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Genotoxicity evaluation of the flavonoid, myricitrin, and its aglycone, myricetin

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ABSTRACT

Myricitrin, a flavonoid extracted from the fruit, leaves, and bark of Chinese bayberry (*Myrica rubra* SIEBOLD), is currently used as a flavor modifier in snack foods, dairy products, and beverages in Japan. Myricitrin is converted to myricetin by intestinal microflora; myricetin also occurs ubiquitously in plants and is consumed in fruits, vegetables, and beverages. The genotoxic potential of myricitrin and myricetin was evaluated in anticipation of worldwide marketing of food products containing myricitrin. In a bacterial reverse mutation assay, myricetin tested positive for frameshift mutations under metabolic activation conditions whereas myricitrin tested negative for mutagenic potential. Both myricitrin and myricetin induced micronuclei formation in human TK6 lymphoblastoid cells under conditions lacking metabolic activation; however, the negative response observed in the presence of metabolic activation suggests that rat liver S9 homogenate may detoxify reactive metabolites of these chemicals in mammalian cells. In 3-day combined micronucleus/Comet assays using male and female B6C3F1 mice, no induction of micronuclei was observed in peripheral blood, or conclusive evidence of damage detected in the liver, glandular stomach, or duodenum following exposure to myricitrin or myricetin. Our studies did not reveal evidence of genotoxic potential of myricitrin *in vivo*, supporting its safe use in food and beverages.

1. Introduction

Flavonoids occur naturally and are a common constituent of higher plants. Myricitrin, a 3-*O*-rhamnoside of myricetin (Fig. 1), is a member of the flavonol class of constituents extracted from the fruit, leaves, and bark of the Chinese bayberry (*Myrica rubra* SIEBOLD) and other edible plants (Chen et al., 2013; Kim et al., 2013; Shimosaki et al., 2011). Myricitrin is thought to offer a variety of potential health benefits, displaying anti-mutagenic (Edenharder and Grunhage, 2003), antioxidant (Chen et al., 2013; Domitrovic et al., 2015), anti-inflammatory (Domitrovic et al., 2015; Kim et al., 2013; Meotti et al., 2006b; Shimosaki et al., 2011), and anti-nociceptive (Meotti et al., 2006a) activities in experimental models. Myricitrin extracted from the Chinese bayberry is used as a flavor modifier in snack foods, dairy products, and beverages in Japan. Myricitrin is listed as “generally recognized as safe” by the U.S. Flavor and Extract Manufacturer Association and was recently judged to be safe at current estimated dietary exposures by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) (JECFA, 2014b). Specifications related to the identity and purity of myricitrin for use as a flavoring agent have been established by JECFA (JECFA, 2014a, b).

Bioavailability of dietary myricitrin occurs in the small intestine upon hydrolysis and release of the aglycone, myricetin (Bravo, 1998). Unabsorbed myricitrin is converted to myricetin by colonic microflora, with urinary excretion of phenylacetic acid metabolites

Abbreviations: EFSA, European Food Safety Authority; FAO, Food and Agriculture Organization of the United Nations; FDA, US Food and Drug Administration; GLP, Good Laboratory Practice; JECFA, Joint FAO/WHO Expert Committee on Food Additives; MN, micronucleus or micronuclei; MN-RET, micronucleated reticulocyte(s); OECD, Organization for Economic Cooperation and Development; RET, reticulocyte(s); WHO, World Health Organization.

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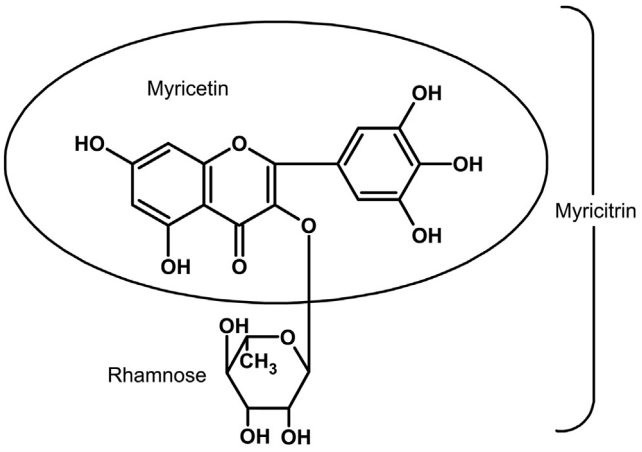

Fig. 1. Chemical structures of the flavonols, myricitrin and myricetin. Myricitrin is a 3-*O*-rhamnoside of myricetin.

(Griffiths and Smith, 1972). The liver is a primary site of metabolism of myricetin absorbed by the gastrointestinal tract (Ong and Khoo, 1997). Myricetin also occurs ubiquitously in plants and is consumed in vegetables, fruits, and beverages such as tea and wine. Like myricitrin, myricetin is purported to have multiple potential therapeutic effects, exhibiting anti-carcinogen, anti-inflammatory, anti-atherosclerotic, antithrombotic, anti-diabetic, and anti-viral properties (Ong and Khoo, 1997). Interestingly, it also has been shown to exert both anti- and prooxidant effects, as well as exhibit mutagenic and anti-mutagenic potential, suggesting a possible dual role in mutagenesis and carcinogenesis (Anderson et al., 1998; Camoirano et al., 1994; Delgado et al., 2008; Duthie et al., 1997a, 1997b; Ong and Khoo, 1997; Sahu and Gray, 1993). Although myricetin is widely available to consumers in some markets, offered as a natural diet supplement, criteria for identity and purity have not been established by any regulatory agency.

