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Lipid-soluble green tea extract: Genotoxicity and subchronic toxicity studies

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ABSTRACT

To assess the potential safety of lipid soluble green tea extract, also referred to as lipid soluble tea polyphenols (LSTP), a series of genotoxicity tests were conducted, including an Ames, *in vivo* mouse micronucleus, and *in vivo* mouse sperm abnormality test. The toxicity of LSTP was evaluated in 90- and 30-day feeding studies. LSTP did not show mutagenic activity in the Ames test and no genotoxic potential in the *in vivo* assays at doses up to 10 g/kg body weight (bw). In the 90-day feeding study, LSTP was given in the diet at levels providing 0, 0.125, 0.25, or 0.50 g/kg bw/day. No significant effects were noted on body weight, food consumption, hematology, clinical chemistry, organ weights, and histopathological examination. The no-observed-adverse-effect level (NOAEL) was therefore considered to be 0.50 g/kg bw/day, the highest dose tested. Likewise, dosing of SD rats by gavage for 30 days also showed no adverse effects of growth, hematology, clinical chemistry, organ weights, or histopathology at doses of 0.58, 1.17, and 2.33 g/kg bw/day. The NOAEL in the 30-day study was considered to be the highest dose tested. These data provide evidence to support the safe use of LSTP in food.

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1. Introduction

Phenolic compounds present in fruits and leafy vegetables have long been studied for their potential health benefits (Williamson et al., 2011; Rosen, 2012; Bhardwaj and Khanna, 2013; Khan and Mukhtar, 2013; Howes and Simmonds, 2014; Khalesi et al., 2014; Vuong, 2014; Butt et al., 2015; Legeay et al., 2015; Matsui, 2015; Li et al., 2016), in particular with regard to antioxidant, anti-inflammatory, anti-carcinogenic, anti-thrombotic, thermogenic, and hepatoprotective activities, among others (Middleton et al., 2000; Scalbert et al., 2005; Soobrattee et al., 2005, 2006). Green tea, and its extracts, is a rich source of polyphenolics. Supplements containing green tea extracts are commonly consumed for weight loss (NTP, 2016).

Tea produced from the leaves of the *Camellia sinensis* (L.) Kuntze (*Thea sinensis* L.) plant is a heterogeneous mixture of polyphenols, with the monomeric flavan-3-ols being the predominant form (Abdel-Rahman et al., 2011; Clifford et al., 2013). The flavan-3-ols present in green tea are often broadly referred to as "green tea catechins". Flavan-3-ols have 2 chiral centers (*i.e.*, at C2 and C3) and therefore can exists as 4 different diastereoisomers (Tsao, 2010). The predominant flavan-3-ols that are present in green tea leaves and their aqueous extracts include (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG) and (+)-catechin (Seto et al., 1997; Feng, 2006; Clifford et al., 2013; Blumberg et al., 2015). Smaller amounts of other flavan-3-ols such as catechin, catechin gallate (CG), gallocatechin (GC) and gallocatechin gallate (GCG) may also be present (Feng, 2006; Clifford et al., 2013; Blumberg et al., 2015).

While there is considerable evidence supporting the potential health benefits of consumption of green tea polyphenolics (Rosen, 2012; Bhardwaj and Khanna, 2013; Khan and Mukhtar, 2013; Howes and Simmonds, 2014; Vuong, 2014; Butt et al., 2015;

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; bw, body weight; DMSO, dimethyl sulfoxide; EGCG, (−)-epigallocatechin gallate; GRAS, Generally Recognized as Safe; HDL, HDL Biological Co. Ltd; HPLC, high-performance liquid chromatography; KBN, Kemin Bioscience (Ningbo) Co., Ltd.; LD_{50}, median lethal dose; LSTP, lipid-soluble tea polyphenols; NCE, normochromatic erythrocytes; NOAEL, no-observed-adverse-effect level; PCE, polychromatic erythrocytes; RBC, red blood cells; WBC, white blood cells.

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Legeay et al., 2015; Matsui, 2015), not just from tea, but from other dietary sources, green tea catechins are water soluble and thus are not soluble in fat-based food matrices. To address this limitation, Kemin Food Technologies and its sister company, Kemin Bioscience (Ningbo) Co., Ltd. (KBN), (formerly known as YuYao HDL Biological Co. Ltd, [HDL]), wherein HDL created a process to react green tea extract with palmitoyl chloride; KBN as the successor corporation to HDL uses the process to manufacture lipid soluble green tea extract for Kemin Food Technologies. This process renders the extract products lipid soluble.

In addition to catechin palmitates, HPLC analysis further identified that the amount of free palmitic acid to be approximately 12–15%. Only small amounts (1–2%) of free catechins, gallic acid, and alkaloids (caffeine, theobromine, and theophylline) are present. The preparation also contains approximately 2–6% moisture, ash and protein.

Lipid soluble green tea extract (FEMA No. 4812) has been reviewed by the Flavor & Extract Manufacturers Association (FEMA) Expert Panel, who determined its use as a flavor modifier in a number of foods to be "Generally Recognized as Safe" (GRAS) (Cohen et al., 2015; FEMA, 2016). Lipid soluble green tea extract is used to modify the overall flavor and/or taste profile in creamy, buttery, and sweet foods (*i.e*., to reduce excessive sweetness or buttery tastes in foods) (FEMA, 2016). Given the lipid soluble nature of this substance, additional uses, including its use as an antioxidant, are expected in the future.

