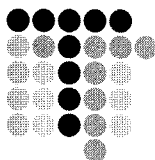

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Evaluation of the Genotoxicity of Decursin and Decursinol Angelate Produced by *Angelica gigas* Nakai

Kang Min Kim[1], Tae Ho Kim[1], Yun Jung Park[1], Ik Hwan Kim[2] & Jae Seon Kang[3]

[1]Department of Smart Foods and Drugs, Inje University, 607 Obang-Dong, Gimhae, Gyeongnam 621-749, Korea
[2]College of Life Sciences and Biotechnology, Korea University, 5-1 Anam-Dong, Seongbuk-Gu, Seoul 136-701, Korea
[3]Department of Pharmacy, Kyungsung University, 314-79 Daeyeon-Dong, Nam-Gu, Busan 608-736, Korea
Correspondence and requests for materials should be addressed to J. S. Kang (jskang28@hanmail.net)

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Abstract

In this study, we assessed the stability and toxicological safety of *Angelica gigas* Nakai (*A. gigas* Nakai) extract, which is comprised of decursin and decursinol angelate (D/DA). D/DA was tested for mutagenicity using Ames *Salmonella* tester strains (TA102, TA1535, and TA1537) with or without metabolic activation (S9 mix). No increase in the number of revertants was observed in response to any of the doses tested (1.25, 12.5, 125, and 1,250 μg/mL). In addition, a chromosome aberration test was conducted in the Chinese hamster lung (CHL) cell line. To accomplish this, cells were treated with D/DA (3.28, 13.12, 52.46, and 209.84 μg/mL) or with Mitomycin C (0.1 μg/mL) as a positive control in the case of no metabolic activation or benzo(a)pyrene (20 μg/mL) in the case of metabolic activation. No significant increase in chromosome aberrations was observed in response to treatment with any of these concentrations, regardless of activation of the metabolic system. According to these results, we concluded that D/DA did not induce bacterial reverse mutation or clastogenicity *in vitro* in the range of concentrations evaluated in these experiments.

Keywords: Mutagenicity, Ames test, Chromosome aberration, *Angelica gigas* Nakai, Decursin, Decursinol angelate

A Korean traditional herbal medicine, *A. gigas* Nakai, is one of the most popular herbal medicines used in Asian countries, including Korea, Japan and China. *A. gigas* Nakai is used under the Korean name Cham-Dang-Gui. In addition, *A. gigas* Nakai is also marketed as a functional food product in Europe and North America. *A. gigas* Nakai can be distinguished from Japan and China Angelica because the former has deep purple flowers, while the latter have white flowers[1]. *A. gigas* Nakai has been studied extensively and shown to contain a variety of substances, including coumarins[2,3], which are comprised of D/DA. Accordingly, *A. gigas* Nakai has been used as a traditional medicine for the treatment of anemia and as a sedative, an anodyne and a tonic agent[4]. Furthermore, *A. gigas* Nakai has been widely used for the treatment of dysmenorrhea, amenorrhea, menopausal syndromes, abdominal pain, injuries, migraine headaches and arthritis, as well as for its antibacterial and antiamnestic effects and its inhibitory effect against acetylcholinesterase, its depression of cardiac contraction, and its ability to activate protein kinase C[5-9].

Many herbal medicines are used as functional food products. Recently, the effects of storage on herbal medicines were evaluated to determine if their quality and circulation could be improved. The results of several genotoxicity assays have shown that the carcinogenicity has generally occurred via DNA damage. For example, bacterial gene mutation chromosomal aberration assays with mammalian cells and micronucleus tests in rodents are commonly used for genotoxicity assays because they are rapid and reliable[10-17]. Currently, three assay systems are commonly utilized to evaluate the genotoxicity of compounds worldwide. Furthermore, these methods are often used as a screening method for the detection of possible carcinogenic substances. Due to the uncertainty associated with herbal medicines, it is important that their extracts be evaluated to determine if they damage DNA. Therefore, in this study, we evaluated the clastogenicity of the extract of *A. gigas* Nakai used in an herbal medicine against several strains of *Salmonella* and Chinese hamster lung cells *in vitro*.

Mutagenicity of D/DA in the Ames *Salmonella* Test

Table 1 summarizes the results of the Ames muta-

Table 1. Number of revertants induced by D/DA as determined by *Salmonella* plate incorporation tests conducted using TA102, TA1535, and TA1537 with or without S9. −S9=without metabolic activation, +S9=with metabolic activation.