Although some bacterial mutagenicity and *in vitro* alkaline DNA unwinding results exist demonstrating the DNA damaging potential of myricetin (Brown and Dietrich, 1979; Camoirano et al., 1994; Hardigree and Epler, 1978; MacGregor and Jurd, 1978; Sahu and Gray, 1993; Uyeta et al., 1981), no comprehensive testing data are available related to the genotoxic potential of highly purified myricitrin and myricetin. The European Food Safety Authority (EFSA) guidances (EFSA, 2010, 2011) recommend a tiered approach for assessing genotoxic potential, typically beginning with a mutagenicity test using multiple strains of bacteria and an *in vitro* test for induction of gene mutation and/or structural and numerical chromosome damage (i.e., micronucleus or chromosomal aberration assay) in mammalian cells. As necessary, follow-up testing should be conducted using a suitable *in vivo* test. For the safety assessment of food ingredients, the US Food and Drug Administration (FDA) also recommends a bacterial gene mutation test and an *in vitro* test for chromosomal damage or gene mutation in mammalian cells, as well as an *in vivo* test for chromosomal damage using mammalian hematopoietic cells (FDA, 2000a), such as the rodent erythrocyte micronucleus (MN) assay (Heddle, 1973; MacGregor et al., 1980; Schmid, 1975). Use of the alkaline (pH > 13) Comet assay (Tice et al., 2000) is typically recommended as a follow-up or supplemental test to measure genotoxicity in a tissue other than bone marrow, with emphasis placed on liver and/or likely targets of exposure to a test chemical or its metabolites (Brendler-Schwaab et al., 2005; EFSA, 2010, 2011; ICH, 2012). This *in vivo* assay is considered a useful indicator test in terms of its sensitivity to substances which cause gene mutations and/or structural chromosome aberrations *in vitro* (EFSA, 2010, 2011; Kirkland and Speit, 2008; Sasaki et al., 2000). Recently, a new Organization for Economic Cooperation and Development (OECD) test guideline (TG 489) was adopted (to take effect in 2016) for utilizing the Comet assay in genotoxicity profiling in rodents (OECD, 2014a). In support of an awaited positive safety opinion from JECFA and the planned eventual global marketing of products containing myricitrin, highly purified myricitrin was evaluated in a Good Laboratory Practice (GLP) test battery compliant with EFSA, OECD, and FDA guidances on genotoxicity testing (EFSA, 2010, 2011; FDA, 2000a, b, c; OECD, 1997a, b, 2010). Since trace amounts of myricetin are present in purified myricitrin and it is a known myricitrin metabolite, its genotoxic potential was also evaluated. Specifically, these flavonols were evaluated in a bacterial reverse mutation assay (Ames et al., 1975; Gatehouse et al., 1994; Maron and Ames, 1983) using *Salmonella* and *E. coli* tester strains and an *in vitro* MN assay (Avlasevich et al., 2011; Bryce et al., 2008) using the human TP53 competent TK6 lymphoblastoid cell line. In addition, as a thorough approach to assessing *in vivo* genotoxicity (Pfuhler et al., 2007; Rothfuss et al., 2011), a combined MN/Comet assay was conducted in male and female B6C3F1 mice (Hobbs et al., 2012; Recio et al., 2010). The MN/Comet assay protocol used in our studies minimizes the use of experimental animals and complies with OECD Test Guideline 474 for the MN assay, as well as recommendations for the conduct of the Comet assay (Burlinson et al., 2007; Tice et al., 2000). The results of this comprehensive battery of genotoxicity tests using highly purified myricitrin and myricetin are reported.

2. Material and methods

2.1. Chemical analysis

All genotoxicity assays were conducted according to OECD guidelines and were GLP-compliant. Samples removed from the top, middle, and bottom fractions of each chemical formulation were submitted for analytical testing (Alera Laboratories, LLC, Durham, NC). All analyzed dose formulations were within 10% of nominal concentrations. Myricitrin (>97% pure; CAS No. 17912-87-7; San-Ei Gen F.F.I., Inc., Osaka, Japan) and myricetin (>98% pure; CAS No. 529-44-2; San-Ei Gen F.F.I., Inc., Osaka, Japan) were determined to be stable for at least 8 days when prepared in corn oil (MP Biomedicals, LLC, Solon, OH) and stored at room temperature and up to at least 4 days when prepared in dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO) and stored refrigerated.

2.2. Bacterial reverse mutation assay

Mutagenicity assays of myricitrin and myricetin, with and without metabolic activation, were conducted as described previously (Ames et al., 1975; Maron and Ames, 1983; Mortelmans and Zeiger, 2000) using the following five *Salmonella* and *E. coli* strains as prescribed in the guideline for the bacterial reverse mutation assay (OECD 471): TA98, TA100, TA97a, TA1535, and *E. coli* WP2 *uvrA* (pKM101). All strains (Moltox, Inc., Boone, NC) were checked for maintenance of genetic markers prior to the study. The results of a 5-strain range-finding assay of myricitrin were negative; therefore, a top concentration of 5 mg/plate, with and without metabolic activation, was chosen as recommended by OECD (OECD, 1997a) and Japanese guidelines (JMHLW, 1996) for non-cytotoxic compounds. In a range-finding assay of myricetin, cytotoxicity was observed at varying dose levels, dependent on the bacterial strain, necessitating the testing of different concentration ranges ± S9 in the mutagenicity study. Myricetin was tested at a top concentration of 3000 μg/plate in TA97a (−S9) and *E. coli* WP2 *uvrA* (pKM101) (±S9) and 2000 μg/plate in TA98, TA100, and TA1535

(−S9). A top concentration of 6000 μg/plate was tested in TA97a, TA100, TA98, and TA1535 (+S9). Because of the varying level of cytotoxicity observed in the range-finder assay, dose spacing for the −S9 condition was tightened at the upper end of the range to ensure identification of the cytotoxic concentration; at the low end of the range, concentration intervals were chosen for practicality of dose formulation rather than increase the spacing to half-log. Concentration levels tested below the lowest toxic level observed in the range-finder assay (1500 μg/plate) were chosen to ensure five analyzable concentrations in the mutagenicity study. The concentrations tested under the +S9 condition were selected to match those for the −S9 condition. Strain specific positive controls tested without metabolic activation were 2-nitrofluorene (TA98), sodium azide (TA100 and TA1535), ICR191 (TA97a), and 4-nitroquinoline-N-oxide (*E. coli* WP2). Benzo[a]pyrene and 2-aminoanthracene were used as the positive controls for TA100, and all other strains, respectively, tested with metabolic activation. Metabolic activation was provided by a 10% phenobarbital/benzoflavone-induced rat liver S9 mix with added cofactors (Regensys™ NADPH Regeneration System Reagents, Moltox, Boone, NC). Test solutions were prepared in DMSO. The assay tubes were pre-incubated at 37 °C for 20 min before plating onto minimal agar. Three test plates per concentration were incubated at 37 °C for 48 h and then counted using the Sorcerer/Ames Study Manager System (Perceptive Instruments, Ltd., Suffolk, UK). To ensure accuracy of the results, reagent sterility and automated scoring checks were conducted. Criteria for a positive response were a ≥ 2-fold increase in the average plate count compared to the solvent control for at least one concentration level and a dose response over the range of tested concentrations in at least one strain with or without metabolic activation. In the case of a positive response, colonies were checked for true revertant status by testing for growth on medium lacking histidine (*Salmonella*) or tryptophan (*E. coli*).

