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Review

Toxicological assessment of Ashitaba Chalcone

Robert R. Maronpot *

Maronpot Consulting LLC, 1612 Medfield Road, Raleigh, NC 27607, United States

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ABSTRACT

The plant *Angelica keiskei* contains two main physiologically active flavonoid chalcones, 4-hydroxyderricin and xanthoangelol. Known as ashitaba in Japan, powder from the sap is widely consumed for its medicinal properties in Asia as a dietary supplement. Limited previously reported mammalian studies were without evidence of toxicity. GLP studies reported here, including a bacterial reverse mutation assay, a chromosome aberration assay, and an *in vivo* micronucleus assay are negative for genotoxicity. A GLP-compliant 90-day repeated oral gavage study of ashitaba yellow sap powder containing 8.45% chalcones in Sprague Dawley rats resulted in expected known physiological effects on coagulation parameters and plasma lipids at 300 and 1000 mg/kg/day. Ashitaba-related pathology included a dose-related male rat-specific alpha 2-urinary globulin nephropathy at 100, 300, and 1000 mg/kg/day and jejunal lymphangiectasia in both sexes at 1000 mg/kg/day. All other study parameters and histopathological changes were incidental or not of toxicological concern. Based on these studies ashitaba chalcone powder is not genotoxic with a NOAEL of 300 mg/kg in male and female rats.

1. Introduction

The plant *Angelica keiskei*, native to the Japanese Izu Islands and the Izu, Bouso, and Miura peninsulas, is known in Japan as ashitaba, and is widely cultivated in Korea where it is known as sinsuncho or tomorrow leaf. The powder derived from the sap contains several flavonoid chalcones. The most abundant and physiologically active ashitaba chalcones are 4-hydroxyderricin and xanthoangelol. These chalcones have myriad health benefits from inducing apoptosis in cancer cells, to anti-oxidant, anti-inflammatory, anti-angiogenic, and anti-diabetic properties (Dimmock et al., 1999; Dorn et al., 2010). They have also been demonstrated to inhibit platelet aggregation (Lo et al., 2009), have vasorelaxant properties (Ko et al., 2004; Lin et al., 2001; Lo et al., 2009), and suppress differentiation of preadipocytes to adipocytes (Zhang et al., 2013). Ashitaba green tea is widely consumed in China, Japan, and India as a health-promoting drink; is a perennial herb in Korea as a vegetable juice ingredient; and ashitaba leaves have been consumed as food and medicine for many years on the Izu Islands in Japan (Chang et al., 2014; Enoki et al., 2007; Raj et al., 2013).

The safety of ashitaba chalcone in a 28-day dietary administration of 17, 170, and 1700 mg/kg body weight of ashitaba powder to male Wistar rats was without evidence of toxicity (Nagata et al., 2007). Absorption and metabolism of 4-hydroxyderricin and xanthoangelol following gavage administration of 50, 100, 200, and 500 mg/kg body weight of ashitaba extract in male ICR mice (Nakamura et al., 2012) was without adverse effect. A feeding study of xanthohumol, a related prenylated chalcone present in hops, was conducted in female BALB/c mice for three weeks, achieving a daily dose of approximately 1000 mg/kg body weight without toxicity (Dorn et al., 2010). To further document the safety profile of ashitaba, data from a battery of toxicity tests are presented.

2. Materials and methods

2.1. Studies reviewed

A battery of genotoxicity assays, an acute oral toxicity study in rats, and a 13-week rat subchronic oral toxicity study were contracted by Japan Bio Science Laboratory (JBSL), Osaka City, Japan. The test substance for all studies was provided by JBSL and prepared under Good Manufacturing Practices from plants organically cultivated in Indonesia. Ashitaba powder is obtained from the sap of cut stems. The sap is pasteurized, mixed with cyclodextrin, sterilized, freeze-dried, shattered and passed through 100 mesh to obtain 8% ashitaba powder. Analytical data from three non-consecutive batches are presented in Table 1.

Data from the following studies are presented in this report by the author at the request of JBSL:

1 Bacterial reverse mutation test conducted at TNO Nutrition and Food Research Laboratory, The Netherlands (TNO Study Number 4405/16; 30 July 2002)
2 Chromosome aberration test conducted at TNO Nutrition and Food Research Laboratory, The Netherlands (TNO Study Number 5002/02; 3 June 2003)
3 *In vivo* mouse micronucleus test conducted at Nucro-Technics, Ontario, Canada (Study Number 282452; 13 June 2014)
4 Acute oral toxicity in rats conducted at TNO Nutrition and Food Research Laboratory, The Netherlands (TNO Study Number 4410/06; 31 May 2006)
5 13-Week rat oral toxicity study conducted at Bozo Research Center, Japan (Study No. B-5530; 31 May 2006) with a subsequent independent pathology peer review (Experimental Pathology Laboratories, Inc., Research Triangle Park, NC; EPL Project No. 946-001; 27 March 2012).

