



## Evaluation of *in vitro* and *in vivo* genotoxicity of *Angelica acutiloba* in a standard battery of assays

Jun-Won Yun<sup>1</sup>, Yun-Soon Kim<sup>2</sup>, Euna Kwon<sup>2</sup>, Seung-Hyun Kim<sup>2</sup>, Ji-Ran You<sup>2</sup>,  
Hyeon Hoe Kim<sup>3</sup>, Jeong-Hwan Che<sup>4,\*</sup>, Byeong-Cheol Kang<sup>2,4,5,6,\*</sup>

<sup>1</sup>Department of Biotechnology, The Catholic University of Korea, Bucheon, Gyeonggi-do, Korea

<sup>2</sup>Department of Experimental Animal Research, Biomedical Research Institute, Seoul National University Hospital, Seoul, Korea

<sup>3</sup>Department of Urology, Seoul National University College of Medicine, Seoul, Korea

<sup>4</sup>Biomedical Center for Animal Resource and Development, Seoul National University College of Medicine, Seoul, Korea

<sup>5</sup>Graduate School of Translational Medicine, Seoul National University College of Medicine, Seoul, Korea

<sup>6</sup>Designed Animal and Transplantation Research Institute, Institute of GreenBio Science Technology, Seoul National University, Pyeongchang-gun, Gangwon-do, Korea

Among three representative species of *Angelica* found in Asian countries, including Korea, China, and Japan, *Angelica acutiloba* (AA) has been used as traditional herbal medicine with antitumor, anti-inflammatory, anti-obesity, and anti-diabetes activities. In this study, the potential genotoxicity and mutagenicity of the AA extract were examined in a battery of *in vitro* and *in vivo* tests (bacterial reverse mutation assay, *in vitro* chromosomal aberrations assay, and *in vivo* micronucleus assay) in accordance with the test guidelines for toxicity testing developed by the Organization for Economic Cooperation and Development. Upon testing in the bacterial mutation assay (Ames test) using five *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537, no significant increase the number of revertant colonies in the metabolic activation system and non-activation system was noted in the AA extract groups. Also, in the chromosome aberration test, the AA extract did not cause chromosomal aberration with or without metabolic activation by S9 mix. A bone marrow micronucleus test of mice demonstrated that the incidence of micronucleated polychromatic erythrocytes in the AA extract groups (500, 1000 and 2000 mg/kg BW) was equivalent to that of the negative control group. Based on these results from a standard battery of assays, the AA extract was concluded to have no genotoxic at the proper dose.

**Keywords:** *Angelica acutiloba*, traditional medicine, genotoxicity, mutagenicity

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Many species *Angelica* belonging to the family Umbelliferae has long been known as traditional herbal medicines that can be found throughout Asian countries, including Korea, China, and Japan. In particular, Danggui (root of *Angelica*) or 'female ginseng' is widely used as a typical therapeutic for treating gynecological disorders such as menoxenia, menstrual pain, anemia, and fatigue/low vitality [1-5]. Despite similarities in shapes, Danggui

has different origin species and scientific names according to geographical locations: *Angelica sinensis* from China (Chinese Danggui), *A. acutiloba* (AA) from Japan (Japanese Danggui), and of *A. gigas* from Korea (Korean Danggui) [6].

Among these three species, AA were reported to exhibit various pharmacological effects including antitumor [7], anti-inflammatory [8-11], immunostimulatory [12],

\*Corresponding authors: Jeong-Hwan Che, Biomedical Center for Animal Resource and Development, Seoul National University College of Medicine, 103 Daehakro, Jongno-gu, Seoul 03080, Korea  
Tel: +82-2-740-8526; Fax: +82-2-743-1839; E-mail: casache@snu.ac.kr  
Byeong-Cheol Kang, Graduate School of Translational Medicine, Seoul National University College of Medicine, 101 Daehakro, Jongno-gu, Seoul 110-744, Korea  
Tel: +82-2-2072-0841; Fax: +82-2-741-7620; E-mail: bckang@snu.ac.kr