2.3. In vitro MN assay

Human TK6 cells (ATCC, Manassas, VA) were cultured and maintained in RPMI 1640 medium containing 10% heat inactivated horse serum plus 1.0% Pluronic F-68, 0.5% sodium pyruvate, and antibiotics (penicillin at 20 Units/mL and streptomycin at 20 μg/mL) at 37 °C, with 6% CO_2. The normal cell cycle time of these cells is approximately 12 h. Metabolic activation was provided using phenobarbital/benzoflavone-induced rat liver S9 (Moltox, Boone, NC) with added Regensys™ cofactors (Moltox, Boone, NC) at a final concentration of 1% S9. The composition of the S9 mix was: 10% S9, 8 mM $MgCl_2$, 32.6 mM KCl, 4.7 mM glucose-6-phosphate, 4 mM NADP, and 0.1 M phosphate buffer. Triplicate cultures of exponentially growing cells seeded at 0.40×10^6 cells/mL in 12-well plates were exposed to myricitrin, myricetin, or controls for approximately 4 h in the presence of metabolic activation (+S9) and approximately 24–29 h in the absence of metabolic activation. Cyclophosphamide and vinblastine were used as the positive controls with and without metabolic activation, respectively. The results of toxicity tests revealed that the concentrations of myricitrin and myricetin required to produce the recommended (OECD, 2010) level of cytotoxicity at the top dose to be quite different. On the basis of several preliminary tests, the doses of myricitrin selected for testing were 5000, 2500, 1500, 1000, 500, and 250 μg/mL for 4 h with S9 and 500, 450, 350, 300, 250, 200, 100, 50, and 20 μg/mL for 24 h without S9; the dose interval in the absence of S9 was tightened because of an apparent steep cytotoxicity response under this condition. Doses of myricetin selected were 75, 50, 20, 10, 5, and 2.5 μg/mL for exposures ± S9. At the end of the 4-h (+S9) treatments, treatment media were replaced with complete medium (−S9) for the remaining culture period; treatment media were left on the cells for the entire culture period for the 24-h (−S9) treatments. At the end of the culture period, cells were analyzed for cytotoxicity and micronucleus induction by flow cytometry using the *In Vitro* MicroFlow™ kit (Litron Laboratories, Rochester, NY) according to manufacturer's instructions. Unless limited by cytotoxicity, 20,000 cells from each sample were analyzed for the frequency of MN using a FACSCalibur™ dual-laser bench top system (Becton Dickinson Biosciences, San Jose, CA).

Cytotoxicity was measured as relative increase in cell count or as relative survival of cells in vehicle control cultures compared to cells in treated cultures using ratios of counted nuclei to counted beads (inert latex microspheres added to each sample). Higher nuclei to bead ratios correspond to greater cell survival. Greater emphasis was placed on relative survival since this flow cytometry-based measurement may provide a more sensitive assessment of cytotoxicity, enhancing assay specificity (Avlasevich et al., 2011). Doses inducing greater than 60% cytotoxicity were excluded from the analysis. An increase in MN frequency ≥ 3-fold over the mean MN frequency in the concurrent vehicle control was considered a positive response.

2.4. Animal husbandry

Male and female B6C3F1 mice (Charles River Laboratories International, Inc.) were 8–10 weeks of age at the time of treatment. Animals were housed in polycarbonate cages with absorbent hardwood bedding in an AAALAC-accredited specific pathogen free facility with a 12 h light/12 h dark cycle. Certified Purina Pico Chow No. 5002 (Ralston Purina Co., St. Louis, MO) and water were provided *ad libitum*. These studies were approved by the ILS, Inc. Institutional Animal Care and Use Committee, and all procedures were completed in compliance with the Animal Welfare Act Regulations, 9 CFR 1–4, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 2011).

2.5. In vivo MN/Comet assay experimental design

Dose range finding studies were conducted to identify appropriate dose levels. Male and female mice were administered myricitrin or myricetin up to and including dose levels of 2000 mg/kg/day (test guideline limit dose) for three consecutive days. No marked changes in body weights were measured in mice administered either chemical and there were no abnormal gross necropsy organ observations. Therefore, for the definitive studies, male and female B6C3F1 mice (5 animals/dose group) were administered myricitrin or myricetin at 2000, 1500, or 1000 mg/kg/day, vehicle (corn oil), or the positive control compound, ethyl methanesulfonate (Sigma–Aldrich, St. Louis, MO) in 0.9% saline (Ricca Chemical Company, Arlington, TX) at 150 mg/kg/day, daily for three days by oral gavage. Three hours after the final dose, peripheral blood was collected for flow cytometric analysis of MN, and liver, duodenum, and stomach (females only) tissues were collected, frozen in liquid N_2, and stored at −80 °C until analysis by the Comet assay (Recio et al., 2012).

2.6. Erythrocyte micronucleus assay

Peripheral blood samples were processed for flow cytometric evaluation of micronucleated reticulocytes (MN-RET) as described previously (Witt et al., 2008). Briefly, cells were fixed and labeled using a MicroFlow^{PLUS} Kit (Litron Laboratories, Rochester, NY) according to manufacturer's directions and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Sunnyvale, CA). For each peripheral blood sample, 20,000 RET were analyzed to determine the frequency of MN-RET. More than 10^6 mature normochromatic

erythrocytes were enumerated concurrently during MN-RET analysis, and the percentage of RET (%RET) among total erythrocytes was calculated as a measure of bone marrow toxicity.

2.7. Comet assay

For each animal, a portion of the left lobe of the liver was placed into a tube containing cold mincing solution [Mg^{2+} and Ca^{2+} free Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA) containing 10% v/v DMSO, and 20 mM EDTA pH 7.4–7.7] and rapidly minced. A portion of the duodenum proximal to the stomach was removed, flushed with mincing solution, and kept cold and moist with mincing solution. The tissue was trimmed and a small section placed in a microcentrifuge tube containing mincing solution and rapidly minced. The stomach was cut open and washed free from food using cold mincing buffer. The glandular stomach was placed into cold mincing buffer and incubated on ice for 15–30 min, then the surface epithelium was gently scraped two times using a scalpel blade. This layer was discarded and the gastric mucosa rinsed with cold mincing buffer. The stomach epithelium was carefully scraped 4–5 times in mincing solution with a scalpel blade to release the cells. The mincing solution containing released epithelial cells was transferred to microfuge tubes. Tissue samples were flash frozen in liquid nitrogen and stored at −80 °C until processed (Recio et al., 2012). A portion of each tissue was fixed in 10% NBF for 24 h, trimmed, and paraffin embedded for possible histopathology evaluation.

