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Evaluation of the Mutagenic Properties of Two Lignans from *Acanthopanax koreanum* Nakai

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Acanthopanax koreanum Nakai, a well known traditional herb grown in Jeju Island, South of Korea, has been used as a tonic and sedative agent, as well as in the treatment of diabetes and immune diseases. Mutagenicity of two lignans, syringaresinol and tortoside A isolated from *A. koreanum*, was assessed using *Salmonella*/microsome (Ames) test. Tester strains used were *Salmonella typhimurium* TA98, TA100, TA1535, and *Escherichia coli* WP2*uvr*A. The mutagenic activity was determined both in the absence or presence of S9 mixture. As a result, tortoside A did not cause any increase in the number of his^+ revertants in *S. typhimurium* and *E. coli* WP2*uvr*A strains in the presence or absence of S9 mix, compared to the controls. Similarly, low concentrations of syringaresinol (750 and 1,500 μg/plate) did not show any mutagenic properties in all bacterial strains, in the presence or absence of S9 mixture. However, in the high concentration of syringaresinol (3,000 μg/plate), the number of revertants were increased in TA1535 strains, in the absence of S9 metabolic activation. Therefore, *in vivo* experiments such as comet assay are needed to further determine the genotoxic/carciogenic potential of syringaresinol isolated from *A. koreanum*.

***Key words*:** *Acanthopanax koreanum*, Ames test, Lignan, Mutagenicity

INTRODUCTION

Acanthopanax species are widely distributed in Korea, Japan, and China, and are used as health supplements. Among them, *A. koreanum* Nakai, a well known traditional herb grown in Jeju Island, south of Korea, has been used as a tonic and sedative agent, as well as in the treatment of diabetes and immune diseases (1). Recently, it was shown that components isolated from *A. koreanum* had various biological activities. For instance, acanthoic acid induced apoptosis of leukemia cells *via* p38 mitogen-activated protein kinase (MAPK)-mediated signaling pathway (2) and inhibited trypsin-induced mast cell activation (3). In addition, extracts of *A. koreanum* attenuated the behavioral and biochemical changes in stressed mice (4). However, compared to other species such as *A. senticosus*, there are still few studies on *A. koreanum*, especially on their safety.

Many conventional drugs used for cancer treatments induce genetic damage which leads to carcinogenesis. Therefore, many researchers are focusing on identifying novel chemotherapeutic agents in plants that does not induce destructive effects of conventional therapeutic cytotoxic drugs. However, many medicinal plants traditionally used have not been scientifically assessed for their safety, thus it may be valuable to assess their safety for continuous use (5). The bacterial reverse mutation test developed by Ames *et al.*, is a biological assay to assess the mutagenic potential of chemicals. It serves as a quick assay to estimate the carcinogenic potential of a compound, and a positive test indicates that the chemical might act as a carcinogen (6). We previously reported that three lignans, eleutheroside E, syringaresinol, and tortoside A isolated from *A. koreanum* have immunomodulating effects in dendritic cells, by enhancing the secretion of interleukin (IL)-12 and interferon gamma (IFN-γ) (1). Herein, we tried to estimate the mutagenicity of syringaresinol and tortoside A isolated from the root of *A. koreanum* using Ames assay.

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Abbreviations: 9-AA: 9-aminoacridine, 2-AA: 2-aminoanthracene, DMSO: dimethyl sulfoxide, AF-2: furylfuramide, IFN-γ: interferon gamma, IL: interleukin, MAPK: mitogen-activated protein kinase, MI mutagenic index, NaN_3: sodium azide

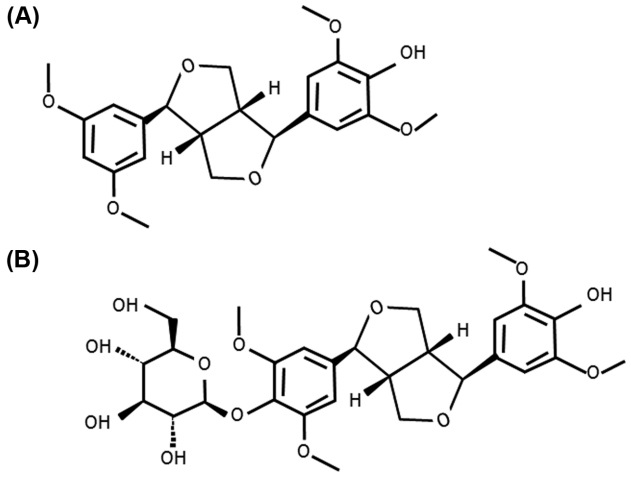

Fig. 1. Chemical structures of (A) syringaresinol and (B) tortoside A.

