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Genotoxicity Study of Polysaccharide Fraction from *Astragalus membranaceus's* Aerial Parts

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Radix Astragali, the root of *Astragalus (A.) membranaceus*, has been applied in a variety of diseases for a long time in Asian countries such as Korea and China. In addition, the aerial parts such as leaves and stems of *A. membranaceus* have received a great deal of attention. Recently, the polysaccharide fraction showing a potent immunomoduating activity was isolated from the aerial parts of *A. membranaceus.* Thus, the aerial parts of *A. membranaceus* would be worthy enough for a food material and a dietary supplement. However, they should be safe even though valuable. In our previous study, it was estimated that NOAEL for female rats are 5000 mg/kg/day of the crude polysaccharide fraction from *A. membranaceus*-aboveground parts. As a series of safety evaluation, genotoxicity test for the crude polysaccharide fraction was carried out in this study. In conclusion, the three genotoxicity assays provided strong overall support that the crude polysaccharide fraction lacks mutagenic and/or clastogenic potential under the GLP-based test conditions. This indicates the aerial parts of *A. membranaceus* would be safe enough for a food material and a dietary supplement.

***Key words*:** *Astragalus membranaceus*, Aerial parts, Genotoxicity test, Polysaccharide

INTRODUCTION

Radix Astragali (RA) is the root of a perennial plant, *Astragalus (A.) membranaceus* Bunge. which is one of the oldest and most frequently used crude herbs for traditional medicine in many asian countries (1,2). More than 100 compounds including polysaccharides, flavonoids, saponins, amino acids and other trace elements exist in Radix Astragali (3). Especially, the polysaccharides found in RA have received a great deal of attention since polysaccharides have been implicated as the main bioactive ingredient of RA and play important pharmacological roles in reducing oxidative stress (4,5).

The polysaccharides found in RA have received a great deal of attention, especially the polysaccharide fraction F3. They have been shown to play a role in immunomodulator actions. Polysaccharides A, B, and C have been identified as glucans, and polysaccharide D as a heteropolysaccharide (5). Polysaccharide fraction of RA (RAP) has been reported to contain a relatively high concentration of β-glucan (6) and have antioxidant, antidiabetic, anti-hypertensive, and immunomodulatory activities (7). There were many research reports on chemical constituents on root parts, but few works have ever been reported on its aerial parts. Recently, the polysaccharides was isolated from the aerial parts (or aboveground parts) such as leaves and stems of *A. membranaceus* having a potent immunomoduating activity (8).

Additionally, the aerial part of *A. membranaceus* is available within a short time for harvest. Indeed, the aerial parts of *A. membranaceus* can be harvested every year, whereas the roots of *A. membranaceus* is harvested 4~6 year after seeding. Thus, the aerial parts of *A. membranaceus* would be worthy enough for a food material and a dietary supple-

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ment in pharmaceutical and economical aspects. However, they should be safe even though the aerial parts of *A. membranaceus are* valuable as a functional material. In our previous study of repeated dose 90-day oral toxicity test for the polysaccharide fraction from *Astragalus membranaceus's* aerial parts, it was estimated that NOAEL (no observed adverse effect level) for rats are 5000 mg/kg/day of the water-extracts from *A. membranaceus*-aerial parts (9). This NOAEL is a dose 30~40 times as high as the effective oral dose in humans. However, genotoxicity test for *A. membranaceus*-aerial parts should be checked whether mutagenic or not. As a series of safety evaluation, GLP (good laboratory practice)-based genotoxicity test through bacterial reverse mutation, in vitro chromosome aberration, and in vivo mammalian erythrocyte micronucleus assay was conducted to assess any mutagenic and clastogenic potential of the polysaccharide fraction (AMA-0) from the aerial parts *A. membranaceus* Bunge.

