA) were grown and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100l g/mL streptomycin. The incubation condition for cell culture was 95% relative humidity and 5% CO_2 at 37°C.

MTT cell proliferation essay and growth morphology observation

Methyl thiazol tetrazolium bromide (MTT) essay was used to quantify cell proliferation of HCT116 and HT29 after treated with emulsion or DMSO-dissolved tangeretin. Briefly, cells that suspended in serum complete medium were seeded into a flat-bottom 96-well clear plate at density of 1 x 10^4 cells/well. The culture media were carefully aspirated and replaced by medium containing emulsion or DMSO-dissolved tangeretin after 24 hours of incubation. Separate sets of negative control (untreated) and blank emulsion vehicle were also cultured for reference value and background subtraction. After 24 hours, the medium contained tangeretin in 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 cause the cell to crystalized within 2 hours of incubation and then be removed from the plate by aspiration. Last, crystals formed were dissolved in 100 μL of DMSO before absorbance was read at 560nm using Microplate Reader (Molecular Devices, Sunnyvale, CA). The relative proliferation values were calculated against the control value. Experiments were conducted in triplicate with 6 repeat in each investigation for avoiding inter- and intra- experiment error. Cells were also observed using an inverted microscope (Leica Microsystems) with 200 times magnification.

Animals

Male ICR mice aged 6 weeks were bought from BioLASCO Experimental Animal Center (Taiwan Co., Ltd). Animals were randomly divided into control and experimental group after 1 weeks of acclimation. All mice were maintained at 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 can liberally access water and food that was replenished everyday. The experimental protocol were approved by Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU)

AOM/DSS-induced colorectal cancer model

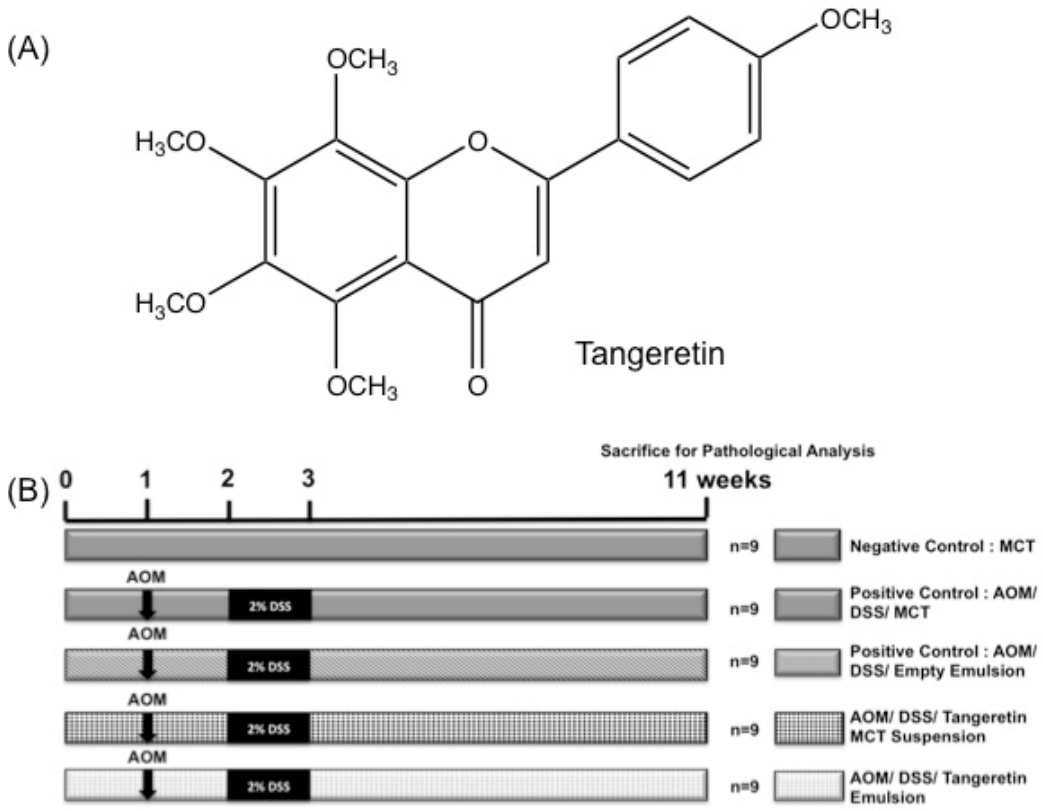

Figure 1: (A) Structure of tangeretin and (B) experimental design for *in vivo* animal

In present study, an AOM/DSS induced colorectal cancer model was conducted in conjunction with a chemopreventive feeding pattern, which contained 1 weeks of pre-induction treatment and 10 weeks of post-induction treatment. A total of 45 male ICR mice were randomly separated into 1 negative control, 2 positive control and 2 experimental groups (Fig.1-B). For 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 while did not receive any carcinogenesis chemical treatment. Starting from week 1, all groups (n = 36) besides 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 were added to drinking water for groups that received AOM ip injection for 7 days and then switch back to normal untreated drinking water. At the end of 11 weeks, all mice were sacrificed by CO_2 asphyxiation and subjected to necropsy inspection. The end body weight, liver, spleen, and kidney were accurately weighted and recorded. Upon necroscopy, colons from mice were carefully removed and flushed with PBS. After weight and length were precisely measure 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 biomarker

evaluation.

Immunoblotting

For immunoblot analysis, the tissues scraped off from 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 interval of 5 minutes. The lysed tissue samples were then centrifuged at 10000g for 30 minutes at 4°C. After centrifugation, the supernatant containing solubilized protein were collected and used for western blot analysis. Fifty micrograms of protein from each samples 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 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) using transfer buffer (25 mM pH 8.9 Tris-HCl, 192 mM glycine, and 20% methanol). The membranes were then blocked with blocking solution consist 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 protein concentration loaded.