MATERIALS AND METHODS

Preparation of syringaresinol and tortoside A. Dried roots of *A. koreanum* were provided by Susin Ogapy Co. (Chungnam, Korea). Two lignans, syringaresinol (Fig. 1A) and tortoside A (Fig. 1B) were isolated from the roots of *A. koreanum* and identified as reported previously (7,8), and were kindly provided by Dr. Jung-Jun Lee at Korea Research Institute of Bioscience & Biotechnology (KRIBB), Daejun, Korea. The isolated lignans were filtered step by step through various membrane filter sizes (0.8, 0.45, and 0.2 μm; Nippon Milipore Ltd., Tokyo, Japan). The samples were stored in −80°C until used.

Chemicals and test bacterial strains. Tester strains used were *Salmonella typhimurium* TA98, TA100, TA1535, and *Escherichia coli* WP2*uvr*A, and all strains were purchased from Korea Collection for Type Cultures (KCTC, Seoul, Korea) except *Salmonella typhimurium* TA1537 that was provided by Moltox (Molecular Toxicology Inc., Boone, NC, USA). Culture stocks were stored below −80°C until used. The tester strain was prepared by adding 20 ml of 2.5% Oxoid Nutrient Broth #2 (Oxoid Ltd., Cambridge, UK) to the permanent culture and incubating for 14 hr at 37°C at 210 rpm, to yield 1×10^8 cells/ml. Histidine requirement, rfa mutation, tryptophan requirement, *uvr*B mutation, R factor, and spontaneous reversion tests were performed according to Maron and Ames (6).

For positive controls, sodium azide (NaN_3), 9-aminoacridine (9-AA), and 2-aminoanthracene (2-AA) were all purchased from Sigma-Aldrich (Poole, UK) and furylfuramide (AF-2) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dimethyl sulfoxide (DMSO, Sigma-Aldrich) was used as a solvent.

Metabolic activation system. Male rat liver S9 fraction was purchased from Wako Pure Chemical Industries, Ltd., and the S9 Cofactor mix was prepared as previously described (9).

Cytotoxicity assay. The cytotoxicity test was performed to determine the highest concentration, and was detected by observing the reduction in the number of reverting colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures (6). Top agar was prepared by dissolving 0.6 g of Bacto agar (BD Biosciences, San Jose, CA, USA) and 0.5 g of NaCl (Sigma) in 100 ml of distilled water and autoclaved for 15 min at 121°C. Overnight bacterial culture (0.1 ml) was added to 0.1 ml of syringaresinol or tortoside A (3000, 1500, 750, 375, 188, 94, 47, and 23 μg/plate) and 2 ml of top agar in the presence or absence of S9 mix. This was placed onto nutritive agar plates and incubated at 37°C for 48 hr.

Mutagenicity assay. The plate incorporation test was performed as previously described by Maron and Ames (6). Briefly, 0.1 ml of three concentrations of syringaresinol and tortoside A (3000, 1500, and 750 μg/plate), 0.1 ml of an overnight bacterial culture (1×10^8 cells/ml), 0.1 ml of positive or negative (phosphate buffered saline, PBS) controls, and 0.5 ml of S9 mix or PBS, were mixed with 2 ml of top agar and poured on minimal glucose agar plates. After incubating for 48 hr at 37°C, the number of his^+ revertants for *S. typhimurium* strains and trp^+ revertants for *E. coli* WP2*uvr*A strains in the sample were compared to the negative control by its mutagenic index (MI) value. MI = (Number of his^+ revertants induced in the sample)/(Number of his^+ revertants induced in the negative control). A sample was considered positive when MI ≥ 2 for at least one of the tested concentrations and there was a reproducible dose response curve. All samples were tested on duplicate plates and the experiments were repeated 3 times.