MATERIALS AND METHODS

Preparation and identification of crude polysaccharide fraction. *A. membranaceus* Bunge were cultivated and harvested at the field of the Jeongsen country, Jeongsen Agricultural technology & Extension Center, in July, 2009 (as 3 years old plants). The harvested was identified and qualified by Sequence characterized amplified region (SCAR) markers to confirm Korean hwanggi medicine selectively (10). A crude polysaccharide fraction was prepared from aerial parts (stems and leaves) of *A. membranceus* by hot water extraction, EtOH precipitation and dialysis. Aerial parts of *A. membranceus* (1,000 g, dry weight) were extracted in boiling distilled H_2O (20 L) until the volume of H_2O was reduced by half. The above procedure was repeated two additional times and the combined extracts were concentrated to 2 L by evaporation. The concentrated extract was stirred at room temperature to which five folds EtOH (10 L) was added to precipitate the polysaccharide fraction. The suspension was stirred overnight and the precipitate was collected by centrifugation (7000 rpm, 30 min, 4°C). The precipitate was dissolved in distilled H_2O, transferred to a cellulose dialysis tubing (molecular weight cut off; 12,000~14,000, Sigma aldrich, USA) and dialyzed against distilled H_2O. After centrifugation (7000 rpm, 30 min, 4°C), the supernatant, called as AMA-0, was lyophilized to obtain a crude water soluble polysaccharide fraction (19.42 g of aerial parts 1,000 g). And then, this crude water soluble polysaccharide fraction was identified further to determine whether it contains polysaccharide or not. The Mw(molecular weight) values of polysaccharides were determined by high performance size exclusion chromatography (HPSEC) using a JASCO model 980 HPLC system equipped with combined columns of Ultrahydrogel™ 2000 and 500 (Waters corporation, USA) in 0.2 M NaCl as the solvent. Relative Mw values of polysaccharides were estimated from the calibration curve obtained by using standard pullulans (Showa Denko Co. Ltd., Tokyo). The contents of β-D-(1 → 3)-galactan in the polysaccharides were analyzed using a single radial gel diffusion using β-D-glucosyl-Yariv antigen according to the procedure of Holst and Clarke (11). A solution (100 ml) of AMA-0 (10 g) was applied to a column (5.6 × 38 cm) of QAE-Sepharose FF (HCO_3), and the column was held with distilled H_2O until non-absorbed portion removed. The absorbed portion was eluted with 0.068 M, 0.115 M, 0.159 M and 0.582 M with NH_4HCO_3 (1000 ml, linear gradient) and four fractions (AMA-1-a, 4.8%; AMA-1-b, 3.2%; AMA-1-c, 3.3% and AMA-1-d, 33.4%) collected after dialysis (MWCO 3500, molecular weight cut off; 12,000~14,000) against distilled H_2O and lyophilization. AMA-1-a-PS1 (Mw 75,000, yield 0.54%) from AMA-1-a was obtained as low molecular weight polysaccharide using Sepharose CL-6B column (2.5 × 94 cm). AMA-1-b was fractionated on Sephacryl S-200 (1.7 × 82 cm) with 0.2 M NaCl to yield intermediate and low molecular weight polysaccharide, AMA-1-b-PS2 (Mw 34,500, yield 0.13%) and AMA-1-b-PS3 (Mw 87,000, yield 0.52%). AMA-1-c was also fractionated on Sephacryl S-300 (2.6 × 90 cm), AMA-1-c-PS4 (Mw 87,000, yield 0.52%) was obtained low molecular weight polysaccharide. The void volume fraction on Sephacryl S-300 was further fractionated on Sepharose CL-6B column using 0.2 M NaCl, AMA-1-d-PS5 (Mw 129,000, yield 2.40%) and AMA-1-d-PS6 (Mw 88,000, yield 2.9%) was obtained as intermediate and low molecular weight polysaccharide. Thus, it was identified that a crude water soluble polysaccharide fraction contains, at least, six polysaccharides with various size. In the present study, the AMA-0 was used for subsequent tests.

Test guidelines. The mutagenic assay was conducted under GLP conditions in accordance with the international guidelines of the Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial reverse mutation test", adopted July 21, 1997. The in vitro clastogenic assay followed the international guidelines of the Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 473, "In vitro Mammalian Chromosome Aberration Test", adopted July 21, 1997. The in vivo clastogenic assay followed the international guidelines of the Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 474, "Mammalian Erythrocyte Micronucleus Test", adopted July 21, 1997. The rodent phase of the in vivo micronucleus study was conducted in accordance with the protocol reviewed by the Animal Welfare Officer in Catholic University of Daegu (Accreditation No. IACUC-2011-010).