While the use of lipid soluble green tea extract is considered GRAS for use as a flavor modifier, additional uses in fat-based foods are expected to lead to increased human exposure to lipid soluble green tea extract. As a result, it was considered prudent to conduct a series of studies to further demonstrate the safety of lipid soluble green tea extract, especially given that there are reports in the literature of green tea or polyphenol-associated cases of hepatotoxicity (Schönthal, 2011; Teschke et al., 2012; García-Cortés et al., 2016). Also, subchronic and chronic toxicity studies in rats and mice administered green tea extract by gavage or in the diet have indicated some potential for liver toxicity at very high doses (*i.e*., ≥ 500–1000 mg/kg bw/day) (Takami et al., 2008; Chan et al., 2010; Wang et al., 2012; Saleh et al., 2013; NTP, 2016) and effects on the nasal cavity at lower doses (*i.e*., 62.5 mg/kg bw/day) (Chan et al., 2010; NTP, 2016). In these studies, the fasted state of animals prior to test article administration was not reported (Takami et al., 2008; Chan et al., 2010; Wang et al., 2012; NTP, 2016) except in the study by Saleh et al. (2013) although the Takami et al. (2008) study dosed green tea extract *via* the feed (i.e., non-fasted). Potential for toxicity appears related to the nature and amount of catechin content contained within the extract.

Given the foregoing, reported herein are the results of several genotoxicity studies, including an Ames assay, an *in vivo* mouse micronucleus test, and an *in vivo* mouse sperm abnormality assay, as well as of repeated dose 90- and 30-day toxicity studies conducted in rats. These studies support the safe use of lipid soluble green tea extract in foods.

2. Materials and methods

2.1. Test substance

LSTP was provided by Yuyao Hudelong Biological Products Co., Ltd. (Batch No. 20090902-2) as a light brown powder and stored at ambient conditions (temperature 20–25 °C, humidity 40–70%) for testing.

Due to the complex nature of plant extracts, the quantification of each single compound in the palmitoylated green tea catechins preparation is not currently feasible. The product tested has been analyzed for the presence of total catechin palmitates by a colorimetric assay utilizing the interaction between ferrous tartrate and tea polyphenol using an external standard, ethyl gallate (Wang et al., 1997). In this assay, it was assumed that ethyl gallate has the same extinction coefficient as catechin palmitates.

Following the initial assay, high-performance liquid chromatography-mass spectrometry (HPLC-MS) was then utilized to identify individual catechin palmitates in the preparation and it was found that lipid-soluble tea polyphenols (LSTP) are composed of catechin mono-, di-, and tri-palmitates, with mono-palmitate being the most abundant form of catechin palmitate rather than dipalmitate. Therefore, the purity calculation was adjusted to convert ethyl gallate to one of the representative mono-palmitates, EGCG mono-palmitate, using the ratio of the molecular weights of the two. Based on the results of these analyses, the typical ranges of the components of the material have been determined and are summarized in Table 1.

2.2. Genotoxicity studies

The series of genotoxicity studies were conducted at the Institute of Health Food, Zhejiang Academy of Medical Sciences, Hangzhou, China.

For the *in vivo* genotoxicity tests, germ-free ICR mice were provided by the Experimental Animal Center of Zhejiang Province [Medical Test Animal Certificate No. SCXK (Zhejiang) 2008-0033 and Medical Test Animal Facility Certificate No. SYXK (Zhejiang) 2005-0074]. Animals were housed individually in stainless steel cages and acclimated to the laboratory conditions (temperature 20–25 °C, humidity 40–70%) for 3 days prior to study initiation.

2.2.1. Bacterial reverse mutation assay (Ames test)

The bacterial reverse mutation assay was performed in *Salmonella typhimurium* TA97a, TA98, TA100, and TA102 provided by the Shanghai Municipal Center for Disease Control (Shanghai, China). The test strains were authenticated through biological identification. The test was conducted using the plate incorporation method, where 0.5 g of LSTP was weighed, ground in a mortar with dimethyl sulfoxide (DMSO), diluted to 10.0 mL, and sterilized in boiling water for 30 min. The sample was diluted to each concentration with sterile DMSO. Five concentrations of 8, 40, 200, 1,000, and 5000 μg/plate were tested in triplicate, with and without S9 metabolic activation based on the results of a preliminary toxicity test (data not shown). The negative control (sterile water), solvent control (sterile DMSO), and positive controls were prepared concurrently with the treatment groups. The positive controls used include 2 μg/plate sodium azide (NaN_3), 50 μg/plate Dexon dimethylamino benzene diazonium sulfonate (fenaminosulf), 10 μg/plate 2-acetylaminofluorene (2-AAF), and 25 μg/plate 2-hydroxyanthraquinone (2-HA). The number of revertant colonies was manually counted. The test was repeated under the same conditions.

2.2.2. In vivo mouse bone marrow micronucleus assay

Five male and 5 female ICR mice, weighing 25–28 g, were randomly assigned to 5 groups. Mice were treated by gavage (20 mL/kg) with either vegetable oil (negative control), 40 mg/kg body weight cyclophosphamide (positive control), or LSTP at a dose of 2.5, 5.0, or 10.0 g/kg body weight. These doses were administered twice, on 2 separate days. Animals were killed by cervical dislocation 6 h following administration of the second dose. The sternal bone marrow was extracted for preparation of bone marrow smears, which were fixed with methanol and stained with Giemsa. For each animal, 1000 polychromatic erythrocytes (PCE) were counted and the incidence of micronucleated PCE and the ratio of

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