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anti-complementary [13], and insecticidal [14]. And, the AA could be an effective ethnomedicine for treating diabetic complications and obesity since it has been reported to attenuate fat accumulation in high fat diet-induced obesity through the up-regulation of lipid metabolism [15] and to ameliorate insulin resistance induced by high-fructose diet in rats through the promotion of glucose homeostasis [4]. In addition, it is reported that the AA inhibited the release of histamine from mast cells and the production of pro-inflammatory cytokines, which contributed to the protection against allergic diseases and inflammatory diseases [16-19]. Additional pharmacological effects of the AA include the enhancement of collagen synthesis and suppression of matrix metalloproteinases, resulting in its use as an anti-wrinkle natural agent [1].

The use of medicinal plants as therapeutics, functional foods, or dietary supplements for health-related issues is increasing as self-medication for symptomatic relief because it is believed that these are natural and free from side effects compared with synthetic chemical drugs [20,21]. However, concerns associated with the lack of quality control (plant origin and method of preparation) and potential risks of self-medication (treatment duration) have been raised [4]. Recently, we have identified that long-term ingestion of well-known medicinal herbs available in market can induce potential toxicity that may be hepatotoxicity or nephrotoxicity. In particular, *Sophorae radix* and vinegar-processed *Genkwa flos* have been found to have clastogenic and mutagenic potentials, respectively, as evidenced by the chromosome aberration assay and the Ames test [22-24].

Along with the current use as an herbal medicine available on the market in Asian countries, many studies have reported various beneficial activities of the AA extract, but little information on risk and safety has been reported. Therefore, we carried out the battery of regulatory genotoxicity studies, including *in vitro* bacterial reverse gene mutation assay (Ames test), *in vitro* chromosome aberration assay, and *in vivo* micronucleus assay in mice, to assess the mutagenicity and clastogenicity of the AA extract according to test guidelines for toxicological studies developed by the Organization for Economic Cooperation and Development (OECD) and the Ministry of Food and Drug Safety (MFDS).

## Materials and Methods

### Test substance and animals

A hot water AA extract was provided by the National Institute of Food and Drug Safety Evaluation (Osong, Korea). AA roots were purchased from an Oriental medicine market in Korea, and an extract of AA was obtained according to a method described previously [25]. In brief, dried AA roots were ground by a mixer, and incubated with distilled water (DW) at 100°C. After filtration through filter paper, the filtrate was freeze-dried and dissolved in DW for oral administration. The extraction yield of the hot water AA extract was 0.153 g of freeze-dried AA extract/g of dried AA root.

ICR mice (Orient Bio, Seongnam, Korea) were used after a week of quarantine and acclimatization. During the studies, the animal facility was maintained under standard conditions (22±2°C, 40-60% humidity, and 12 h light/dark cycle). The animals were fed a rodent diet (LabDiet 5002 Certified Rodent Diet, PMI Nutrition International, St. Louis, MO, USA) and tap water *ad libitum*. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Biomedical Research Institute at the Seoul National University Hospital, and this study was performed in compliance with the guidelines published by the OECD as well as the guidance for the GLP for toxicity tests issued by the Ministry of Food and Drug Safety [26].

### *In vitro* bacterial reverse gene mutation assay (Ames test)

Five characterized histidine-dependent strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535, TA1537; MFDS, Osong, Korea) were utilized for bacterial reverse mutation assay (Ames test) in accordance with OECD guideline 471 [27]. *S. typhimurium* strains were incubated with the AA extract with or without an S9 mix in the dark at 37°C for 48 h. The standard mutagens (2-nitrofluorene, sodium azide, mitomycin C, 9-aminoacridine, and 2-aminoanthracene; Sigma-Aldrich, St. Louis, MO, USA) were used as positive controls. The extract was considered to be positive if there was a two-fold increase relative to negative control or a dose-dependent increase in the number of revertant colonies.