Reverse mutation (RM) test. Five strains such as TA98, TA100, TA1535, TA1537 of *Salmonella typhimurium*, and

WP2uvrA of *Escherichia coli* were used for test. Each cell line was tested after determination on its genetic characteristics, such as histidine-deficiency, crystal violet sensitivity, UV sensitivity, tolerance against ampicillin, and frequency of spontaneous reverse-mutation. Test for dose determination was conducted from the highest 5 μl/plate divided by 2. Also, tests for dose determination and main test were conducted by metaphase analysis both in the absence and presence of S9 fraction. In the case without S9, sodium azide (NaN_3), 9-aminoacridine (9-AA), 4-nitroquinoline 1-oxide (4-NQO), and 2-aminoanthracene (2-AA) and in the case with S9 fraction, 2-AA were used for the positive controls. Each 5 strain was put in a test tube and blended with 0.1 ml fermentation solution, 0.05 ml AMA-0 and 0.5 ml phosphate buffer saline (0.2 M, pH 7.4) (0.5 ml S9 mix in case of metaphase analysis) and then pre-incubated (37°C, 30 min). After the pre-incubation, 2 ml top agar was added and double laid on minimal glucose agar plate, and then incubated (37°C, 48 hr). Reverse mutated colonies were counted. The colony counts were the average values of triplicates. When the reverse-mutated colonies increase dose-dependently or more than twice, it is positive as the mutation inducer (12). No statistical skill was used for discussion of this result.

Chromosome aberration (CA) test. Cultured cells of Chinese hamster ovary fibroblast cell line (CHO-K1) purchased from Korea Cell Line Bank (KCLB, Seoul, Korea) were used for test. Cells were cultured on Eagles minimum essential media (EMEM) including 10% fetal bovine serum (FBS), penicillin, and streptomycin, subcultured with 0.5% trypsin-EDTA per every 2 or 3 days. Chromosomal number of cell is basically 22 and it takes about 15 hr for twice. After removal of the culture media, 0.05 ml AMA-0 and 0.5 ml S9 mix were poured into 4.45 ml fresh culture media (37°C) for a total of 5 ml. Culture time with test material of metaphase analysis in the presence of S9 was 6 hr followed by 18 hr in normal medium and without S9 was 24 hr. Test for dose-determination was conducted by 7 concentration steps (0.08, 0.16, 0.32, 0.63, 1.25, 2.5, and 5.0 μl/ml) from the highest 5.0 μl/plate divided by 2. The inhibitory rate against cell proliferation was calculated by mitotic index from chromosomal sample against negative control (100%). The inhibitory rate was also determined by metaphase analysis with and without S9 fraction. Positive controls of the analyses were treated with mitomycin C (MMC) 0.15 μg/ml and cyclophosphamide (CPA) 15 μg/ml, respectively. Sterile distilled water was used as a negative control. Cells treated with AMA-0 were cultured for 24 hr and then treated with 0.2 μg/ml of Colcemid for 2 hr followed by centrifugation (160 g × 5 min) and collected. The upper layer of the cell solution was removed, then it was treated with 75 mM KCl store solution (37°C) for 20 min and fixed in Carnoy's solution (acetic acid : methanol = 1 : 3). Chromosome sample was dyed with 5% Giemsa solution for 5 min and then CAs of 100 medium cells were counted. Two pieces of sample plates were made per every concentration. The CA was grouped by structural and numerical aberration through microscopic observation of 200 medium cells as affected by concentration. In case of structural aberration, break and exchange of chromosome and chromatid were counted and both cases with and without gap were also recorded (13,14). Numerical aberration was defined from the frequency of diploid, polyploidy, and endo duplication classified by chromosomal number. Each result was determined on the frequency rate of CA as affected by background level as follows;

(1) Cases where CA are found in test groups while less than 0.5% CA is found in negative control.

a. It is positive that CA cells are significantly increased dose-dependently ($p = 0.01$).

b. It is strong positive that CA cells of all test groups, which are no dose-dependent or more than 2 groups are significantly increased ($p = 0.01$) and it is weak positive that parts of the test groups are significantly increased ($p = 0.01$).

(2) Cases where CA is found in test group while 0.5~5.0% CA is found in negative control.