For processing, cells were partially thawed in a warm water bath and placed on ice until slide preparation. Cell samples were empirically diluted with 0.5% low melting point agarose (Lonza, Walkersville, MD) dissolved in Dulbecco's phosphate buffer (Ca^{2+}, Mg^{2+} and phenol free) at 37 °C, layered onto each well of a 2-well CometSlide™ (Trevigen, Gaithersburg, MD), and immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, containing freshly added 10% DMSO and 1% Triton X-100) overnight. After rinsing in 0.4 M Trizma base (pH 7.5), slides were treated with alkali (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 20 min, then electrophoresed at 4 °C for 20 min at ~1.0 V/cm, 300 mA. After electrophoresis, slides were neutralized with 0.4 M Trizma base (pH 7.5) for 5 min, incubated for 5 min in ice-cold 100% ethanol (Pharmco-AAPER, Shelbyville, KY) and allowed to air-dry. Slides were stored at room temperature in a desiccator until stained and scored. After staining slides with SYBR Gold™ (Molecular Probes, Invitrogen, Carlsbad, CA), 100 cells were scored per sample at 20x magnification without knowledge of sample identity using Comet Assay IV Imaging Software, Version 4.3.1 (Perceptive Instruments, Ltd., Suffolk, UK). The extent of DNA migration was characterized using the % tail DNA endpoint measurement (intensity of all tail pixels divided by the total intensity of all pixels in the comet, expressed as a percentage). NaCl, Na_2EDTA, Triton X-100, and Trizma base were purchased from Sigma–Aldrich (St. Louis, MO); NaOH and DMSO were purchased from Fisher Scientific (Pittsburgh, PA).

2.8. Statistical analyses

For the *in vivo* MN/Comet assay, body weight data were analyzed using Statistical Analysis System version 9.2 (SAS Institute, Cary, NC). Homogeneity of the data was assessed using the Levene's test with a 95% confidence level. If data were heterogeneous, then appropriate transformation of the data was performed and the data re-analyzed for homogeneity of variance. Data were then analyzed using a one way analysis of variance (ANOVA) and treated groups compared to the appropriate control group using Dunnett's test. Dose-dependent changes were evaluated using

Table 1
Bacterial reverse mutation assay results with and without metabolic activation − myricetin.

Dose (μg/plate)	Mean revertants/plate (±SD) without rat liver S9					Mean revertants/plate (±SD) with rat liver S9				
	TA97a	TA100	TA98	TA1535	WP2 *uvrA*	TA97a	TA100	TA98	TA1535	WP2 *uvrA*
0	105.3 ± 5.8	95.7 ± 15.3	20.7 ± 2.5	10.3 ± 4.0	132.0 ± 10.5	155.0 ± 3.6	94.7 ± 6.5	18.0 ± 4.0	10.0 ± 5.6	171.7 ± 18.5
25	–	87.7 ± 10.1	23.7 ± 2.1	12.3 ± 3.8	–	–	–	–	–	–
50	98.0 ± 7.9	82.7 ± 8.5	29.3 ± 7.5	12.0 ± 1.7	149.7 ± 8.1	–	–	–	–	185.0 ± 17.3
100	104.3 ± 11.7	79.3 ± 15.8	29.0 ± 4.4	7.0 ± 2.0	150.3 ± 16.8	–	–	–	–	195.0 ± 14.5
200	101.3 ± 12.6	80.3 ± 15.5	38.0 ± 3.6	7.7 ± 1.2	148.3 ± 11.0	293.0 ± 24.0	149.0 ± 9.6	59.3 ± 11.5	27.0 ± 14.0	206.7 ± 10.1
500	101.0 ± 4.2	53.3 ± 14.0	37.7 ± 5.1	6.0 ± 4.0	153.7 ± 11.0	235.7 ± 14.0	132.7 ± 26.5	57.0 ± 8.9	7.0 ± 2.6	218.7 ± 14.7
1000	101.7 ± 11.7	0.0 ± 0.0	20.7 ± 3.5	0.0 ± 0.0	114.3 ± 14.6	152.7 ± 15.5	109.0 ± 19.0	72.3 ± 5.5	7.7 ± 4.6	146.7 ± 16.4
2000	70.0 ± 7.9	0.0 ± 0.0	6.7 ± 2.1	0.0 ± 0.0	34.3 ± 1.2	179.3 ± 22.2	75.7 ± 4.2	69.0 ± 26.9	6.0 ± 1.7	98.3 ± 4.6
3000	83.7 ± 9.9	–	–	–	7.3 ± 8.4	332.0 ± 12.1	80.3 ± 25.7	149.0 ± 14.1	6.0 ± 3.6	24.0 ± 24.1
6000	–	–	–	–	–	147.0 ± 33.5	217.3 ± 27.6	199.3 ± 33.4	10.7 ± 2.1	–
Positive control	1986.0 ± 195.8[a]	600.3 ± 36.0[b]	511.7 ± 24.7[c]	588.0 ± 37.2[b]	2761.7 ± 38.7[d]	2105.7 ± 363.9[e]	384.0 ± 35.8[f]	1408.3 ± 116.1[g]	262.0 ± 39.9[e]	1168.7 ± 15.5[h]

[a] ICR191 administered at 0.25 μg/plate.
[b] Sodium azide administered at 1 μg/plate.
[c] 2-Nitrofluorene administered at 3 μg/plate.
[d] 4-Nitroquinoline-N-oxide administered at 0.25 μg/plate.
[e] 2-Aminoanthracene administered at 2.5 μg/plate.
[f] Benzo[a]pyrene administered at 2 μg/plate.
[g] 2-Aminoanthracene administered at 2 μg/plate.
[h] 2-Aminoanthracene administered at 20 μg/plate.

Table 2
Bacterial reverse mutation assay results with and without metabolic activation − myricitrin.