Immunohistochemical staining

The colon mucosa samples that was fixed in 10% buffered formalin were cut into 3mm section, deparaffinied, rehydrated, and treated with 0.3% hydrogen peroxide (H_2O_2) for 15 min to block endogenous peroxidase. To unmask epitopes, sections were pressure-cooked four times at 7 minutes each in pH 6.0 10 mM citrate buffer, pH 6.0 (Immuno DNA retriever with citrate, BIO SB, Inc., Santa Barbara, CA). 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 min each. The positive signal was detected as the substrate, 3,3'-Diaminobenzidine tetrahydrochloride (0.05%, DAB), presented as brown color under a light microscope. The procedure for analyzing the stained tissue was reported in previous published literatures(*11*). 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 CO_2 anesthetization through cardio puncture. Blood collected were

mixed with heparin and centrifuged at 3000g for 15min at 4°C. After centrifugation, serum were collected and stored at -80°C before subjected to clinical chemistry analysis. Serum triglycerides (TG) and total cholesterol (TCHO) were evaluated using commercial available kits (BioVision Incorp., Mountain View, CA, USA) and Fuji DRI-CHEM40000 (FUJIFILM).

Statistical analysis

Cell line studies and biochemistry analysis for animal study were performed in at least triplicate. Results were expressed as means ± standard deviation. One-way student t-test were performed using Sigmaplot 10.0 software to examining the bioactivity difference between unformulated and emulsion tangeretin. A p value < 0.05 was considered statistically significant.

Results and Discussions

Emulsion particle size and loading analysis

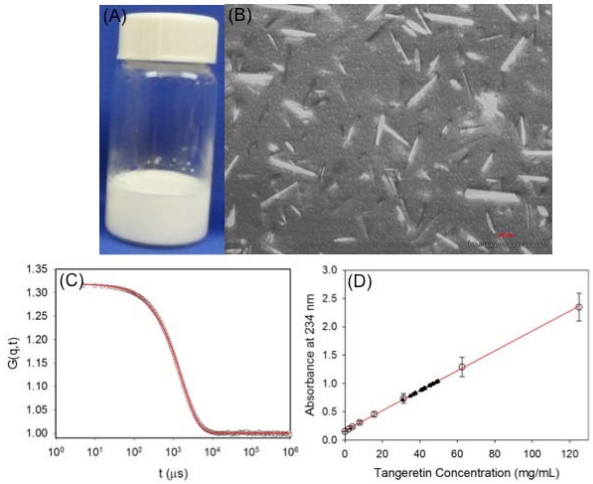

Figure 2: (A) tangeretin emulsion at room temperature. (B) Microscopic picture of tangerein emulsion at 500x magnification. (C) The DLS autocorrelation function curve and single stretched exponential fitting of diluted tangeretin emulsion (1:5000 diluted by DI water). (D) The loading of tangeretin samples (black triangles) were measured using standard curve constructed at 234nm absorbance.

In this study, an emulsion-based system was successfully formulated as delivery vehicle for tangeretin. The tangeretin emulsion formulation composed of 2.1% tangeretin, 50.4% MCT, 1.5% lecithin, and 46% DI water was used for present study. To control the formulation quality and confirm compound loading, emulsion stability, particle size and precise loading was evaluated on each production batches through out the study. For each processing batch, approximately 30g of tangeretin emulsion will be deposited into a clean glass bottle with cap and store in dark under room temperature until used (Fig. 2A). Daily observation of stored emulsion samples were conducted and the storage stability was good with no signs of phase separation and precipitation before consumed by later experiments. The morphology of undiluted tangeretin emulsion sample was further observed using optical microscope with 500x maginification (Fig 2B). As shown in the picture, tangeretin emulsion system is rather a mix system with part of the compounds either encapsulated in the emulsion droplet or homogeneously entrapped in the viscous emulsion systems. According to our previous investigations, the size of emulsion droplet plays critical role in the bioavailability and efficacy of included compound(*12, 13*), the

size of emulsion droplet and entrapped tangeretin crystal were precisely measured. With the image processing software (Image J, nih.gov) and correct reference scale, tangeretin crystals present in the system were measured in average as 9.33±1.3μm. The mean hydrodynamic diameter of tangeretin emulsion was evaluated using DLS method at room temperature. The DLS autocorrelation function curve and single stretched exponential fitting of diluted tangeretin emulsion (1:5000 in DI water) was shown in figure 1B. The mean hydrodynamic diameter of emulsion droplet was determined as 482.3±1.8 nm. The particle size determined using DLS method include the surrounding hydrated layer, which may in term give a systematic higher value than otherwise measurement using dried method. The polydispersity of each sample were measure at 0.127±0.007 indicating a narrow size deviation within each tangeretin emulsion samples. Moreover, the particle sizes of each production batches were relatively constant with a standard deviation value of ± 50nm.

In vitro lipolysis study to predict the effect of emulsion based delivery system on bioavailability.

The therapeutic dosages of many health beneficial bioactives are difficult to reach as a result of low aqueous solubility. It was stated in many literatures that the oral bioavailabilities of lipophilic compounds could be greatly increased when consumed with lipids (*14-18*). 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 *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(*15, 19-21*).