### **In vitro chromosomal aberration assay**

An *in vitro* chromosomal aberration test using Chinese hamster lung (CHL) fibroblast cells was conducted in accordance with OECD guideline 473 [28]. The cells were incubated in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37°C, high humidity) with the AA extract in the presence or absence of an S9 mix for 6 h or 24 h. Mitomycin C and cyclophosphamide (Sigma-Aldrich) were used as positive controls. After colcemid (0.2 µg/mL, GIBCO, Carlsbad, CA, USA) was added for 2 h, the cells were treated with hypotonic solution, fixed in 3:1 methanol/glacial acetic acid, and stained with 4% Giemsa.

### **In vivo bone marrow micronucleus assay**

An *in vivo* bone marrow micronucleus test was conducted in accordance with OECD guideline 474 [29]. 8-week-old male ICR mice were orally treated with the AA extract at 0, 500, 1000, and 2000 mg/kg of body weight (BW) once daily for 4 d. Mitomycin C (2 mg/kg BW) served as a positive control and was intraperitoneally injected. The clinical signs and body weight were evaluated once daily. The mice were euthanized at 24 h after the last dose. The femoral bone marrow cells were isolated, centrifuged, smeared on the slides, and dried. After fixation with methanol and 5% Giemsa staining,

the number of micronucleated polychromatic erythrocytes (MNPCEs) was counted from 2000 PCEs. In addition, the PCE/(PCE+NCE) ratio, where the NCEs indicate the normochromatic erythrocytes, was calculated to detect the possibility of cytotoxicity [30].

### **Statistical analysis**

All of the values are expressed as mean±SD. The statistical analysis was performed using a one-way ANOVA, followed by a multiple comparison procedure with a Tukey/Duncan test using SPSS software version 19 (SPSS Inc., Chicago, IL, USA). *P* values of less than 0.05 were considered to be statistically significant.

## **Results and Discussion**

### **In vitro bacterial reverse gene mutation assay (Ames test)**

Among the battery of three genotoxicity tests, the Ames test is known as the most accurate and commonly used *in vitro* method developed by Ames and coworkers in the early 1970s for an initial detection of genotoxicants that induce mutations in the DNA, particularly point mutation induction [31,32]. The mutagenic activity of the AA extract was investigated by Ames test using

**Table 1.** Results of *S. typhimurium* reversion assay with *Angelica acutiloba* extract

S9	Chemical	Dose (µg/plate)	His <sup>+</sup> revertant colonies/plate				
			TA98	TA100	TA102	TA1535	TA1537
-	Distilled water <sup>a</sup>	0.0	22.0±5.3	279.3±30.6	487.0±45.6	21.3±2.5	73.7±18.4
	2-nitrofluorene <sup>b</sup>	10	285.7±13.3*	-	-	-	-
	Sodium azide <sup>b</sup>	5	-	1578.0±321.5*	-	-	-
	Mitomycin C <sup>b</sup>	0.5	-	-	1906.7±179.9*	-	-
	Sodium azide <sup>b</sup>	0.5	-	-	-	453.3±47.2*	-
	9-aminoacridine <sup>b</sup>	80	-	-	-	-	2440.0±322.0*
		312.5	24.0±2.6	255.7±16.7	463.7±18.0	19.7±1.5	59.0±1.0
		625	26.0±7.2	283.3±22.3	452.7±12.9	21.7±5.5	61.0±11.4
	<i>Angelica acutiloba</i>	1,250	31.0±1.7	311.7±5.9	506.7±58.2	17.0±1.0	75.3±18.6
		2,500	28.7±6.4	309.0±19.1	533.0±29.5	20.7±3.5	71.3±9.2
	5,000	23.0±4.6	366.7±42.2	598.7±96.2	28.0±2.6	59.7±6.0	
+	Distilled water <sup>a</sup>	0.0	31.0±4.6	213.3±22.9	543.0±15.9	10.3±1.2	61.7±8.5
	2-aminoanthracene <sup>b</sup>	2	263.3±18.8*	641.7±25.0*	-	-	-
		5	-	-	1438.0±244.2*	239.0±18.4*	866.3±64.7*
		312.5	29.0±7.2	214.7±15.0	531.7±28.6	9.3±0.6	59.3±5.5
		625	31.7±2.1	235.0±7.8	534.0±8.7	14.3±0.6	60.0±8.5
	<i>Angelica acutiloba</i>	1,250	29.7±7.2	214.0±18.2	557.7±49.9	9.7±4.5	51.7±5.5
		2,500	31.7±6.1	219.7±30.0	587.3±80.8	14.7±6.7	53.0±2.6
		5,000	34.7±3.8	186.7±60.4	653.7±71.7	11.3±1.5	46.7±4.6