Dose (μg/plate)	Mean revertants/plate (±SD) without rat liver S9					Mean revertants/plate (±SD) with rat liver S9				
	TA97a	TA100	TA98	TA1535	WP2 *uvrA*	TA97a	TA100	TA98	TA1535	WP2 *uvrA*
0	111.0 ± 9.2	72.0 ± 1.0	19.3 ± 4.2	6.7 ± 3.5	141.3 ± 13.0	161.7 ± 6.8	85.7 ± 8.5	30.0 ± 9.9	8.3 ± 3.1	192.3 ± 25.1
250	99.0 ± 4.6	62.7 ± 4.9	31.7 ± 6.0	11.0 ± 2.6	159.3 ± 6.1	115.3 ± 5.9	74.7 ± 6.8	23.7 ± 8.1	9.3 ± 0.6	193.0 ± 9.8
500	94.0 ± 17.7	67.0 ± 6.0	29.0 ± 11.8	7.0 ± 2.0	167.3 ± 39.6	127.3 ± 8.7	89.7 ± 6.5	28.3 ± 3.8	13.0 ± 4.0	184.3 ± 21.5
1000	89.3 ± 4.9	70.3 ± 9.5	23.3 ± 9.5	9.0 ± 7.2	141.3 ± 11.2	115.3 ± 10.7	89.7 ± 11.7	27.3 ± 5.9	7.3 ± 1.2	170.7 ± 21.6
1500	101.0 ± 9.8	72.0 ± 10.5	17.3 ± 4.0	6.3 ± 4.2	163.0 ± 21.7	111.0 ± 9.2	76.7 ± 2.3	32.0 ± 8.9	7.0 ± 2.0	171.0 ± 4.6
2500	103.7 ± 2.5	74.3 ± 12.3	20.3 ± 5.1	11.7 ± 4.6	163.3 ± 18.3	123.3 ± 7.6	77.0 ± 10.1	26.3 ± 8.5	10.7 ± 2.5	176.3 ± 19.7
5000	92.0 ± 5.6	71.7 ± 4.9	18.3 ± 4.0	9.0 ± 3.6	160.3 ± 12.7	135.0 ± 11.5	92.7 ± 6.5	34.3 ± 3.5	9.0 ± 6.0	175.0 ± 8.7
Positive control	1169.7 ± 194.2[a]	697.7 ± 49.9[b]	715.0 ± 140.5[c]	614.7 ± 29.4[b]	2680.7 ± 67.7[d]	2293.0 ± 171. 1[e]	368.0 ± 18.7[f]	1960.7 ± 233.1[g]	293.3 ± 12.2[e]	1055.0 ± 61.2[h]

[a] ICR191 administered at 0.25 μg/plate.
[b] Sodium azide administered at 1 μg/plate.
[c] 2-Nitrofluorene administered at 3 μg/plate.
[d] 4-Nitroquinoline-N-oxide administered at 0.25 μg/plate.
[e] 2-Aminoanthracene administered at 2.5 μg/plate.
[f] Benzo[a]pyrene administered at 2 μg/plate.
[g] 2-Aminoanthracene administered at 2 μg/plate.
[h] 2-Aminoanthracene administered at 20 μg/plate.

linear regression. The normality of the MN and Comet data was verified using the Shapiro–Wilk test with a confidence level of 95%. Linearity and variance in the % RET, MN-RET frequency, and DNA migration (% tail DNA) data between treatment groups were evaluated using linear regression and one-way ANOVA analyses using Analyse-it® software for Microsoft Excel. The Dunnett's multiple comparison test was used to determine if a treatment group was significantly different ($p < 0.05$) from vehicle controls. Data that were not normally distributed ($p < 0.05$) were analyzed using the Mann–Whitney test comparing each dose level to the concurrent control and the Kendall rank correlation test to determine the presence of a dose response. For all *in vivo* endpoints, a one-tailed t-test was used to verify a positive response to the reference compound, ethyl methanesulfonate ($p < 0.05$). Criteria for a positive result in the MN and Comet assays were at least one statistically significant dose group ($p < 0.05$), a dose group falling outside the range of laboratory historical control data, and a statistically significant trend test ($p < 0.05$). A test was considered equivocal if only one or two of these conditions were met (OECD, 2014a, b).

3. Results

3.1. Results of the bacterial reverse mutation assays

Mutagenicity assays were conducted to assess the potential of myricitrin and myricetin to induce gene mutations in bacteria. Mutagenic effects of exposure to myricitrin were assessed up to concentrations exceeding the recommended maximum for non-cytotoxic chemicals; no evidence of general toxicity or precipitation was noted in any of the test plates. Myricetin was cytotoxic in all five strains without metabolic activation, and in *E. coli* WP2 *uvrA* pKM101 with metabolic activation. Myricetin precipitated in the assay tubes at doses of 3000 and 6000 μg/plate with S9; the precipitate interfered slightly with plate counts at 6000 μg/plate but not at 3000 μg/plate. Average plate counts for each set of replicate plates are provided in Tables 1 and 2.

Myricetin was not mutagenic up to toxic doses in TA97a, TA100, TA1535, or *E. coli* WP2 *uvrA* (pKM101) with or without metabolic activation; myricetin was also not mutagenic in TA98 without metabolic activation. Myricetin did test positive in TA98 with metabolic activation, demonstrating a dose response and producing revertant counts of more than twice the corresponding vehicle control at all doses tested. True revertant status of the colonies was confirmed. These results indicate that myricetin is mutagenic in the bacterial reverse mutagenicity assay under the conditions of the assay. A positive mutagenic response to myricitrin was not produced in any of the five *Salmonella* or *E. coli* strains tested either with or without metabolic activation. Average revertant values for positive control chemicals, both with and without metabolic activation, were at least 2-fold above concurrent solvent controls. The lack of induction of an increase in revertants or any apparent concentration-dependent response indicates that, under the assay conditions tested, myricitrin is not mutagenic in the bacterial reverse mutation assay.

3.2. Results of the in vitro MN assays

MN are identified by flow cytometry using a combination of characteristics of size (as measured by light scatter) and fluorescence (based on differential staining) that differentiates debris and necrotic and apoptotic cells from healthy cells containing micronuclei (Avlasevich et al., 2006; Bryce et al., 2007). MN frequency and cell viability data for cultures exposed to myricitrin are summarized in Table 3. After approximately 4 h in the presence of metabolic activation, cytotoxicity was less than 50%, within the

Table 3
Micronucleus assay results in TK6 cells exposed to myricitrin.