Decursin & decursinol angelate (μg/plate)	Number of revertants/plate	
	+S9 (Mean±S.D.)	−S9 (Mean±S.D.)
TA102		
Control (1% DMSO)	136±7	127±38
1250	138±13	127±17
125	121±9	131±14
12.5	125±8	135±15
1.25	143±2	136±6
Positive control		
2-AA (1 μg/plate)	1113±10*	
MMC (1 μg/plate)		1132±6*
TA1535		
Control (1% DMSO)	151±32	137±14
1250	154±18	133±12
125	160±18	145±3
12.5	133±22	138±13
1.25	140±28	159±29
Positive control		
2-AA (1 μg/plate)	1627±212*	
SA (1 μg/plate)		4535±158*
TA1537		
Control (1% DMSO)	108±6	98±13
1250	124±6	138±6
125	110±5	131±11
12.5	139±21	113±12
1.25	122±16	138±4
Positive control		
2-AA (1 μg/plate)	1878±109*	
ICR-191 (1 μg/plate)		1124±129*

Mean±S.D. of three plates; The results of each concentration were compared with the solvent control by Dunnett's multiple comparison; $^*P<0.01$

genicity assay conducted using *S. typhimurium* strains TA102, TA1535, and TA1537. No significant increases in the number of revertant colonies were observed in response to treatment with any of the concentrations tested for any of the tester strains when compared with the solvent control. The positive control compounds, 2-AA, SA, MMC, and ICR-191, showed very high frequencies of revertant colonies when compared to the solvent control and treated cultures.

Carcinogenicity of D/DA in the Chinese Hamster Lung Fibroblast Cell Line

The IC_{50} value was 209.84 μg/mL for D/DA. As shown in Table 2, there was relatively low cytotoxicity, regardless of whether the metabolic system was activated. Furthermore, the use of 1% DMSO as a control revealed no clastogenicity (Table 2). Moreover, the use of mitomycin C (0.1 μg/mL) as a positive control without metabolic activation and benzo(*a*)pyrene (20 μg/mL) as positive control with metabolic activation resulted in approximately 77 and 55% chromosome aberration, respectively, in CHL cells. Taken together, these findings suggest that D/DA exerts no clastogenicity in CHL fibroblast cells *in vitro* at the range of concentrations evaluated in this study, regardless of whether the metabolic system was activated.

Discussion

Tables 1 and 2 show the results of an Ames mutagenicity assay conducted using *S. typhimurium* strains TA102, TA1535, and TA1537 and the chromosome aberration assay system using CHL. Several short term methods have been developed, used and found to be capable of predicting the carcinogenicity of chemicals. For some compounds, mutagens and certain non-mutagenic carcinogens have also been utilized to evaluate chromosomal rearrangement[10,11,19,20]. There are also several screening methods for evaluating DNA-attacking substances to determine if they are carcinogenic and to validate the genotoxicity of antimutagenicity[10-12,21-26]. The detection and regulation of synthetic chemicals are the subjects of concern due to the correlation between environmental contamination and human health. In this study, we evaluated the genotoxicity of D/DA.

We used *S. typhimurium* strains and CHL cells in the present study. It was reported that there were no differences in the sensitivity of CHL and CHO (Chinese hamster ovary) cells to various mutagens in an *in vitro* chromosome aberration study[27]. No significant increase in the number of revertant colonies of *S. typhimurium* was observed in response to treatment with any of the concentrations of D/DA evaluated in this study (1.25, 12.5, 125, and 1,250 μg/plate) when compared with the solvent control. Furthermore, the IC_{50} value of D/DA for cytotoxicity against CHL cells was found to be 209.84 μg/mL. Moreover, D/DA revealed no clatogenicity in CHL cells when evaluated at concentrations of 3.28, 13.12, 52.476, and 209.84 μg/mL *in vitro* with or without metabolic activation. The results of this study are similar to those of a study conducted by Yu *et al.*[28] The results of a *Salmonella* reversion assay (TA98 and TA100) and micronucleus test conducted to evaluate the *in vitro* genotoxicity of γ-irradiated *A. gigas* Nakai revealed that it was similar to that of non-irradiated *A. gigas* Nakai, and that mutagenicity did not occur in the two assays, with or without metabolic activation[28]. These results and the results of the present study suggest that the main compounds of *A. gigas* Nakai should be stable and

Table 2. Chromosome aberration test of D/DA in the CHL cell line.

Treatment	Dose (μg/mL)	S9 mix	Aberrations/cell[a)						Frequence of cells with total aberration (%)
			Chromatid type			Chromosome type			
			Gap	Break	Exchange	Gap	Break	Exchange	
Control (1% DMSO)			1	1	1	1	1	1	6±0.48
Mitomycin C	0.1		37	18	4	4	11	3	77±2.45*
Decursin & decursinol	209.84		3	1	1	3	1	0	9±0.65
angelate	52.46	−	1	1	0	1	1	0	4±0.65
	13.12		1	1	1	1	1	1	6±0.55
	3.28		1	0	1	1	0	1	4±0.58
Control (1% DMSO)			1	1	1	1	1	1	6±0.79
Benzo(*a*)pyrene	20		23	11	11	5	4	1	55±2.31*
Decursin & decursinol	209.84		2	1	1	1	0	1	6±0.55
angelate	52.46	+	1	0	1	0	0	0	2±0.48
	13.12		1	1	1	1	1	1	6±0.58
	3.28		1	0	0	0	0	0	1±0.29

[a)Three sample slides were prepared for each concentration and 100 cells were counted per slide
Significantly different from the negative control ($P < 0.01$)

that the safety of *A. gigas* Nakai can be revealed in further tests conducted *in vivo*.