RESULTS AND DISCUSSION

Salmonella/microsome (Ames) test is widely used in investigating the mutagenic effects of chemicals (6). If the test chemical is mutagenic to any particular strain of bacterium, the number of histidine-independent colonies arising on those plates will be significantly greater than the corresponding control plates for that strain of bacteria (10). When the toxicity tests were carried out with TA100 by the plate incorporation method, using NaN_3 (in the absence of S9 mix) and 2-AA (in the presence of S9 mix) as positive controls, results showed that syringaresinol and tortoside A did not cause any decrease in the number of his^+ revertant colonies compared to the negative controls (Table 1 and Table 2). Therefore, we chose 3,000 μg/plate as the highest concentration for syringaresinol and tortoside A, and a total

Table 1. Toxicity of syringaresinol in *S. typhimurium* TA 100 strain in the absence or presence of a metabolic-activating enzyme (S9)

Metabolic activation system	Test substance	Part	Conc. (μg/plate)	Number of colonies (Mean ± SD)
S9(−)	PBS	-	100 μl/plate	160 ± 11
	A. koreanum	Syringeresinol	3,000	192 ± 13
			1,500	187 ± 6
			750	143 ± 11
			375	161 ± 9
			188	191 ± 8
			94	148 ± 12
			47	177 ± 14
			23	186 ± 5
	NaN_3	-	-	988 ± 23
S9(+)	PBS	-	100 μl/plate	184 ± 9
	A. koreanum	Syringeresinol	3,000	182 ± 7
			1,500	191 ± 6
			750	166 ± 2
			375	172 ± 5
			188	179 ± 14
			94	197 ± 10
			47	201 ± 9
			23	194 ± 17
	2-AA	-	-	1011 ± 21

Values are mean ± S.D. of three plates. Negative control (NC): PBS (100 μl/plate); positive control (PC): for TA 100/−S9, NaN_3 (5 μg/plate); for TA100/+S9, 2-AA (5 μg/plate).
NaN_3: sodium azide, 2-AA: 2-aminoanthracene. A thinning of auxotrophic background growth was not shown.

Table 2. Toxicity of tortoside A in *S. typhimurium* TA 100 strain in the absence or presence of a metabolic-activating enzyme (S9)

Metabolic activation system	Test substance	Part	Conc. (μg/plate)	Number of colonies (Mean ± SD)
S9(−)	PBS	-	100 μl/plate	144 ± 8
	A. koreanum	Tortoside A	3,000	137 ± 5
			1,500	130 ± 6
			750	143 ± 5
			375	122 ± 6
			188	129 ± 8
			94	148 ±17
			47	160 ± 9
			23	151 ± 12
	NaN_3	-	-	1191 ± 34
S9(+)	PBS	-	100 μl/plate	162 ± 13
	A. koreanum	Tortoside A	3,000	174 ± 16
			1,500	168 ± 5
			750	155 ± 11
			375	183 ± 8
			188	191 ± 12
			94	180 ± 3
			47	147 ± 6
			23	161 ± 19
	2-AA	-	-	956 ± 27

Values are mean ± S.D. of three plates. Negative control (NC): PBS (100 μl/plate); positive control (PC): for TA 100/−S9, NaN_3 (5 μg/plate); for TA100/+S9, 2-AA (5 μg/plate).
NaN_3: sodium azide, 2-AA: 2-aminoanthracene. A thinning of auxotrophic background growth was not shown.

of 3 concentrations (3,000, 1,500, and 750 μg/plate) were tested. The mutagenic activity was determined both in the absence or presence of S9 mixture. The Ames test without S9 metabolic activation can only detect direct mutagens (NaN_3, AF-2, and 9-AA), while with S9 metabolic activation allows the detection of indirect mutagens (2-AA) (5). As a result, tortoside A did not cause any increase in the number of his^+ revertants in *S. typhimurium* and *E. coli* WP2*uvr*A strains in the presence or absence of S9 mix, compared to the controls. Similarly, low concentrations of

Table 3. Revertants in five **s**train**s** of *S. typhimurium* and *E. coli* WP2*uvr*A treated with different concentration**s** of syringaresinol in the absence or presence of a metabolic-activating enzyme (S9)

Metabolic activation system	Test substance	Part	Conc. (μg/plate)	Number of colonies (Mean ± SD)				
Type culture strain				TA100	TA98	TA1535	TA1537	*E. coli*
S9(−)	PBS	-	100 μl/plate	212 ± 25	21 ± 2	14 ± 1	11 ± 2	20 ± 1
	A. koreanum	Syringeresinol	3,000	203 ± 16	14 ± 2	34 ± 3	9 ± 1	27 ± 4
			1,500	231 ± 17	19 ± 4	16 ± 3	19 ± 1	35 ± 2
			750	213 ± 26	26 ± 4	15 ± 1	15 ± 3	32 ± 2
	NaN_3	-	-	1150 ± 45	-	375 ± 18	-	
	AF-2	-	-	-	511 ± 13	-	-	399 ± 26
	9-AA	-	-	-	-	-	213 ± 11	-
S9(+)	PBS	-	100 μl/plate	170 ± 10	27 ± 4	20 ± 3	11 ± 2	22 ± 4
	A. koreanum	Syringeresinol	3,000	294 ± 11	37 ± 3	20 ± 9	15 ± 1	30 ± 4
			1,500	173 ± 22	31 ± 7	25 ± 4	16 ± 1	24 ± 7
			750	144 ± 18	28 ± 3	30 ± 2	9 ± 2	27 ± 1
	2-AA	-	-	1059 ± 39	643 ± 30	417 ± 25	366 ± 19	586 ± 31