Dose (μg/mL)	Micronucleus frequency (%)		Apoptotic/Necrotic cells (%)		Relative survival (%)	RICC (%)
	Mean	Fold change	Mean	Fold change	Mean	Mean
4 h with S9						
DMSO	0.54	1.00	14.34	1.00	100.00	100.00
0	0.53	0.98	13.67	0.95	92.94	82.05
250	0.50	0.93	15.11	1.05	95.88	79.68
500	0.54	1.00	14.03	0.98	103.18	99.32
1000	0.54	1.00	14.69	1.02	100.05	85.78
1500	0.54	1.00	15.46	1.08	91.00	97.63
2500	0.59	1.09	20.40	1.42	72.81	65.42
5000	0.73	1.35	28.84	2.01	54.93	54.48
CP	6.50	12.04	29.83	2.08	41.37	25.97
24 h without S9						
DMSO	0.49	1.00	1.96	1.00	100.00	100.00
0	0.54	1.10	2.35	1.20	104.14	93.35
20	0.40	0.82	1.76	0.90	104.67	83.77
50	0.41	0.84	1.74	0.89	92.49	78.59
100	0.39	0.80	1.88	0.96	90.50	89.39
200	0.89	1.82	3.03	1.55	90.85	112.72
250	1.02	2.08	3.90	1.99	84.81	91.63
300	1.80	3.67	7.57	3.86	81.99	69.27
350	2.28	4.65	13.06	6.66	54.79	60.32
400	NS	–	NS	–	NS	25.11
450	NS	–	NS	–	NS	17.00
500	NS	–	NS	–	NS	21.02
VIN	5.02	10.24	10.62	5.42	64.90	62.56

RICC = relative increase in cell count; CP = cyclophosphamide administered at 3.125 μg/mL.
VIN = vinblastine administered at 0.5 ng/mL; NS = not scored due to excessive cytotoxicity.

recommended OECD guideline levels, up to the limit dose of 5000 μg/mL. MN frequencies measured at all tested doses were less than 3-fold higher than the vehicle control. Therefore the overall response was considered negative. The percent of apoptotic and necrotic cells at 5000 μg/mL was approximately 2-fold greater than that measured for the vehicle control cells. Exposure to cyclophosphamide, used as a positive control chemical requiring metabolic activation, resulted in a 12-fold induction of micronuclei as compared to the vehicle control. Cytotoxicity of cultures exposed to myricitrin for approximately 24 h in the absence of metabolic activation was 40–45% at the highest analyzable concentration (350 μg/mL). MN frequencies increased in a concentration-dependent manner and were greater than 3-fold over the corresponding vehicle control at the top two analyzable doses. Therefore, the overall response was considered positive. The percent apoptotic and necrotic cells was 3.9- and 6.7-fold higher than was measured for vehicle control cells at 300 and 350 μg/mL, respectively. Treatment with vinblastine, included as a direct-acting positive control chemical, led to a 10.2-fold higher induction of MN as compared to the vehicle control (Table 3).

The results of exposing TK6 cells to myricetin are summarized in Table 4. Four hours in the presence of metabolic activation resulted in excessive cytotoxicity at the top two doses of 50 and 75 μg/mL and could not be scored. Relative survival at 20 μg/mL was 41.2%; this dose was considered to be the highest analyzable dose for the +S9 test condition. Measured MN frequencies at all analyzable concentrations of myricetin were less than 3-fold higher than the vehicle control. Therefore, the overall response with metabolic activation was considered negative. The percent apoptotic and necrotic cells at 20 μg/mL myricetin was 4.1-fold higher than the vehicle control. Exposure to the positive control, cyclophosphamide, led to a 27.3-fold induction of MN. Exposure of TK6 cells to myricetin at concentrations of 50 μg/mL and above for 24 h in the absence of metabolic activation were excessively cytotoxic and could not be scored. Relative survival at 20 μg/mL was 51.2% with a relative increase in cell count of 47.7%; therefore, 20 μg/mL was considered to be the highest analyzable dose for the −S9 test condition. A dose-dependent increase in MN frequency was observed, up to a 3.7-fold response over the corresponding vehicle control at 20 μg/mL myricetin. Although the response was considered positive without S9, the highest level of MN induction measured falls within the laboratory historical negative control range for the assay. The positive control, vinblastine, induced a 14.2-fold increase in MN formation.

3.3. Results of the in vivo MN/Comet assays

Based on the results of preliminary dose setting studies, combined MN/Comet assays were conducted in which male and female B6C3F1 mice were administered myricitrin or myricetin at 1000, 1500, and 2000 mg/kg/day for 3 consecutive days. No exposure-related clinical signs were noted during the course of the studies with either chemical. There were no statistical differences in final body weight or body weight gain between the animals administered myricitrin or myricetin and the concurrent control animals. A statistically significant decreasing trend in body weight gain in males, but not females, administered myricitrin is not biologically relevant. No dose-dependent body weight change was observed for animals administered myricetin. The positive reference chemical, ethyl methanesulfonate, did induce a statistically significant loss in mean body weight in male, but not female, mice as compared to the vehicle control groups.

Results of flow cytometric analysis of MN-RET and RET frequencies are summarized in Tables 5 and 6. Under the conditions used in the MN/Comet study, no increase in the frequency of MN-RET was observed for male or female mice administered either myricitrin or myricetin. No decrease in the % RET was measured in mice administered these flavonols, indicating a lack of bone marrow cytotoxicity at the tested doses, although female mice administered myricetin exhibited a small, but statistically significant, increase in % RET. There were statistically significant increases in MN-RET in both male and female animals administered ethyl

Table 4
Micronucleus assay results in TK6 cells exposed to myricetin.

Dose (μg/mL)	Micronucleus frequency (%)		Apoptotic/Necrotic cells (%)		Relative survival (%)	RICC (%)
	Mean	Fold change	Mean	Fold change	Mean	Mean
4 h with S9						
DMSO	0.56	1.00	5.93	1.00	100.00	100.00
0	0.56	1.00	4.09	0.69	115.90	113.25
2.5	0.55	0.98	6.86	1.16	105.47	65.42
5	0.57	1.02	7.37	1.24	92.82	54.71
10	0.78	1.39	11.27	1.90	76.06	46.86
20	1.17	2.09	24.42	4.12	41.21	35.18
50	NS	–	NS	–	NS	23.41
75	NS	–	NS	–	NS	26.92
CP	15.28	27.29	46.75	7.88	7.10	–
24 h without S9						
DMSO	0.30	1.00	3.24	1.00	100.00	100.00
0	0.47	1.57	3.05	0.94	98.34	103.91
2.5	0.48	1.60	2.60	0.80	105.98	113.91
5	0.48	1.60	2.73	0.84	97.22	98.26
10	0.64	2.13	3.79	1.17	90.71	76.75
20	1.11	3.70	12.9	3.98	51.23	47.67
50	NS	–	NS	–	NS	1.74
75	NS	–	NS	–	NS	−7.39
VIN	4.25	14.17	23.63	7.29	40.54	–

RICC = relative increase in cell count; CP = cyclophosphamide administered at 12.5 μg/mL.
VIN = vinblastine administered at 1 ng/mL; NS = not scored due to excessive cytotoxicity.