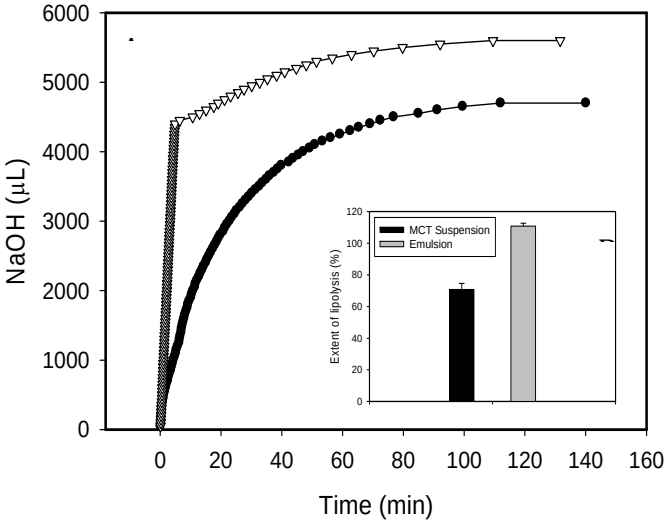

Figure 2: In vitro lipolysis (A) digestion kinetic curve of blank emulsion (▽) and blank MCT oil (●). (B) Extent of lipolysis at 30 min for blank emulsion and blank MCT oil.

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 min (Fig. 3A). 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 100% lipid digestion, the extent of lipolysis for emulsion and unformulated oil samples were compared at the 30 minutes time point. The extent of lipolysis, defined as percentage of lipid digested, exhibited great difference between emulsion (>100%) and crude MCT sample (64.5%) (Fig. 3B). Since digested lipids are constantly adapted into mixed micelle during lipolysis, any nearby lipophilic compounds may as well be incorporated into the micelle available for intestinal absorption. Since intestinal activity is a dynamic process of digestion, absorption, and excretion, lipophilic compounds will have longer intestinal retention time and, thus, a higher chance to be absorbed if it is rapidly incorporated into the mix micelle and become soluble in the intestinal lumen.

To be bioaccessible for intestinal up take, 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 mix micelle. During the *in vitro* lipolysis study, the lipid is simultaneously digested to form mix micelle, which imitates the component of intestinal lumen where lipophilic compound 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. In comparison to the MCT suspension, tangeretin emulsion increased the percent of total bioaccessibility from 23.0% to 30.7% and the concentration of total solubilized PMF from 1.75±0.39 mg to 2.27±0.20 mg. The result from percent bioaccessibility evaluation was consistent with previous reported literatures that faster lipid digestion kinetic will result in higher bioaccessibility of lipophilic compounds(*22, 23*).

In vivo pharmacokinetic study

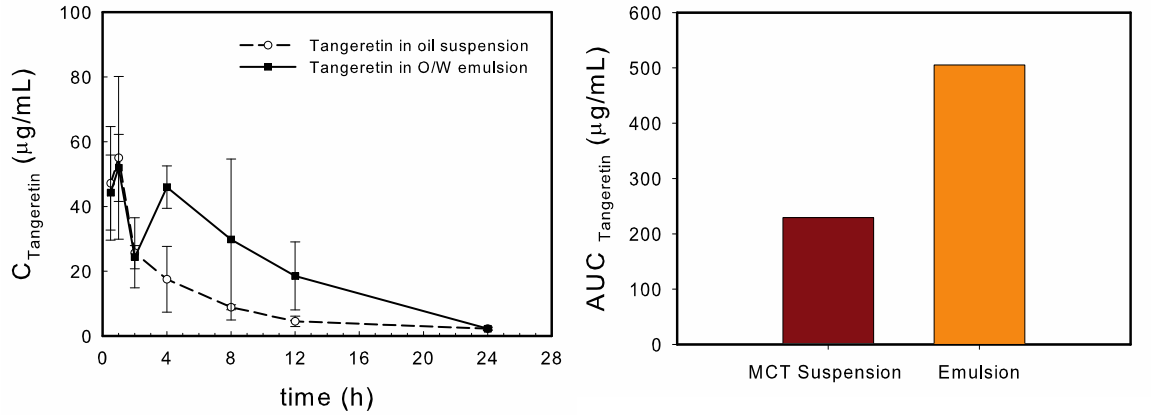

Figure 4: Result from tangeretin pharmacokinetic study.

To directly investigate the oral bioavailability of tangeretin in the emulsion-based delivery system, pharmacokinetics analysis was performed on mice after oral

administration with tangeretin MCT suspension or tangeretin emulsion. To obtain a HPLC detectable level, tangeretin from the serum were first extracted using 800µL ethyl acetate, concentrated by nitrogen drying and then re-dissolved in 100µL of ethanol. As shown in Figure 3, the plasma concentrations of total tangeretin was all apparently higher than unformulated tangeretin MCT susepsion. The C_{max} and T_{max} were similar for both oral formulations. However, a second concentration peak was observed from mice treated with tangeretin emulsion potentially due to the control release mechanism that may delay the release profile of tangeretin. The $AUC_{0-\infty}$ of tangeretin in emulsion-based delivery system increased 120% than the tangeretin MCT suspension demonstrating that the nanoemulsion formulation was able to improve the oral bioavailability (BA) of tangeretin.