<sup>a</sup>Negative control

<sup>b</sup>Positive control

\*Significantly different from negative control group (*P*<0.05).

**Table 2.** Results of MTT assay in CHL cells treated with *Angelica acutiloba* extract

Substance	Dose (µg/mL)	Absorbance	Survival rate (%)
Distilled water	0.0	0.53±0.07	100.0
	39.1	0.51±0.16	96.5
	78.1	0.50±0.05	93.9
	156.3	0.50±0.09	94.3
<i>Angelica acutiloba</i>	312.5	0.60±0.10	113.7
	625.0	0.49±0.17	93.5
	1250.0	0.42±0.06	79.3
	2500.0	0.51±0.06	96.6
	5000.0	0.52±0.06	98.1

histidine requiring strains of *S. typhimurium*, such as the frameshift tester strain TA98 and TA1537, the base-pair substitution tester strain TA100 and TA1535, or oxidative and cross-linking tester strain TA102 [33]. The AA extract treatments exerted no significant increases in the number of revertants per plate in the absence and presence of the metabolic activation system at all concentration tested (312.5, 625, 1250, 2500 and 5000 µg/plate) relative to the negative control group (Table 1). In contrast, large increase in the number of revertant colonies were seen for the various mutagens (positive controls) with or without of S9 mix, indicating that the test system conducted in the present study responded appropriately under acceptable experimental condition. Therefore, the results of this assay indicated that the AA extract did not show mutagenicity in the tester strains under these experimental conditions.

### *In vitro* chromosomal aberration assay

The chromosomal aberration test is an *in vitro* screening assay that has been used to detect chromosomal damage in cultured mammalian cells [34-36]. At first, the effect

of the AA extract on the proliferation of CHL cells were analyzed using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (Table 2), and we decided to set 5000 µg/mL of the AA extract as the highest exposure level for *in vitro* chromosomal aberration assay based on the MTT assay resulting to 98.1% viability at 24 h incubation. The positive controls (mitomycin C without the S9 mix and cyclophosphamide with the S9 mix) resulted in significant increases in the incidence of structural chromosome aberrations, supporting the validity of the study (Table 3). In contrast, the number of cells with chromosomal aberrations (breaks, fragments, and exchanges) in the AA extract groups was not significantly different from the negative control group after short-term treatment for 6 h with or without the S9 mix and after continuous treatment for 24 h without the S9 mix. Our current data show that the AA extract did not induce chromosomal aberrations in CHL cells under the conditions used in this assay.

### *In vivo* bone marrow micronucleus assay

Micronuclei, also known as Howell-Jolly bodies, are chromatin particles from damaged chromosome fragments that were not incorporated into the nucleus after mitosis [37]. In this study, the presence of micronuclei, which is regarded as an indirect indicator of quantitative and structural chromosomal aberrations [38], was evaluated to determine the genotoxic property of the AA extract. During the study, no abnormal clinical signs of toxicity in general appearance were detected in any of the mice following the AA extract administration. And, there were no statistically significant differences in body weights across all groups (Table 4). The mean ratio of PCEs to total erythrocytes were 50.9, 51.6, 47.4, 53.9, and 28.5% for the negative control, at 500, 1000, 2000