Values are mean ± S.D. of three plates. The negative control (NC) consisted of 100 μl PBS/plate. The positive control (PC) in the −S9 plate consisted of TA98, AF-2 (0.01 μg/plate); for TA100 and TA1535, NaN_3 (5 μg/plate); for TA1537, 9-AA (50 μg/plate); for WP2*uvr*A, AF-2 (0.01 μg/plate). The positive control (PC) in the +S9 plate consisted of 2-AA (5 μg/plate).
NaN_3: sodium azide, 9-AA: 9-aminoacridine, AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide, 2-AA: 2-aminoanthracene.

Table 4. Revertants in five strains of *S. typhimurium* and *E. coli* WP2*uvr*A treated with different concentrations of tortoside A in the absence or presence of a metabolic-activating enzyme (S9)

Metabolic activation system	Test substance	Part	Conc. (μg/plate)	Number of colonies (Mean ± SD)				
Type culture strain				TA100	TA98	TA1535	TA1537	*E. coli*
S9(−)	PBS	-	100 μl/plate	130 ± 5	20 ± 5	13 ± 2	6 ± 1	21 ± 3
	A. koreanum	Tortoside A	3,000	177 ± 11	21 ± 2	17 ± 3	6 ± 2	31 ± 2
			1,500	192 ± 12	26 ± 2	18 ± 3	5 ± 1	25 ± 5
			750	194 ± 16	24 ± 1	13 ± 2	4 ± 1	17 ± 4
	NaN_3	-	-	964 ± 31	-	591 ± 12	-	
	AF-2	-	-	-	475 ± 11	-	-	433 ± 38
	9-AA	-	-	-	-	-	261 ± 12	-
S9(+)	PBS	-	100 μl/plate	165 ± 14	33 ± 7	19 ± 2	9 ± 1	40 ± 4
	A. koreanum	Tortoside A	3,000	178 ± 32	28 ± 9	12 ± 1	7 ± 1	35 ± 8
			1,500	181 ± 16	33 ± 4	24 ± 2	12 ± 1	47 ± 5
			750	189 ± 18	30 ± 8	30 ± 2	8 ± 2	29 ± 2
	2-AA	-	-	1116 ± 64	587 ± 32	519 ± 17	336 ± 25	662 ± 43

Values are mean ± S.D. of three plates. The negative control (NC) consisted of 100 μl PBS/plate. The positive control (PC) in the −S9 plate consisted of TA98, AF-2 (0.01 μg/plate); for TA100 and TA1535, NaN_3 (5 μg/plate); for TA1537, 9-AA (50 μg/plate); for WP2*uvr*A, AF-2 (0.01 μg/plate). The positive control (PC) in the +S9 plate consisted of 2-AA (5 μg/plate).
TA: tortoside A, NaN_3: sodium azide, 9-AA: 9-aminoacridine, AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide, 2-AA: 2-aminoanthracene.

syringaresinol (750 and 1,500 μg/plate) did not show any mutagenic properties (Table 3 and Table 4) in all bacterial strains, in the presence or absence of S9 mixture. However, in the high concentration of syringaresinol (3,000 μg/plate), the number of revertants were increased in TA1535 strains, in the absence of S9 metabolic activation (Table 3). Since TA1535 has a base substitution that produces a missense mutation in the gene coding, where the mutant enzyme has a proline instead of leucine, it would be interesting to perform *in vivo* experiments such as comet assay for further studies.

In conclusion, tortoside A from the roots of *Acanthopanax koreanum* showed a negative result in the bacterial reverse mutation test, indicating that it is not mutagenic. However, mutagenic activity was observed in the high concentration of syringaresinol without metabolic activation for TA1535 strains. Hence, further experiments are needed to study the genotoxicity potential of syringaresinol in humans.

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