In vitro proliferation evaluations and morphology observations

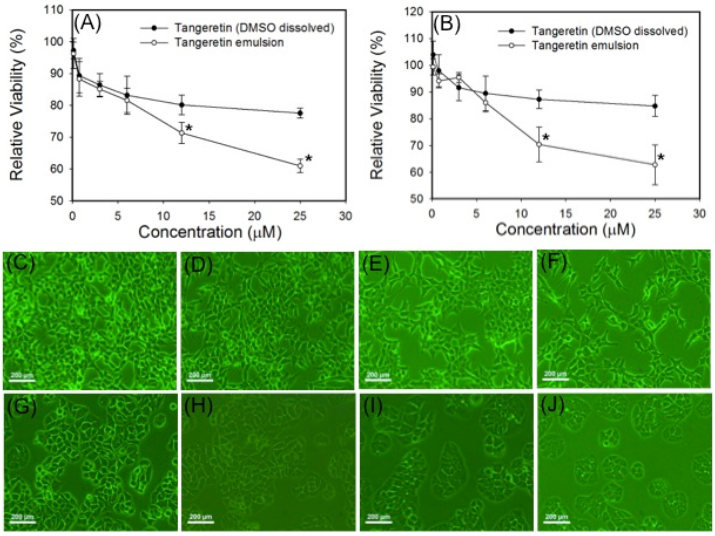

Figure 5: Relative cell viability of (A) HCT116 and (B) HT29 from MTT essay. Microscopic observation of cell morphologies: HCT116 (C) untreated control (D) blank emulsion treated (E) treated with DMSO solubilizaed tangeretin and (F) tangeretin emulsion. HT29 (G) untreated control (H) blank emulsion treated (I) treated with DMSO solubilizaed tangeretin and (J) tangeretin emulsion.
* $P< 0.05$

The mechanisms that bioactive compounds undertaken 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 was documented to inhibit cell proliferation through inducing G_0/G_1 cell cycle arrest(*6, 7*). Due to the lipophilic nature, higher dosing concentration was generally required for tangeretin to produce meaningful anti-proliferation effect in cancer cells. Therefore, to investigate the effectiveness of emulsion-based delivery system to improve the bio-efficacy of tangeretin, an *in vitro* MTT essay was performed as efficient assessment before executing the subsequent *in vivo* animal study, which may be much more complex and time consuming. In this study, two colorectal carcinoma cell lines, HCT116 and HT29, seeded in 96-well plates in density of $1x10^4$ cells/well were treated with serum complete medium containing tangeretin either dissolved by DMSO or dispersed by emulsion delivery system. The potency of 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 on cell proliferation in all treatment concentrations (3.125, 6.25, 12.5, and 25 μM) as shown in figure 5A and B. Cells treated with tangeretin emulsion showed statistically significant reductions in cell proliferation when compared with DMSO dissolved ones. Moreover, the improvement in anti-proliferative activities by emulsion-based delivery system was higher in HT29 than HCT116 cell line. HT29 cell line, in comparison with HCT116 cell line, are consider as a more malignant colorectal carcinoma cells that contain a mutated *p53* tumor suppressor gene, which is responsible for many anti-cancer function, such as regulating normal DNA repair, cell cycle, normal cell apoptosis, and autophagy. Higher proliferative reduction rate promoted by tangeretin emulsion in HT29 than HCT116 cell line indicates emulsion system may be an effective method to enhance the anti-cancer efficacy of tangeretin.

To have a direct observation of cell conditions, cell treated by tangeretin in both systems (solubilized in DMSO or incorporated in emulsion) were examined using optical microscope under 100x magnification. The effectiveness of tangeretin to inhibit cell growth and proliferation was discussed in terms of the observed cell size and density in relative to the untreated control. While cells treated with blank emulsion (Fig. 5D and H) shows no difference in cell density to the untreated control (Fig. 5C and G), the cells treated by tangeretin in both systems showed various degree of growth inhibitions. As similar trend was seen, tangeretin emulsion presented the lowest cell density in both colorectal cancer cell lines (Fig. 5F and J). Moreover, the cell sizes were also smaller in tangeretin emulsion treated groups than groups treated with DMSO solubilized tangeretin. The microscope examinations allowed simple and direct realization that emulsion-based delivery system amended tangeretin efficacy on reducing cancer cell growth and proliferation. Together with the result from MTT essay and microscopic observation, emulsion-based was proven as an effective and efficient means to augment the *in vitro* anti-proliferative of tangeretin.

General observation of in vivo AOM/DSS-induced mice colorectal cancer model

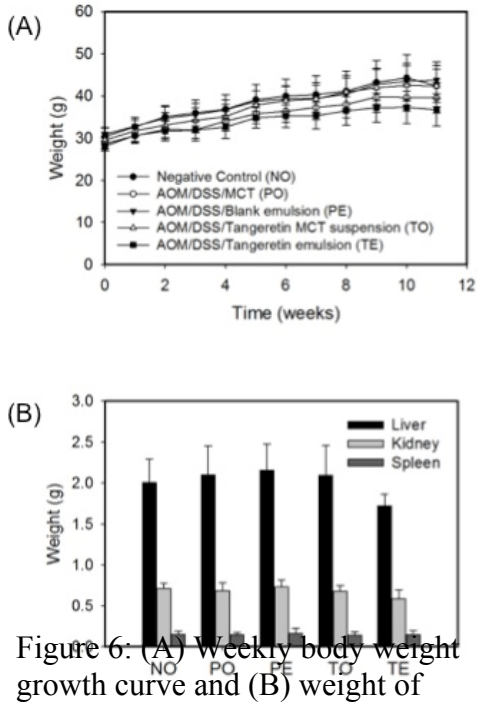

Figure 6: (A) Weekly body weight growth curve and (B) weight of liver, kidney, and spleen at the end of experiment.

As discussed in previous section, the emulsion-based delivery system was an effective means to enhance the *in vitro* anti-proliferative activity of tangeretin, the effect of emulsion processing on the *in vivo* oral bioefficacy of tangeretin was also evaluated using a colitis related mice colorectal cancer model. Among the experimentally induced CRC models, AOM/DSS model mimics the accelerated development of CRC induced by inflammatory stimulant. After single injection of AOM, 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 as 8 weeks(*24*). In present study, the experimental period last for 11 weeks was determined to allow consequential tumor development. During the

11-week study, the means body weight of mice from all groups increased steadily with no observable phenotypic characteristic of toxicity at any point during the experiment (Fig. 6A). By the end of the experiment, all groups of mice presented approximately 80% body weight increment since the beginning of 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 liver average weight of mice fed with tangeretin emulsion was lower than other groups due to the lower average body weight (Fig. 6B). 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 cause by toxicity as the relative liver weight (g/100 g body weight) showed no significant difference among all experimental groups. Overall, dosing level of 100mg/kg produced no conspicuous sign of toxicity during and at the end of the animal study.