**Table 3.** Results of chromosomal aberration induced by *Angelica acutiloba* extract

Substance	Dose (µg/mL)	Number of cells scored	No. of cells with aberrations		
			-S9		+S9
			6h	24h	6h
MEM <sup>a</sup>	0	200	4.5±2.1	2.5±3.5	5.5±0.7
Mitomycin C <sup>b</sup>	0.1	200	38.0±7.1*	66.5±9.2*	-
Cyclophosphamide <sup>b</sup>	10	200	-	-	98.0±1.4*
Distilled water	0	200	4.5±3.5	2.5±0.7	7.0±2.8
	1,250	200	1.5±2.1	2.5±3.5	2.0±2.8
	2,500	200	3.5±5.0	2.5±0.7	5.0±1.4
	5,000	200	4.0±2.8	7.5±5.0	6.0±4.2

<sup>a</sup>Minimum essential medium (negative control)

<sup>b</sup>Positive control

\*Significantly different from negative control group ( $P < 0.05$ ).

**Table 4.** Body weight changes following treatment with *Angelica acutiloba* extract

Day after administration	<i>Angelica acutiloba</i> (mg/kg BW)				Mitomycin C <sup>b</sup> (mg/kg BW)
	0 <sup>a</sup>	500	1,000	2,000	0.002
1	34.5±1.1	34.3±1.3	34.2±1.2	34.3±0.9	-
2	35.2±1.6	34.7±1.5	34.9±1.3	34.6±0.8	-
3	35.3±1.5	34.9±1.7	35.4±1.5	34.9±1.0	-
4	35.9±1.8	35.6±1.5	36.1±2.1	35.4±1.1	36.2±1.2
5	35.7±1.7	35.3±1.6	35.8±1.8	34.6±1.2	35.9±1.9

<sup>a</sup>Negative control<sup>b</sup>Positive control**Table 5.** Micronucleated polychromatic erythrocytes (MNPCEs) in mice bone marrow following treatment with *Angelica acutiloba* extract

Substance	Dosage (mg/kg BW)	Number of mice	PCE/(PCE+NCE) <sup>c</sup>	MNPCE <sup>d</sup>
Distilled water <sup>a</sup>	0.0	5	50.9±6.5	1.0±1.2
	500	5	51.6±7.4	0.6±0.9
<i>Angelica acutiloba</i>	1,000	5	47.4±9.0	0.8±0.5
	2,000	5	53.9±4.5	1.4±1.1
Mitomycin C <sup>b</sup>	0.002	5	28.5±9.0	78.6±36.9*

<sup>a</sup>Negative control<sup>b</sup>Positive control<sup>c</sup>The ratio of polychromatic erythrocytes to all erythrocytes (polychromatic+normochromatic) (%)<sup>d</sup>Polychromatic erythrocyte with micronuclei was calculated from 2,000 polychromatic erythrocytes (%)\*Significantly different from negative control group ( $P < 0.05$ ).

mg/kg BW of the AA extract, and the positive control, respectively (Table 5), indicating that this study was valid since no decrease in the PCE/(PCE+NCE) ratio (greater than 20%) reflects a lack of toxic effects of the AA extract (Heddle *et al.*, 1984). The incidence of micronuclei showed no significant difference between the negative control group and the AA extract groups (500, 1000, or 2000 mg/kg BW). In contrast, the number of micronuclei in the mitomycin C-treated groups was significantly higher than that of the negative control group.

In conclusion, these fundamental toxicology data from the Ames assay, *in vitro* chromosome aberration assay, and *in vivo* micronucleus assay clearly suggest that the AA extract is safe in terms of genotoxicity. To our knowledge, this is the first comprehensive study assessing the potential genotoxic effects of the AA extract as a traditional medicine with beneficial activities in accordance with the OECD and the GLP Regulations.

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**Conflict of interests** The authors declare that there is no financial conflict of interests to publish these results.

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