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Safety evaluation of tangeretin and the effect of using emulsion-based delivery system: Oral acute and 28-day sub-acute toxicity study using mice

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ABSTRACT

Polymethoxyflavones, found widely in the peel of citrus fruits, is an emerging group of bioactive compounds with wide arrays of disease prevention functionalities. To understand the potential oral toxicity, tangeretin, being one of the most abundant polymethoxyflavones from natural sources, was used as model compound for the safety evaluation. Acute oral toxicity study was conducted using both male and female mice giving 1000, 2000, or 3000 mg/kg body weight (bw) of tangeretin in oil suspension from single gavage administration. No evidence of death was observed during 14-day post-administration period. Alterations of the hepatic cell and clinical chemistry profile increased dose dependently and exhibited distinct injury recovery pattern among different sexes. To determine the potential safety concern related to emulsification, the sub-acute toxicity of tangeretin in emulsion was evaluated and compared with un-processed oil suspension when conducting the sub-acute toxicity study over 28 days. In the sub-acute study, emulsion system did not induce a significant increase of toxicity response. However, the daily low-dose application of tangeretin showed U-shaped dose–response pattern in regard to hepatic alteration. The result from this study can serve as a good safety reference for future application of polymethoxyflavone as a functional ingredient in food.

1. Introduction

As significant effort has been dedicated to promote better human health, numerous active compounds derived from dietary sources have been identified as potential agents to prevent or treat human diseases (Chen et al., 2014; Chiou et al., 2014). However, the actual therapeutic effectiveness of these bioactive ingredients is often reduced by their unfavorable interactions with the physiological environment after they are consumed. That is, depending on the chemical structures, different limiting factors, such as pH instability, rapid degradation, low aqueous solubility, poor membrane permeability, and extensive metabolism by the human body, have been identified to prevent bioactives from being efficiently utilized by the biological system. In this sense, much research is now devoted to formulating various delivery systems for overcoming the potential biological challenges that each active compound may encounter. Various processing technologies and ingredients have been used to produce tailored delivery vehicles of various sizes, chemical and physical properties that could effectively change the biological fate of targeted active ingredients (Ting, Jiang, Ho, & Huang, 2014a).

Tangeretin, one of the polymethoxyflavones isolated from citrus peel, is recognized for its broad health benefits including anti-inflammation (Chen, Weng, & Lin, 2007), anti-proliferation (Pan, Chen, Lin-Shiau, Ho, & Lin, 2002), anti-carcinogenesis, neuro-protection (Datla, Christidou, Widmer, Rooprai, & Dexter, 2001), and inhibition of cardiovascular diseases (Kurowska, Manthey, Casaschi, & Theriault, 2004) and diabetes (Sundaram, Shanthi, & Sachdanandam, 2014). However, despite its health-promoting properties, the application of tangeretin as a potential oral therapeutic agent is greatly attenuated due to its low oral bioavailability (Manthey, Cesar, Jackson, & Mertens-Talcott, 2010). The hydrophobic chemical structure of tangeretin results in its poor solubility in the aqueous environment of the gastrointestinal tract and reduces the absorption of tangeretin by intestinal enterocytes.

Due to its high melting point, tangeretin appears crystallized at room temperature and is difficult to remain stable in oral formulations without sufficient viscosity (Li, Zheng, Xiao, & McClements, 2012). Thus, in our previously published research, an optimized lecithin-based viscoelastic emulsion (LE) system composed solely of generally recognized as safe (GRAS) ingredients was formulated for the oral delivery of tangeretin (Ting, Xia, Li, Ho, & Huang, 2013). Due to the viscoelastic

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property, lecithin-based emulsion system was able to contain more than 2.5% of tangeretin with exceptional storage stability (Ting et al., 2013). Therefore, before proceeding to clinical evaluation of such formulation, it is necessary to evaluate the potential changes in toxicity and side effects that could be imposed by this emulsion system. To serve this purpose, a 28-day sub-acute oral toxicity study was conducted using both male and female ICR mice fed with tangeretin in either oil suspension or lecithin emulsion. In addition, to better define the safety profile of tangeretin, the present investigation also included a 14-day acute toxicity study at a dose up to 3000 mg/kg bw tangeretin as medium-chain triglyceride (MCT) suspension. To the best of our knowledge, this is the first report on the toxicity of tangeretin as well as the first assessment of sub-acute safety on the emulsion delivery system for dietary bioactives.

