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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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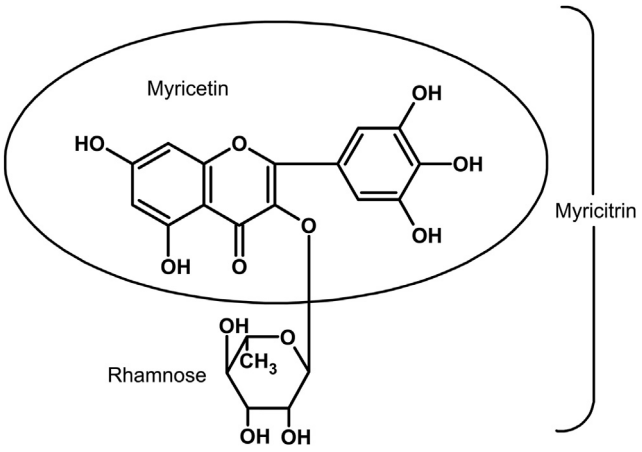

Fig. 1. Chemical structures of the flavonols, myricitrin and myricetin. Myricitrin is a 3-*O*-rhamnoside of myricetin.

(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