* Maronpot Consulting LLC, 1612 Medfield Road, Raleigh, NC 27607, United States. Tel.: +1 919 818 9964.
E-mail address: Maronpot@me.com.

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2.2. Animal husbandry and care

The animal care and use committee at each performing laboratory approved study protocols. Animals were housed and maintained according to the AAALAC International Guide for the Care and Use of Laboratory Animals and CCAC Guidelines for Care and Use of Experimental Animals.

2.3. Study details

2.3.1. Bacterial reverse mutation assay

The bacterial reverse mutation assay was carried out in compliance with Good Laboratory Practices and OECD Guideline 471 utilizing *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100 with tryptophan-requiring *Escherichia coli* strain WP2 *uvrA*. All assays were conducted with and without metabolic activation (S9 mix from liver homogenate from rats treated with Aroclor 1254). Ashitaba chalcone powder was cytotoxic in *Salmonella* strain TA 100 and negative for other strains tested both with and without metabolic activation. The positive controls (see Table 2) gave the expected response in revertant colonies with and without S9-mix. The highest concentration of test agent that could be dissolved in DMSO was 125 mg/mL. Given the 8% purity of chalcone, the highest concentration of chalcone was 10 mg/mL resulting in a highest concentration of 1000 μg/plate. The assay used 5 different concentrations of test substance from 12.3 to 1000 μg/plate. Assay results were considered positive if the mean number of revertant colonies had a concentration-dependent increase or if there was a reproducible two-fold increase over negative control.

2.3.2. Chromosome aberration

Two chromosome aberration tests were conducted in compliance with Good Laboratory Practice and OECD Guideline 473. Tissue culture media was purchased from Life Technologies (Gibco, The Netherlands) and Chinese hamster ovary cells (CHO K-1 line) were obtained from Prof. Dr. A.T. Natarajan, University of Leiden, The Netherlands. Other reagents included S9 mix (as above), colcemid (Fluka AG, Switzerland), DMSO (Sigma Chemical Company, USA), and methanol, acetic acid, and Giemsa stain (Merck-Darmstadt, FRG). The purity of the ashitaba powder was set at 100% (actual concentration of chalcone was 8%) and final concentrations of test substance in culture media ranged from 10 to 2500 μg/mL. Positive controls consisted of mitomycin C in the absence of S9 and cyclophosphamide in the presence of S9 mix. All cells at the highest concentrations (2500, 1250, 625 and 313 μg/mL) died before the end of a 4-hour treatment and these concentrations were considered too toxic for further study. For non-cytotoxic dose concentrations, a two-sided Fisher's exact probability test was used to compare treated versus control. Study details are as follows:

Test 1. In the absence of S9 metabolic activation, treatment time was 4 hours (pulse treatment) and 18 hours (continuous treatment) while in the presence of S9 metabolic activation treatment time was 4 hours (pulse treatment). Harvesting of cells was 18 hours after onset of treatment.

Test 2. In the absence of S9 continuous treatment was for 18 hours with harvesting at 18 hours and at 32 hours. In the presence of S9 there was a 4-hour pulse exposure followed by harvesting at 18 and 32 hours. A repeat assay was done when the expected cyclophosphamide positive control did not yield expected results at a 32-hour sampling period.

2.3.3. In vivo MN assay

An *in vivo* micronucleus assay using ashitaba chalcone powder following OECD guideline 474 and in compliance with Good Laboratory Practices was conducted in Swiss mice (CD-1, Charles River, Canada). The range-finding study used groups of 3 male and female mice at doses of 500, 1000, and 2000 mg ashitaba chalcone power/kg body weight. The ashitaba yellow powder consisted of 30% sap and 70% branched cyclodextrin and contained analytically confirmed 7.2% chalcone. Based on no sex difference in response in the range-finding study, the main study was done in groups of 14 male mice at the limit dose of ashitaba (2000 mg/kg), with a methylcellulose vehicle control, and 70 mg cyclophosphamide/kg body weight as a positive control. Seven mice in each group were terminated 24 and 36 hours post-dosing, harvested bone marrow was stained with Giemsa, and 2000 polychromatic erythrocytes were blindly scored by high magnification microscopy for the presence of micronuclei. The ratio of normochromatic to polychromatic erythrocytes was determined based on 500 cells and the number of micronuclei in normochromatic erythrocytes was also determined.