2. Materials and methods

2.1. Materials

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

2.2. Preparation of tangeretin viscoelastic emulsion

Emulsified tangeretin was produced using the method described in our previously published paper (Ting et al., 2013). To describe the process briefly, the dispersed phase of LE was prepared by placing tangeretin and emulsifier (lecithin) into the carrier oil (100% MCT) that was pre-heated to 130 °C. Until all materials were completely solubilized, the dispersed phase was immediately cooled to 70 °C and the pre-heated aqueous phase (100% double deionized water at 70 °C) was added. Subsequently, the mixture was continuously stirred on a heated plate (70 °C) until a crude emulsion was formed. To avoid blockage of the high-pressure homogenization device (EmulsiFlex-C6, Avestin Inc., Ottawa, Canada), high-speed homogenization (ULTRA–TURRAX T-25 basic, IKA Works Inc., Wilmington, NC, USA) was first applied to the crude emulsion at a speed of 24,000 rpm, which resulted in the reduction of emulsion viscosity. Finally, approximately 25–30 g of stable LE samples was gathered from each processing batch after undergoing pressure treatment at 500 bar under elevated temperature (55 °C). Similarly, the tangeretin oil (MCT) suspension was prepared by directly mixing tangeretin with MCT oil, and then preheated to 130 °C before cooled to ambient temperature. The tangeretin oil suspension was vortexed for 5 min before being given to the mice.

2.3. Loading concentration analysis of tangeretin emulsion

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

2.4. Experimental animals

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

2.5. Oral acute toxicity assay

After 1 week of acclimation, mice were randomly divided into control and experimental groups receiving different treatment doses. The animals were divided into five groups containing 5 male and 5 female mice. The oral acute toxicity of tangeretin was conducted with procedures according to the Organization for Economic Co-operation and Development (OECD) Guidelines 423. All animals were fasted for 12 h before dose administration. Taking into account that oil might affect the physiological condition of the mice, 2 out of 5 groups of mice were used as control groups, in which one of the groups was fed with 200 μL of DI water and the other with 200 μL of MCT. Three dose levels of tangeretin at 1000 mg/kg bw, 2000 mg/kg bw, 3000 mg/kg bw were administered to mice at equal feed volume (200 μL). Due to limitations on the loading of LE, tangeretin tested for acute toxicity was only administered to mice as suspension in MCT. Food was returned to the animal approximately 2 h after treatment. Following administration, mice were closely observed every 30 min during the first 6 h, and, subsequently, checked daily for 14 days, for signs of toxicity, recovery from toxic effect and incidence of mortality. Body weights of mice were measured every two days until they were sacrificed at day 14.

2.6. Oral sub-acute toxicity

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

2.7. Clinical test parameters

At the end of the study, blood samples were collected via cardio puncture after sacrifice using CO_2. To prepare the blood sample for clinical testing, blood samples were mixed with 10 μL of sodium heparin (5000 I.U./mL) and centrifuged for 10 min at 5000 *g* and 4 °C. After centrifugation, the clear plasma sample was carefully withdrawn and stored at −80 °C until further analysis. Glutamic–oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), triacylglycerol (TG), total cholesterol (TCHO), and high-density lipoprotein (HDL) were analyzed by an enzyme method using Fuji DRI-CHEM4000 (Fujifilm) and Fuji DRI-CHEM slides (GOT/AST-P3, GPT/ALT-P3, TG-P3, TCHO-P3, and HDL-P3 FUJIFILM). For the clinical test, 10 μL of plasma was used in each analysis.

2.8. Necropsy

Upon sacrifice, the vital organs from each group of the mice were collected. Liver, heart, spleen, lung and kidney were obtained, weighed, photographed, and examined for signs of toxicity. The data (expressed