Less than 5%	− (negative)
More than 5% and less than 10%	± (pseudo positive)
More than 10%	+ (positive)

Micronucleus (MN) test. This test was performed in a specific pathogenfree (SPF) area (room temperature 23 ± 3°C, relative humidity 50 ± 10%, ventilation frequency 10~20 times/hr, lighting duration 12 hr, and luminous intensity 150~300 lx). Mice were fed the irradiation-sterilized pellet food (Zeigler Bros. Inc. USA) ad libitum. Water was disinfected by UV sterilizer and ultrafiltration and made available ad libitum using a water bottle. The main test was conducted in 3 treated groups, which consisted of 2,000, 1,000, and 500 mg/kg, respectively. MMC, 2 mg/kg was commonly used as a positive control in this kind of study. MMC was dissolved in sterile distilled water before use. Dead mice were not found through limit test feeding 2,000 mg/kg. Also, no pathogenic difference was found in every male and female mouse after feeding the limit dose. Therefore, a male mouse was used in main test with the highest 2 g/kg of the limit dose and 2 additional lower doses divided by 2. AMA-0 was fed for 24 and, 48 hr. After feeding the AMA-0, the cervical vertebra was disarticulated, and femur was extracted with no blood contamination. Myelocyte was washed with 500 μl FBS and then centrifuged (160 ×g, 5 min). After discard of upper layer, the myelocyte was resuspended with FBS. The solution was smeared on a slide glass, dried at an ambient temperature, and fixed with methanol for 5 min. The fixed sample was dyed with acridine

orange (40 μg/ml), covered with a cover glass, then bone marrow cell proliferation, and micronucleated polychromatic erythrocytes (PCE) created in PCE were observed. To find out proliferating phenomena, about 200 of total erythrocyte (polychromatic and normochromatic erythrocytes) selected in good smeared area were observed and determined the rate of PCE. MN cells were counted from about 2,000 of PCE to check out the MN frequency. Among dyed samples, PCE was the erythrocyte emitting red fluorescence without nucleus and normochromatic erythrocyte (NCE) was sorted by shade without fluorescence. Significant differences of both the frequency of micronucleated PCE of AMA-0 fed group against negative control and the ratio of PCE against total erythrocyte were determined by one-way analysis of variance (ANOVA), Dunnett's T test and Duncan test ($p = 0.05$) of multiple comparison through SPSS 14.0K (SPSS Inc., Chicago, IL, USA).

RESULTS

RM test. In dose-determination test (data not shown), conducted from the highest 5.0 μl/plate divided by 2 (5.0, 2.5, 1.25, 0.625, 0.3125, and 0.0 μl/plate), no growth inhibitory effect by the AMA-0 was found in every concentration compared to the result of control. Thus, the tested doses were applied for the following main test. The results of RM by AMA-0 with and without S9 mix are shown in Table 1. In the main test conducted by metaphase analysis without S9, TA 100 cell line showed 112 ± 9.5, 107 ± 4.6, 123 ± 3.2, 138 ± 20.4 and 144 ± 5.8 colonies/plate in the negative control and each 5 test group, respectively. TA1535 showed the range from 23 ± 3.5 to 32 ± 4.2 colonies/plate and WP2uvrA was from 31 ± 1.7 to 36 ± 2.7 colonies/plate. No dose dependence was found in these base-substitution type strains. In the case of frameshift type strains, TA98 showed 43 ± 3.2, 42 ± 5.3, 50 ± 5.6, 44 ± 3.0, 42 ± 1.2 and 47 ± 2.0 colonies/plate and TA1537 showed the range from 13 ± 4.2 to 21 ± 2.9 colonies/plate and the positive control was 187 ± 13.2 colonies/plate. In metaphase analysis with S9, TA100 cell line showed 94 ± 11.7, 96 ± 14.7, 95 ± 6.6, 93 ± 12.0, 104 ± 8.1 and 103 ± 13.1 colonies/plate in the negative control and each 5 test group, respectively. TA1535 showed the range from 18 ± 2.0 to 28 ± 6.1 colonies/plate and WP2uvrA was from 32 ± 2.0 to 36 ± 2.7 colonies/plate without any dose dependence. TA98 showed the range from 39 ± 2.3 to 48 ± 4.9 colonies/plate and TA1537 which had the range from 13 ± 6.1 to 21 ± 1.2 colonies/plate with no dose-dependence and the positive control was 135 ± 3.0 colonies/plate. Strains and dose groups which was increased more than twice, compared to that of the negative control (a determination criterion in the main test) were not found in metaphase analysis both with and without S9 mix. Reverse-mutated colonies in positive control groups were induced within the standard range of positive determination, so this test was appropriately done.