Table 5
Micronucleus assay results in mice administered myricitrin.

Dose (mg/kg/day)	% RET[a]	MN-RET/1000[a]
Males		
0	1.58 ± 0.01	2.58 ± 0.12
1000	1.37 ± 0.15	2.09 ± 0.11
1500	1.65 ± 0.19	2.69 ± 0.19
2000	1.71 ± 0.16	2.54 ± 0.26
EMS	1.11 ± 0.05	11.58 ± 2.19[b]
Females		
0	1.17 ± 0.12	1.71 ± 0.13
1000	1.00 ± 0.10	1.87 ± 0.16
1500	1.01 ± 0.05	1.72 ± 0.17
2000	1.30 ± 0.08	2.02 ± 0.23
EMS	0.77 ± 0.09	13.01 ± 0.62[b]

EMS = ethyl methanesulfonate administered at 150 mg/kg/day.
[a] Group mean ± standard error of the mean.
[b] Significant at $p < 0.05$.

Table 6
Micronucleus assay results in mice administered myricetin.

Dose (mg/kg/day)	% RET[a]	MN-RET/1000[a]
Males		
0	1.49 ± 0.07	2.22 ± 0.10
1000	1.35 ± 0.08	2.75 ± 0.10
1500	1.60 ± 0.18	2.07 ± 0.14
2000	1.54 ± 0.15	2.76 ± 0.32
EMS	1.32 ± 0.14	12.89 ± 0.99[b]
Females		
0	0.98 ± 0.08	2.28 ± 0.12
1000	1.28 ± 0.02[b]	2.35 ± 0.23
1500	1.19 ± 0.11[b]	2.22 ± 0.12
2000	1.32 ± 0.15[c]	2.69 ± 0.23
EMS	0.79 ± 0.10	15.44 ± 0.90[b]

EMS = ethyl methanesulfonate administered at 150 mg/kg/day.
[a] Group mean ± standard error of the mean.
[b] Significant at $p < 0.05$.
[c] Significant trend at $p < 0.05$.

methanesulfonate as a concurrent positive control for myricitrin and myricetin.

The results of the assessment of DNA damage in several potential target organs of mice administered myricitrin or myricetin, as measured by the Comet assay, are provided in Tables 7 and 8. Under the conditions of the assay, there was no evidence of DNA damage in the liver, duodenum, or stomach of male or female mice exposed to myricitrin as assessed by % tail DNA. In male mice administered myricetin, no significant increase in DNA damage was measured in the liver or duodenum of any of the dose groups as compared to the concurrent vehicle control groups; however, the trend test for DNA damage in the liver was statistically positive ($p < 0.05$). Similar to male mice, a dose-dependent increase in % tail DNA in the liver of female mice administered myricetin was observed without a corresponding increase in damage in any of the dose groups. Although a statistically significant increase in % tail DNA was measured in the duodenum of female mice administered 1000 mg/kg/day myricetin compared to the vehicle control group, a significant increase was not observed in the 1500 or 2000 mg/kg/

Table 7
Comet assay results in mice administered myricitrin.

Dose (mg/kg/day)	% Tail DNA[a]		
	Duodenum	Liver	Stomach
Males			
0	3.99 ± 0.37	4.68 ± 1.18	–
1000	2.96 ± 0.45	4.59 ± 0.35	–
1500	3.58 ± 0.42	4.54 ± 0.80	–
2000	3.82 ± 0.98	4.68 ± 0.43	–
EMS	10.35 ± 1.04[b]	15.84 ± 0.95[b]	–
Females			
0	3.27 ± 0.43	5.89 ± 0.81	13.84 ± 1.43
1000	3.65 ± 0.39	6.19 ± 1.35	9.71 ± 1.23
1500	3.42 ± 0.77[c]	6.49 ± 1.21	11.52 ± 1.09
2000	3.29 ± 0.56	6.74 ± 0.80	12.09 ± 1.61
EMS	12.97 ± 1.12[b]	21.26 ± 1.29[b]	24.95 ± 2.26[b]

EMS = ethyl methanesulfonate administered at 150 mg/kg/day.
[a] Group mean ± standard error of the mean.
[b] Significant at $p < 0.05$.
[c] n = 4 animals.

Table 8
Comet assay results in mice administered myricetin.

Dose (mg/kg/day)	% Tail DNA[a]		
	Duodenum	Liver	Stomach
Males			
0	8.73 ± 1.37	4.04 ± 0.81	–
1000	7.38 ± 0.77	4.07 ± 0.49	–
1500	8.07 ± 1.09	5.53 ± 1.48	–
2000	8.22 ± 0.49	6.79 ± 0.77[c]	–
EMS	18.06 ± 1.38[b]	12.97 ± 1.35[b]	–
Females			
0	3.79 ± 0.38	4.23 ± 0.58	15.79 ± 5.28[d]
1000	7.45 ± 0.86[b]	5.95 ± 0.58	15.49 ± 2.41
1500	4.98 ± 0.30	5.45 ± 0.54	14.94 ± 1.63
2000	6.32 ± 1.22	5.97 ± 0.49[c]	11.83 ± 2.52
EMS	24.75 ± 6.97[b]	14.99 ± 1.05[b]	26.69 ± 4.19[b]

EMS = ethyl methanesulfonate administered at 150 mg/kg/day.
[a] Group mean ± standard error of the mean.
[b] Significant at $p < 0.05$.
[c] Dose dependent response at $p < 0.05$.
[d] Potential outlier included in calculation.

Table 9
Summary of genotoxicity assessments of myricetin and myricitrin.

Assay	Myricetin	Myricitrin
Bacterial mutagenicity	Positive (TA98 +S9)	Negative
In vitro micronucleus	Positive (−S9)[a]	Positive (−S9)
In vivo micronucleus	Negative	Negative
In vivo Comet		
Liver	Equivocal (♂ and ♀)[b]	Negative
Duodenum	Equivocal (♀)[c]	Negative
Stomach[d]	Negative	Negative

[a] Highest value falls within laboratory historical negative control data.
[b] Positive trend; group means fall within laboratory historical negative control data.
[c] Positive low group; group means fall within laboratory historical negative control data.
[d] Stomach not tested in males.

day dose groups, nor was there indication of a dose response.