Pathological findings

Table 1: body weight, colon weight and length, adenoma incidence and multiplicity of mice.

Treatment	Body Weight (g)	Colon			Adenomas in colon	
		Weight (g)	Length (cm)	W/L ratio	Incidence	Multiplicity
Negative Control	42.85 ± 5.20	0.147 ± 0.028	7.182 ± 0.751	0.021 ± 0.004	0%	0
AOM/DSS/MCT	40.13 ± 3.18	0.235 ± 0.134	6.15 ± 0.626	0.038 ± 0.020[a]	82%	6.22 ± 6.16
AOM/DSS/Blank emuslion	43.76 ± 4.54	0.268 ± 0.079	6.500 ± 1.453	0.041 ± 0.011[a]	90%	7.25 ± 3.92
AOM/DSS/Tangeretin MCT Suspension	42.33 ± 4.97	0.244 ± 0.084	6.700 ± 0.978	0.036 ± 0.018[a]	73%	5.80 ± 3.36
AOM/DSS/Tangeretin Emulsion	36.69 ± 3.91	0.236 ± 0.060	6.900 ± 1.197	0.034 ± 0.011[ab]	64%	4.60 ± 3.27

The progression of colitis related CRC could be assessed by several characteristic large bowl symptoms. On the basis, the degree of emulsion-based delivery system to improve the protective efficacy of tangeretin in treatment of AOM/DSS induced colorectal tumor development was evaluated. In AOM/DSS induced colitis CRC model, the increased weight and shortened length of colon are the most common pathological features pinpointed as the disease proceed. Mice colons from all experimental groups were carefully excised and measured since DSS stimulated colon inflammation result in the reduced colon length. When compare to the non-treated control (group 1), AOM/DSS induced mice showed significant reduction in length ($P<0.05$). The oral administration of tangeretin in form of MCT oil suspension (TO) or emulsion (TE) resulted in longer colon than groups fed with blank MCT and emulsion (Table 1). Due to uncontrollable cell proliferation, the process of developing colorectal tumor directly increase the cell density, mucosal thickness and, thus, the colon weight. The calculation of colon weight and length

(W/L) ratio was then established based on the rationality that heavier and shorter colons are viewed as a sign of greater malignant gradient of CRC. While significant increase of colon W/L ratio was seen in positive controls (group 2 and 3, $p < 0.001$), 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 compare to positive control group fed with empty emulsion (PE). On the contrast, only mild improvement was seen in the mice fed with TO in relative to the blank MCT positive control (PO) mice.

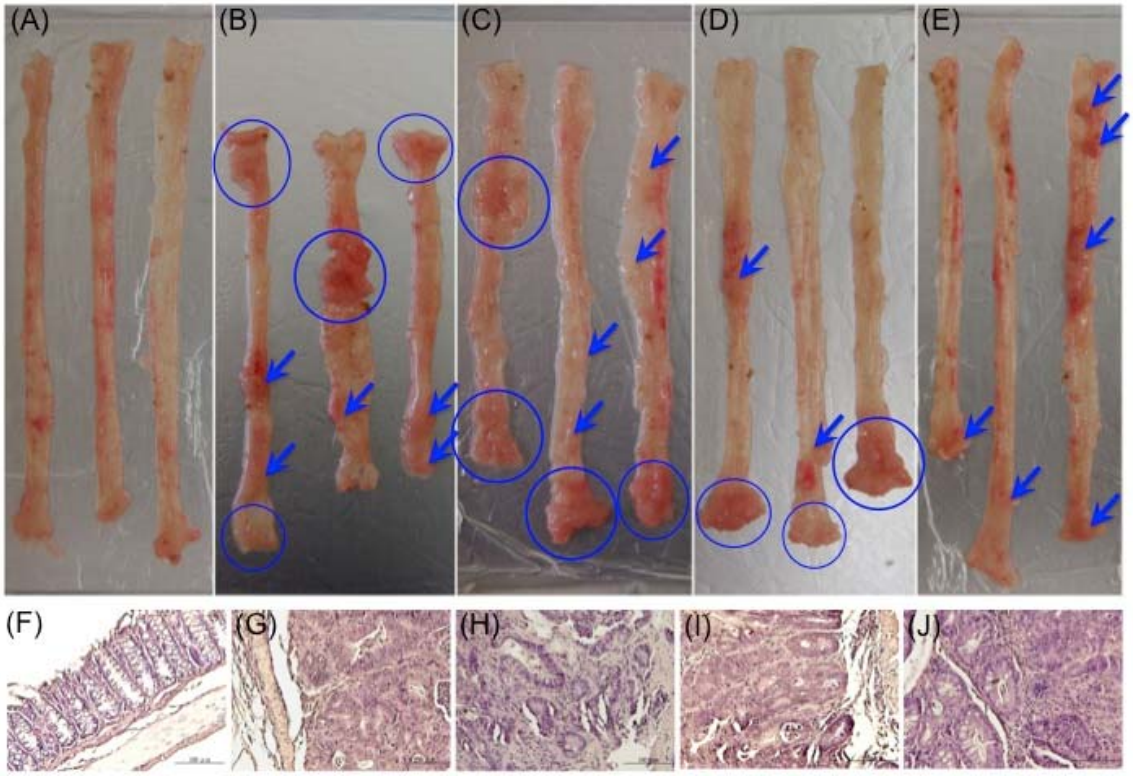

Figure 7: Picture observation of mice colon from AOM/DSS induced tumeregenesis study: (A) negative control: MCT (B) positive control: AOM/DSS/MCT (C) positive control: AOM/DSS/Empty emulsion (D) AOM/DSS/Tangeretin MCT suspension (E) AOM/DSS/Tangeretin emulsion. The blue circles represent the occurance 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.