Any intergroup difference in mean number of polychromatic erythrocytes with micronuclei was analyzed using ANOVA on ranks (Kruskal–Wallis One Way Analysis of Variance on Ranks) at $p < 0.05$. To isolate the group(s) that differed from others, All Pair-wise Multiple Comparison Procedures (Dunn's Method and Duncan's Method) were used.

2.3.4. Acute toxicity

An acute gavage toxicity study of ashitaba chalcone powder (8% as chalcone) following OECD Guideline 423 and in compliance with Good Laboratory Practice was conducted in three male and three female Wistar outbred rats (Charles River, Germany) at a dose level of 2000 mg/kg following an overnight fast. Clinical signs and body weight were monitored during a 14-day observation period and rats were examined for macroscopic changes at study termination.

2.3.5. 90-day repeated dose oral toxicity study

A 90-day repeated dose oral gavage study of ashitaba chalcone was conducted in Sprague Dawley rats (Atsugi Breeding Center, Charles River, Japan) at 0, 100, 300 and 1000 mg/kg/day using 12 rats per dose per sex. Dose levels were selected based on result from a preliminary 14-day oral toxicity study. The ashitaba yellow sap powder (30.8% solids and 69.2% branched cyclodextrin) contained 8.45% chalcone and was suspended in olive oil for daily (7 days per week) gavage delivery using a dose volume of 5 mL/kg body weight. The study was done in compliance with Good Laboratory Practice and followed repeated dose toxicity study guidelines (No. 655, Ministry of Health and Welfare, Japan, April 5, 1999). Study parameters included daily clinical observations, frequent body weight measurements, food and water consumption, ophthalmological examinations, urinalysis, hematology, and clinical chemistry analyte measurements. At necropsy abnormal macroscopic findings were recorded and organ weights were obtained for brain, pituitary, thyroids (including parathyroids), adrenals, thymus, spleen, heart, lung, submandibular and sublingual salivary glands, liver, kidneys, testes, prostate, seminal vesicles, ovaries, and uterus. Histopathology slides for gross abnormalities and normal appearing tissues were stained with hematoxylin and eosin. Histopathology slides were peer reviewed by an experienced independent toxicologic pathologist (Seely, 2012).

Microscopic examination was done on gross lesions and all tissues from the high doses and controls. Potential test-article-related tissues examined microscopically

Table 1
Ashitaba chalcone powder specifications and data from three non-consecutive batches.

	Specification	Batch 1	Batch 2	Batch 3	Method
Color/State	Yellow Powder	IACA8Y20	IACA1825	IACB1119	
Total chalcones[a]	NLT 8%	8.33%	8.76%	8.00%	HPLC
Xanthoangelol		11.00 mg/200 mg	11.18 mg/200 mg		
4-Hydroxyderricin		5.66 mg/200 mg	6.34 mg/200 mg		
Other chalcones		7.34 mg/200 mg	7.28 mg/200 mg	2.8 mg/200 mg	
Protein-crude (as is, Nx6.25)		0.6%	0.4%	0.5%	Kjeldahl
Carbohydrates (by difference)		83.9%	83.7%	88.8%	
Moisture	<8.0%	3.0%	2.9%	0.6%	1 g, 105 °C, 4 hr
Fat		12.0%	12.4%	9.4%	Soxhlet
Ash		0.5%	0.6%	0.7%	Ashing to 550 °C
Arsenic	NMT 1 ppm				ICP/MS
Cadmium	NMT 1 ppm				ICP/MS
Lead	NMT 1 ppm				ICP/MS
Mercury	NMT 1 ppm				ICP/MS
Total viable aerobic count	NMT 1000 CFU/g				U.S. FDA BAM (Ch. 3)
Salmonella	Negative/25 g				AOAC Sec. 967.26
E. coli	Negative/g				U.S. FDA BAM (Ch. 4a)
Coliform	NMT 30 CFU/g				ISO 4382.1991
Yeast/Mold	NMT 100 CFU/g				U.S. FDA BAM (Ch. 18)

[a] Total chalcone as the sum of xanthoangelol and 4-hydroxyderricin.