CA test. Cell growth rate: Cell growth (CG) rates were measured in both dose determination test and main test

Table 1. Reverse mutation with and without metabolic activation by AMA-0

		Dose (μg/plate)	No. of reverse mutation colonies/plate				
			Base-substitution type			Frame-shift type	
			TA100	TA1535	WP2urA	TA98	TA1537
w/o S-9Mix		0.0	121 ± 8.4	28 ± 3.6	34 ± 2.0	43 ± 3.2	19 ± 0.6
		0.3125	112 ± 9.5	28 ± 2.0	31 ± 1.7	42 ± 5.3	13 ± 4.2
		0.625	107 ± 4.6	27 ± 3.6	35 ± 4.4	50 ± 5.6	17 ± 6.8
		1.25	123 ± 3.2	23 ± 3.5	33 ± 1.0	44 ± 3.0	21 ± 2.9
		2.5	138 ± 20.4	27 ± 5.5	35 ± 2.3	42 ± 1.2	17 ± 0.6
		5.0	144 ± 5.8	32 ± 4.2	36 ± 2.7	47 ± 2.0	20 ± 1.2
w/ S-9Mix		0.0	94 ± 11.7	20 ± 4.0	32 ± 2.0	44 ± 8.7	20 ± 6.7
		0.3125	96 ± 14.7	25 ± 1.5	36 ± 2.7	45 ± 6.7	13 ± 6.1
		0.625	95 ± 6.6	18 ± 2.0	35 ± 4.9	39 ± 2.3	16 ± 6.9
		1.25	93 ± 12.0	22 ± 5.0	36 ± 2.7	48 ± 4.9	21 ± 1.2
		2.5	104 ± 8.1	22 ± 1.7	32 ± 2.5	45 ± 6.7	18 ± 4.0
		5.0	103 ± 13.1	28 ± 6.1	34 ± 2.9	44 ± 2.9	20 ± 5.1
Positive control	w/o S-9Mix	Chemicals	NaN_3	NaN_3	4-NQO	4-NQO	9-AA
		Dose (μg/plate)	1.5	1.5	0.5	0.5	80
		No. of colonies/plate	331 ± 10.5*	258 ± 5.1*	311 ± 9.5*	162 ± 4.4*	187 ± 13.2*
	w/ S-9Mix	Chemicals	2-AA	2-AA	2-AA	2-AA	2-AA
		Dose (μg/plate)	1.0	2.0	10	0.5	2.0
		No. of colonies/plate	345 ± 13.1*	130 ± 7.6*	168 ± 6.0*	167 ± 16.1*	135 ± 9.0*

*Significantly different from the control (0.0 dose) at $p < 0.05$.

Table 2. Cytotoxicity data for dose-determination and in the main study of CA test

	Concentration (μl/ml)	Dose-determination	Main study
		Cell growth rate (% of negative control)	Cell growth rate (% of negative control)
		24 hr treatment	24 hr treatment
w/o S9Mix	0.0 (negative control)	100	100
	0.08	100	NT[1)]
	0.16	94	NT
	0.31	100	NT
	0.63	98	NT
	1.25	95	100
	2.5	83	97
	5.0	75	85
	Concentration (μl/ml)	Dose-determination	Main study
		Cell growth rate (% of negative control)	Cell growth rate (% of negative control)
		24 hr treatment	24 hr treatment
w/ S9Mix	0.0 (negative control)	100	100
	0.08	96	NT
	0.16	98	NT
	0.31	100	NT
	0.63	97	NT
	1.25	100	97
	2.5	93	93
	5.0	97	89

[1)]; NT: not tested.