As mentioned above, the anomalous cell proliferation cause the mucosal thickening and eventually leads to occurrence of colorectal adenomas. To efficiently study and compare the anti-tumoregenesis efficacy of both tangeretin formulations, the incidence and multiplicity of colon adenomas was correspondingly summarized in table 1. Single ip injection of AOM followed by one weeks exposure of drinking water containing 2% DSS resulted in 82% and 92% of large bowl adenoma incidence in PO and PE respectively. Oppose to the mice that does receive any preventive treatment, mice that treated by either TO or TE decrease the frequency of colorectal adenomas existence to 73% and 64%

respectively. Besides looking at the incidence of large bowl tumor occurrence, the number of adenomas presented 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 compare to group that consumed only blank emulsion sample (4.6±3.27 vs. 7.25±3.92, $p< 0.01$). While the tangeretin emulsion showed the lowest number of adenoma per mice, TO only insignificantly decrease the multiplicity 7% in relative to PO (5.8±3.36 vs. 6.22±6.16).

In figure 7A, the macroscopic pictures of representative colons from each group were presented for direct gross morphology observation. According to earlier discussion, a healthy mice colon should be reasonably long and lean as represented by picture from untreated negative control (Fig 7A). However, in picture of positive controls (AOM/DSS treated with oral administration of blank MCT or emulsion), the large bowl become brittle and appeared as short and flappy as the incidence of abnormal colonial mucosal inflammation and proliferation arised (Fig 7B and C). The change in colon morphology was in agreement with the previously reported pathological signs of AOM/DSS murine model(*24*). The colonic adenomas observed were distributed unevenly along the colon with higher frequency in the lower rectal part. The advancement of colonic disease was indicated when colonic adenomas appeared as large clusters result from higher tumor density. In comparison to MCT suspension (Fig. 7D), preventive treatment of Tangeretin emulsion (Fig. 7E) for total of 11 weeks more effectively alleviated the symptomatic morphological change and suppressed the number of adenoma. Moreover, the histological examination of colonial adenoma showed serious disruption of mucosal architecture as the goblet cell structure in the normal intestinal mucosa (Fig. 7F) was generally disappeared in the AOM/DSS treated positive control groups (Fig 7G and H). The mucosal retained its architecture in various degrees when tangeretin was consumed orally in 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 was proven effective to prevent the colitis related colon adenoma progression and alleviate the symption related. Nevertheless, the utilization of emulsion-based delivery system was more significantly improve the dose efficiency and potency of the therapeutic efficacy of tangeretin as evidenced by the experimental results.

Effect of emulsion-based delivery system on the efficacy of tangeretin to inhibit colitis related colon tumoregenesis.

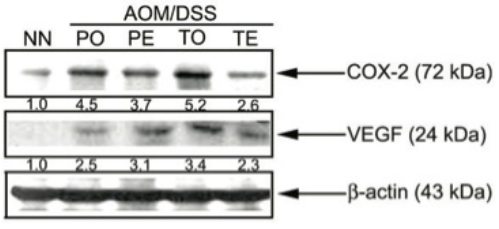

Figure 8: The result from immunoblotting. Tangeretin emulsion treatment effectively decreased the expression of COX-2 inflammation cytokine and VEGF.

The combination of AOM/DSS administration mimicked the human colitis-related colorectal carcinogenesis through stimulating a state of chronic inflammation(*24*). Therefore, the suppression of large bowl inflammation by

bioactives is then considered a potential AOM induced colorectal lesions(*25, 26*). A cytoplasmic protein, COX-2, is the common biomarker used for evaluation of inflammatory stress of many *in vivo* models. While normal epithelial cells show negative expression, anomalous COX-2 expression plays critical role in the development of the colitis-associated CRC. In this study, the effectiveness of tangeretin to regulate the expressions of COX-2 pro-inflammatory cytokine was evaluated using immunoblotting methods. After challenged by AOM/DSS, the expression of COX-2 markedly increased in PO and PE treated group as denote by the relative band intensity score of 4.5 and 3.7 correspondingly. While TO did not show any suppression effect, COX-2 expression was successfully lowered in the mice treated by TE (Fig. 8). The efficacy of tangeretin to regulate the *in vivo* COX-2 production was notably enhanced by emulsion delivery system due to enhanced dose efficiency result from better solubility, release profile, intestinal uptake, and transportability. Similar phenomenon was seen when we further examine the expression of protein related to the pathogenesis of tumor growth and metastasis. VEGF is the common cell signaling protein that mediate the formation of new blood vessel (angiogenesis) in the endothelial cells and become excessively expressed during tumoregenesis(*27, 28*). In previous literatures, inhibition of VEGF is an effective approach to suppress pathological angiogenesis and associated tumor growth(*27, 29*). Since the VEGF expression is positively related to the tumor formation, the efficacy of tangeretin to reduce tumor growth was assessed by immunodetection of VEGF level in colonic epithelial cell collected from all groups of mice studied. According to the immunoblotting result, the level of VEGF expression was decreased appreciably in the group treated by TE when compare to emulsion treated positive control. However, the group treated by TO presented the highest level of VEGF expression with no evident of inhibition activity when compare to the positive controls (Fig.8). That is, the unformulated tangeretin suspend in MCT was not capable to produce significantly *in vivo* anti-coloreactal ctumoregensis activity in AOM/DSS murine model.