In these studies, the time interval from obtaining tissue until the lysing step of the Comet assay was controlled by flash freezing the tissue samples in a buffered solution containing DMSO, thereby minimizing a potential source of sample variation that could become significant over the course of a necropsy (Recio et al., 2012). Mean values for % tail DNA generally fell within a 95% confidence interval and the minimum–maximum range of laboratory historical control data for liver, stomach, and duodenum samples frozen in this manner. However, there was a spuriously high stomach % tail DNA result for one vehicle control female mouse (366% of historical mean) in the myricetin study. Statistical analyses were performed with the potential outlier value included and excluded (mean ± standard error of the mean with animal excluded = 11.0 ± 2.93); under both scenarios, there was no significant increase in % tail DNA of female mice administered myricetin as compared to the vehicle control group. The positive control, ethyl methanesulfonate, demonstrated a statistically significant DNA damage response when the animal with the abnormally elevated result was excluded from the analysis whereas inclusion of this animal did not result in a significant positive control test ($p = 0.0723$). Otherwise, administration of ethyl methanesulfonate resulted in a significant increase in % tail DNA in all tissues analyzed for both male and female mice.

4. Discussion

Evaluation of genotoxic potential is an important consideration for regulating the use of food flavoring agents intended for human consumption. These studies were conducted to produce additional information regarding the safety of myricitrin in support of recent FAO/WHO scientific review (JECFA, 2014a, b) and regulatory acceptance of the marketing of products containing myricitrin to countries beyond Japan. The ability of highly purified myricitrin to induce mutations, chromosomal abnormalities, and DNA damage was evaluated using a battery of GLP-compliant *in vitro* and *in vivo* genetic toxicology assays. Trace amounts of myricetin, present as a by-product of the hydrolytic processing of myricitrin, and a known metabolite of ingested myricitrin, was also tested for its genotoxic potential. The results of these tests are summarized in Table 9.

In a bacterial reverse mutation assay, myricitrin did not produce a positive response in any of the five test strains, either with or without metabolic activation, up to the OECD-recommended limit dose. In contrast, myricetin tested positive for mutagenicity in the TA98 strain under conditions of metabolic activation. Our results are generally consistent with previously published results (Brown and Dietrich, 1979; Camoirano et al., 1994; Hardigree and Epler, 1978; MacGregor and Jurd, 1978; Uyeta et al., 1981), although the mutagenic potential of myricetin sometimes reported for the TA100 *Salmonella* tester strain was not observed in our study. Although both myricitrin and myricetin induced MN formation in human TK6 lymphoblastoid cells following a 24 h exposure without metabolic activation, neither compound induced MN following exposure for 4 h in the presence of metabolic activation. The negative response in the presence of liver homogenate suggests that S9 may be capable of detoxifying the effects of these compounds in mammalian cells. Interestingly, there are numerous reports in the literature to suggest that myricetin and myricitrin possess both genotoxic and anti-genotoxic activities in mammalian cells (Anderson et al., 1998; Delgado et al., 2008; Duthie et al., 1997a, 1997b; Edenharder and Grunhage, 2003; Ong and Khoo, 1997; Sahu and Gray, 1993). The anti-genotoxic property of these flavonoids has been primarily linked to a role as an anti-oxidant, largely attributed to an ability to scavenge reactive oxygen species (Chen et al., 2013; Domitrovic et al., 2015; Ong and Khoo, 1997). The biological impact of the opposing genotoxic/anti-genotoxic activities of these flavonols, particularly in concert with effects of other natural dietary flavonoids, is assumed to be quite complex.

In our combined MN/Comet assays in which male and female B6C3F1 mice were administered myricitrin or myricetin for three consecutive days, there was no increase in micronucleated reticulocytes in peripheral blood. Administration of 1000 mg/kg/day myricetin (low dose), but not higher dose levels, caused a statistically significant increase in DNA damage in the duodenum of female mice as measured by the Comet assay. There was not a positive trend and the mean % tail DNA was within our laboratory historical negative control data. Notably, this equivocal result for duodenum of female mice was not reproduced in male mice. Since no evidence of increased sensitivity of female mice to effects of myricetin exposure was detected in a dose setting study, the lack of a DNA damage response in male mice effectively negates the equivocal response measured in the duodenum of female mice. No DNA damage was measured in the stomach of female mice administered myricetin (stomach was not tested in male mice). Although there was a statistically significant positive trend test for DNA damage in the liver of both male and female mice administered myricetin, the lack of any significant pair-wise responses and the results falling within the range of laboratory historical control data lead us to conclude that the equivocal response in the Comet assay is probably not biologically relevant. Moreover, the expected

level of myricitrin consumption (≤3000 μg/day or 50 μg/kg bw per day) (JECFA, 2014b) should result in a concentration of myricetin metabolite orders of magnitude lower than the level tested in our studies, thereby likely posing no significant human health risk.

Importantly, there was no evidence of DNA damage, as assessed by the Comet assay, detected in male or female mice administered the glycosylated compound, myricitrin, up to the limit dose of the assay. The lack of induction of MN or DNA damage in mice in multiple tissue types suggests that mice detoxify any genotoxic metabolite of myricitrin in a manner that is not mimicked in cultured cells, perhaps by a complement of liver CYP450 isozymes. Although we did not directly measure exposure in our mouse studies, evaluation of toxicokinetic parameters in Sprague Dawley rats revealed quantifiable concentrations of myricitrin and myricetin in the blood up to at least 24 h following administration of myricitrin; however, in a parallel study, no measurable myricetin was detected in animals administered myricetin at a concentration equivalent to the trace amount (1.6 mg/kg) present at the top dose level (1000 mg/kg) of myricitrin tested in the toxicokinetics study (Maronpot et al., manuscript submitted). It has been estimated that the no observable adverse effect level (NOAEL) of 884 mg/kg bw per day as determined by a 52-week study in rats is ~18,000 times the highest estimated human dietary exposure to myricitrin when used as a flavoring agent (JECFA, 2014b). This large margin of exposure supports the expectation of minimal risk contributed by trace amounts of myricetin in myricitrin manufactured as a flavoring agent.

In summary, although there is some *in vitro* indication of genotoxic potential, our acute studies in mice do not provide evidence of genotoxicity resulting from exposure to myricitrin, supporting its safe use in food and beverages. Our results reinforce the recent opinion of JECFA (JECFA, 2014b) that myricitrin poses no safety concern for humans when consumed at current estimated dietary exposures.

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