Table 3. Meaphase analysis in the absence of S9 mix

Group	Treated time (hr)	Dose (μl/ml)	No. of observed cells	Polyploid		No. of aberrant cells								Incidence (%) (−g)
						Gap	Chromatid type		Chromosome type		Others	Total no. of aberrant cells		
				No.	Incidence (%) / Decision	g	ctb	cte	csb	cse		−g[1)]	+g	Decision
Negative control	24	0.0	100	0.0 ± 0.00[2)]	Control	0.5	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.00	0.5 ± 0.71	Control
Test substance	24	1.25	100	0.0 ± 0.00	Negative	1.0	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.00	1.0 ± 0.00	Negative
		2.5	100	0.0 ± 0.00	Negative	0.5	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.00	0.5 ± 0.71	Negative
		5	100	0.0 ± 0.00	Negative	0.5	0.5	0.0	0.0	0.0	0.0	0.5 ± 0.71	1.0 ± 1.41	Negative
Positive control (CPA)	24	0.15	100	0.0 ± 0.00	Negative	5.0	3.0	15.0	2.5	1.5	0.0	22.0 ± 0.00	27.0 ± 1.41	Positive

[1)]; g, gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange.
[2)]; Mean ± SD (n = 2).

(Table 2). In the test without S9 and 24 hr treatment, the CG rates were 100, 94, 100, 98, 95, 83 and 75% in 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, and 5.0 μl/ml treated groups, respectively. In the test with S9 and 6 hr treatment, the CG rate was 100% in negative control, 96, 98, 100, 97, 100, 93 and 97% in 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, and 5.0 μl/ml treated groups, respectively. According to the Organization for Economic Co-operation and Development (OECD) guideline, the highest dose in the main test is the concentration of test material inhibiting CG 50% or more. But the inhibitory activity against CG was less than 25% at the highest 5.0 μl/ml in metaphase analyses both with and without S9 mix. Thus, CA test were conducted by 3 step doses of 5.0, 2.5 and 1.25 μl/ml. In the main test, the CG rates were 100, 97 and 85% in 1.25, 2.5 and 5.0 μl/ml treated groups for 24 hr without S9, respectively and they were 97,

Table 4. Metaphase analysis in the presence of S9 mix

Group	Treated time (hr)	Dose (μl/ml)	No. of observed cells	Polyploid		No. of aberrant cells								Incidence (%) (−g)
						Gap	Chromatid type		Chromoseome type		Others	Total no. of aberrant cells		
				No.	Incidence (%) Decision	g	ctb	cte	csb	cse		−g[1)]	+g	Decision
Negative control	6	0.0	100	0.5 ± 0.71[2)]	Control	1.5	1.0	0.0	0.0	0.0	0.0	1.0 ± 0.00	2.5 ± 0.71	Control
Test substance	6	1.25	100	0.0 ± 0.00	Negative	1.0	0.0	1.0	0.0	0.0	0.0	1.0 ± 0.00	2.0 ± 0.00	Negative
		1.25	100	0.0 ± 0.00	Negative	1.5	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.00	1.5 ± 0.71	Negative
		5	100	0.5 ± 0.71	Negative	1.0	0.5	0.0	0.0	0.0	0.0	0.5 ± 0.71	1.5 ± 0.71	Negative
Positive control (CPA)	6	15	100	0.0 ± 0.00	Negative	5.5	3.5	20.5	0.5	1.5	0.0	26.0 ± 1.41	31.5 ± 2.12	Positive

[1)]; g, gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange.
[2)]; Mean ± SD (n = 2).