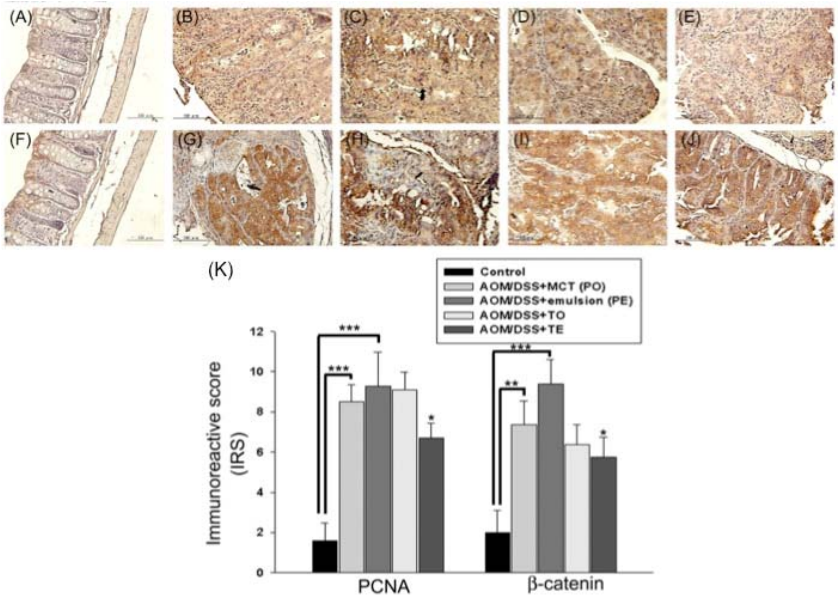

Figure 9: Microscopic picture of immunohistological study. Mice treated by tangeretin MCT suspension and emulsion decrease the expression of PCNA (A-E) and β-catenin (F-J). ***P<0.001, **P<0.01, *P<0.05

Since the occurrence of tumor is the result of unregulated cell growth, the expression of proliferative related protein can serve as important indicator to estimate the progression of tumoregenesis. Under normal condition, PCNA present in the cell nucleus of proliferating cell situated in various part 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%(*30*). In this study, the degree of PCNA elevation in 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, the proportion of browned nucleus was significantly elevated in positives controls (Fig. 9 B and C) when compared to untreated negative control (Figure 9A). As discussed earlier, tangeretin was reported to serve as anti-proliferative agent through withholding cell at G1 phase. The results from our study were well agreed with the previous finding since the administrations of tangeretin considerably lessen the appearance of PCNA in the colonic epithelial cells (Fig 9 D and E). Moreover, the IRS for TE treated group was significantly lowered when reference to the positive controls ($P<0.05$) indicating the reduced proliferative activity within the cells (Fig 9 K).

In present study, the downstream tumoregenesis-related protein expression was found to be variably regulated by treating with tangeretin in both administrated oral formulations. To further elucidate the mechanism that underlies the efficacy of tangeretin in preventing the colitis-related colorectal cancer, the nucleus existence of β-catenin, the well-known regulator protein for inflammation related protein expression(*31*), was studied by IHC method. Being part of the Wnt signaling pathway, β-catenin proteins are involved in the progression of cell cycles that includes 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 condition(*32*). However, abnormal cell phosphorylation cause by chronic inflammation induce the release of β-catenin into cytoplasmic and nucleus. Once β-catenin migrates into the nucleus, it tends to bind to the Tcf DNA binding protein and cause the transcription of down stream carcinogenesis gene expressions. As the positive link between the overexpression of β-catenin to CRC formation had been widely studied by prevailing researches, the modulation of β-catenin mediated signaling pathway then become a major target for CRC prevention(*31-35*). Here, the AOM/DSS treated groups (Fig. 9 G and H) significantly boosted the nucleus concentration of β-catenin indicating higher transcription activities of downstream tumoregenesis genes. After dietary administration 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) presented. Once again, tangeretin emulsion result in significant reduction of β-catenin IRS when compare to 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 tumoregenesis.

Clinical chemistry

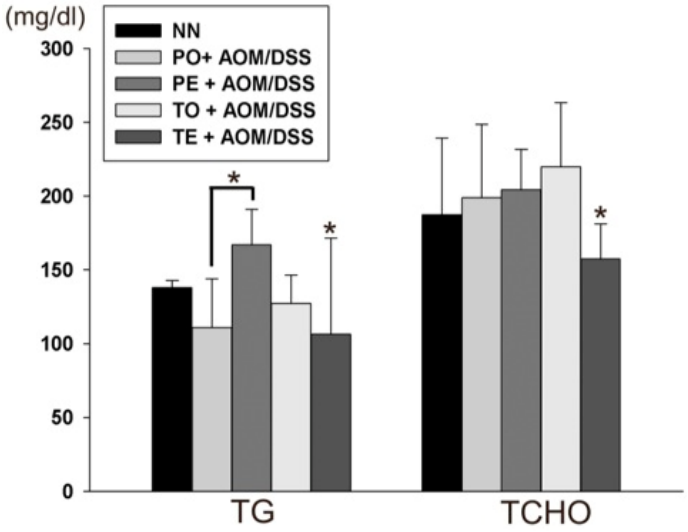

Figure 8: Clinical chemistry analysis on serum TG and TCHO level of experimental mice from AOM/DSS induced colon tumeregenisis study. Tangeretin emulsion significantly

In this study, both MCT suspension and emulsion system used for tangeretin oral administration were primarily composed of lipid. The association of lipid metabolic syndrome to the colorectal carcinogenesis was well investigated(*36-39*). From earlier discussion, mice group fed with TE exhibited the lowest average weight among all experimental groups through out the study. To reveal whether the ability of tangeretin to prevent colonic tumoregenesis also correlated to the regulation of serum lipid metabolism, the serum TG and TCHO was analyzed (Fig. 8). While the dietary administration of blank emulsion produce 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, TE treated group exhibited noteworthy drop ($P<0.05$) when compare to 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 tangeretin. Moreover, interesting decrease in the serum TG and TCHO level after tangeretin oral application suggested that blood lipid regulation ability of tangeretin might be worthwhile for future investigation.