Materials & Methods

Chemicals and Reagents

Colcemid, Trypsin-EDTA, and Fetal bovine serum were purchased from Gibco BRL (Gland Island, NY, USA). 2-Aminoanthracene (2-AA), Sodium azide (SA), Acridine mutagen ICR-191 (ICR-191), Mitomycin C (MMC), Benzo(*a*)pyrene, S9 fraction, cofactor, and other reagents were purchased from Sigma (St. Louis, MO, USA). The purity of D/DA, which was extracted from *A. gigas* Nakai, was at least 95%.

Bacterial Strains and Cell Culture

Salmonella typhimurium (*S. typhimurium*) tester strains TA102, TA1535, and TA1537 were obtained from the Korea Culture Center of Microorganisms (KCCM) or the Korean Collection for Type Cultures (KCTC). A clone sub-line of CHL was obtained from the Korea Institute of Science and Technology (Seoul, Korea). The karyotype of CHL cells consisted of 25 chromosomes. All cells were maintained by 3-4 day passages and grown in a monolayer with Eagles' minimum essential medium (GIBCO, 41500-034) supplemented with 10% fecal bovine serum (GIBCO, 26140-020) at 37°C under 5% CO_2.

Mutagenicity Assays

Quantitative salmonella mutagenicity plate tests employing tester strains TA102, TA1535, and TA1537 were prepared as described by Maron *et al.*[11], with and without metabolic acid (S9 mix). Test chemicals and D/DA were dissolved in 1% DMSO, after which different concentrations (1.25, 12.5, 125, and 1,250 μg/plate) were tested for mutagenicity. Briefly, plates were inverted within an hour and then placed in a dark vented incubator at 37°C for 48 h. For experiments with and without the S9 mixture, positive controls (1 μg/plate 2-AA for TA102, TA1535, and TA1537, 1 μg/plate SA for TA1535, 1 μg/plate MMC for TA102, and 1 μg/plate ICR-191 for TA1537) and negative controls (1% DMSO) were run concurrently in all experiments. Three plates were used for each concentration tested and for both positive and negative controls. All the experiments were analyzed for each concentration tested. After 48 h of incubation, the revertant colonies on each plate were counted.

Determination of The 50% Growth Inhibition Concentration (Cytotoxicity)

Growth inhibition tests were conducted on each sample before the chromosome aberration tests were conducted as follows. For the growth inhibition assay, 6 well plates were seeded with CHL cells at a density of 1.76×10^6 cells/5 mL. Twenty-four hours after seeding, several different doses of each sample were added separately and the plates were then incubated for an additional 24 hr. The cells in the monolayer cultures that developed were then washed, fixed with methanol, and stained with 5% Giemsa's buffered solution (pH 6.8, Sigma, G4507) for 10 min. The 50% inhibition concentration (IC_{50}) values were then calculated.

Chromosome Aberration Test

Chromosome preparations were created by incubat-

ing 3-day-old cultures (about 10^6 cells/60 mm dish) with positive controls (mitomycin C 0.1 μg/mL) or D/DA (3.28, 13.12, 52.46, and 209.84 μg/mL) for 22 h in the case of no metabolic activation. In the case of metabolic activation, chromosome preparations were created by incubating 3-day-old cultures (about 10^6 cells/60 mm dish) with positive controls (benz(*a*) pyrene 20 μg/mL) and cofactor : S9 (7 : 3) for 6 hr. The cells were then treated with colcemid (0.2 μg/mL) for 2 hr, after which they were trypsinized and harvested by centrifugation. Next, the cell pellets were incubated in 4 mL of 0.075 M KCl hypotonic solution for 20 min at 37°C, after which they were centrifuged and the pellets were fixed with ice-cold fixative (methanol : glacial acetic acid=3 : 1 (v/v)) twice and then allowed to stand overnight in a refrigerator (−4°C). The samples were then centrifuged (1,000 rpm, 5 min), after which the fixative was removed and the cell pellet solutions were prepared by pipetting gently. A few drops of cell pellet suspension were then placed on precleaned dry slides, after which the chromosomes were spread onto the slides by allowing them to dry while slanted. The slides were then stained with 5% Giemsa's buffered solution (pH 6.8) for 10 min, after which the number of cells with chromosomal aberrations was determined based on evaluation of 100 well-spread metaphases at a magnification of 1,000 using an Olympus DP-70 microscope (Olympus, Tokyo, Japan). Cells that were treated with solvents alone served as controls.

Statistical Analysis

The results of the Ames mutagenicity assay were analyzed using an analysis of variance test with Dunnett's multiple comparison test to identify differences relative to the controls[18]. Untreated CHL cells usually have less than 3.0% cells that exhibit spontaneous chromosome aberrations. Data regarding the metaphase stages of the 100 well-spread chromosomes were expressed as percentages. Aberration frequencies were defined as the number of aberrations observed divided by the number of cells counted. Dose-dependent responses and the statistical significance in *P*-value were considered as positive results.

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