Conclusion

The result from in this study clearly indicated that the emulsion-based delivery system was an effective application method to enhance the bioavailability and efficacy of tangeretin as shown in the *in vitro* and *in vivo* studies. Resulting from the lipophilic nature, the insufficient oral bioavailability of tangeretin leads to the low *in vitro* and *in vivo* correlation in terms of the biological efficacy. Herein, the emulsion system served as a platform to increase the efficacy correlation between the *in vitro* and *in vivo* investigations. Depends on the design of different emulsion systems, the strategy that each system utilized to enhance the efficacy of bioactive compounds varies considerably. In this study, the applied emulsion system was designed to improve tangeretin efficacy through improving the aqueous solubility, gastric retention time, intestinal uptake, and metabolic stability. As shown in present study, the unformulated tangeretin showed insignificant dose dependent reduction in HT29 and HCT116 colon cancer cell lines. After incorporation of emulsion delivery system, the dose efficiencies of tangeretin were significantly improved at higher application concentrations. Moreover, the *in vivo* anti-tumoregenesis efficacy of tangeretin also significantly enhanced when emulsion oral formulation was applied.

As chronic inflammation was reported to be one of the leading causes of CRC, the AOM/DSS-induced colitis related colon carcinogenesis model was developed as pre-clinical animal study for therapeutic evaluation. The regulation of β-catenin expression and translocation was an established pathway to inhibit the transcription of downstream inflammation and proliferation related gene expression. Despite of many in vitro investigation reported anti-cancer efficacy, the oral ingested 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 seen when tangeretin was orally applied as part of the emulsion delivery system. In emulsion treated mice groups, the increased β-catenin regulation result in lower expression of COX-2 inflammation cytokine and proliferative related proteins expression. In summery, this study clearly indicated that applied emulsion-based delivery system was an effective approach to increase the bioefficacy of tangeretin. Moreover, to best of our knowledge, this is the first study of long-term *in vivo* evaluation on the effect of oral delivery system on the efficacy of nutraceuticals. The result from this study showed that the delivery systems are indeed worthwhile investigations that will allow the efficient oral application of bioactive compound.

Future Work

The potential toxicity of the nanoemulsions-encapsulated tangeretin using in vivo animal model is still undergoing in PI's laboratory, and is expected to finish by the end of 2013.

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Final Project Report

Development of a Center of Excellence for Food Safety Risk Analysis Decision Making Tools

Dr. Donald Schaffner

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Rutgers University

Center for Advanced Food Technology

Cooperative Research & Development

I. Project Title

Development of a Center of Excellence for Food Safety Risk Analysis Decision Making Tools

II. Personnel

Don Schaffner (Rutgers University) in the project director.

Marc Knowlton (RU) the systems administrator who runs the Rutgers University IIS webserver platforms.

Rob Muldowney (RU) will support the software.

Todd Ruthman (RSI) is the programmer who built T2P2.

Greg Burnham (Natick) is the one who commissioned the software (retired)

Steve Moody (Natick) is the project officer.

III. Summary

The project start was delayed by adverse weather in the Fall of 2012. The required software was ordered November 17, 2012. The change from the originally proposed project (Development of a generic risk assessment for managing *Salmonella* risk in low moisture foods) to the new project (Development of a Center of Excellence for Food Safety Risk Analysis Decision Making Tools) required a change to the budget allocation. This was accomplished December 11, 2012. The software license code was obtained

January 4, 2013. Final changes to the software on the RSI server were accomplished in February 2013. Testing of the installation on the Rutgers University server took place in March 2013. The software was tested by a small group of beta testers on the Rutgers University server.

IV. Background

The aim of the project is to deliver on-line hosting for predictive modeling tool (T2P2) that enables users to describe the time-temperature chain of a food being handled, stored and prepared (for example X days of refrigeration at specified temperatures, followed by Y hours of cooking etc.). Using predictive microbiology models, the tool will generate an estimate of the overall log changes in selected pathogens that would be predicted to occur given the time-temperature chain described by the user.

The current application requires the following:

- Windows Server 2003 with IIS 6.0 and the Microsoft .NET Framework Version 3.5 (the application should run on more recent versions of Windows server as well)
- Microsoft SQL Server Express or Microsoft SQL Server. The application currently uses SQL Server Express 2005 but could be upgraded if required.
- Lumina's Analytica Decision Engine 4.4 (32 bit version) (www.lumina.com)

V. Specific Objectives

Initial scoping meeting: Will occur prior to funding - Complete

Trial version up and running: Within 3 month of funding - Complete

Final risk models up and running: Twelve months after funding - Complete

VI. Materials and Methods

This project uses three software programs (Windows Server 2003 with IIS 6.0 and the Microsoft .NET Framework Version 3.5; Microsoft SQL Server Express; and Lumina's Analytica Decision Engine 4.4 - 32 bit version) to deliver on-line hosting for predictive modeling tool (T2P2) that enables users to describe the time-temperature chain of a food being handled, stored and prepared.

VII. Results and Discussion:

N/A