Table 5. In vivo micronucleus test

Treatment	Treatment time (hr)	Dose (mg/kg)	PCE observed	MNPCE observed	MNPCE (%)	PCE/(PCE+NCE) counted	PCE counted	PCE/(PCE+NCE) (%)
Negative control	24	0	2009 ± 7.62	3.0 ± 1.22	0.15 ± 0.062	200 ± 0.00	82.0 ± 4.18	41.0 ± 2.09
	24	500	2027 ± 12.6	3.4 ± 0.89	0.17 ± 0.044	200 ± 0.00	81.4 ± 4.04	40.7 ± 2.02
Test substance	24	1000	2059 ± 62.8	1.6 ± 0.55	0.08 ± 0.027	200 ± 0.00	86.0 ± 2.74	43.0 ± 1.37
	24	2000	2047 ± 46.4	1.6 ± 1.82	0.08 ± 0.090	200 ± 0.00	80.8 ± 3.83	40.4 ± 1.92
Positive control	24	2	2094 ± 107.0	22.4 ± 2.51	1.08 ± 0.161*	200 ± 0.00	87.2 ± 6.57	43.6 ± 3.29
Negative control	48	0	2013 ± 7.3	1.8 ± 1.30	0.09 ± 0.065	200 ± 0.00	80.2 ± 5.07	40.1 ± 2.53
	48	500	2015 ± 8.2	1.2 ± 0.45	0.06 ± 0.022	200 ± 0.00	82.6 ± 4.22	41.3 ± 2.11
Test substance	48	1000	2009 ± 10.8	2.0 ± 1.00	0.10 ± 0.050	200 ± 0.00	83.2 ± 3.42	41.6 ± 1.71
	48	2000	2014 ± 12.7	1.6 ± 1.95	0.08 ± 0.096	200 ± 0.00	79.0 ± 2.83	39.5 ± 1.41
Positive control (MMC)	48	2	2017 ± 23.1	6.0 ± 2.00	0.30 ± 0.096*	200 ± 0.00	81.6 ± 2.30	40.8 ± 1.15

* Significantly different from other groups.

93 and 89% in the same amount-treated groups for 6 hr with S9, respectively. The frequency of CA in the absence of S9 was shown in Table 3. The frequencies of structural CA, when gap(g) was not included, were 0.0 ± 0.00, 0.0 ± 0.00, and 0.5 ± 0.71 in 1.25, 2.5 and 5.0 μl/ml, respectively and the negative control was 0.0 ± 0.00. No polyploid in the numerical CA was shown in the all AMA-0-treated groups and negative control. The frequency of CA with S9 was shown in Table 4. The frequencies of structural CA were 1.0 ± 0.00, 0.0 ± 0.00 and 0.5 ± 0.71 were 1.25, 2.5 and 5.0 μl/ml, respectively and the negative control was 1.0 ± 0.00. Polyploid was not shown in all test groups including the negative control. The frequency of CA was not dose-dependent in the tests both with and without S9. The frequencies of structural CA of the positive control by metaphase analysis with and without S9 were significantly different ($p < 0.01$) with those of the negative control. Therefore, it was evaluated that the conditions and conclusion of these tests were suitable.

MN test. According to the pretest for single dose oral toxicity test (data not shown), approximate lethal dose (ALD) on mice was estimated to be more than 2.0 ml/kg, a limit dose. Therefore, three dose steps were applied from the highest 2.0 ml/kg divided by 2. Three dose steps were applied from the highest 2.0 ml/kg divided by 2. AMA-O was fed for 24 and 48 hr and then, the mice were observed (Table 5). Polychromatic erythrocytes (PCE) among total erythrocytes were 41.0 ± 2.09, 40.7 ± 2.02, 43.0 ± 1.37 and 40.4 ± 1.92% in each negative control, 0.5, 1.0, and 2.0 ml/kg fed groups for 24 hr, respectively. The mean frequencies were not significantly different ($p > 0.05$). Mean frequencies of micronucleated PCE (MNPCE) among PCE more than 2,000 were 0.15 ± 0.062, 0.17 ± 0.044, 0.08 ± 0.027 and 0.08 ± 0.090 in the negative control and 0.5, 1.0, 2.0 ml/kg fed group for 24 hr, respectively. Mean frequencies

were not significantly different and they were not dose-dependent ($p > 0.05$). PCEs among total erythrocytes were 40.1 ± 2.53, 41.3 ± 2.11, 41.6 ± 1.71, 39.5 ± 1.41% in each negative control, 0.5, 1.0, and 2.0 ml/kg fed group for 48 hr, respectively and the mean frequencies were not significantly different ($p > 0.05$). Mean frequencies of micronucleated PCE among PCE more than 2,000 were 0.09 ± 0.065, 0.06 ± 0.022, 0.10 ± 0.050 and 0.08 ± 0.096% in each negative control, 0.5, 1.0, and 2.0 ml/kg fed groups for 48 hr, respectively. Mean frequencies were not significantly different and they were not dose-dependent ($p > 0.05$). These results showed that the frequencies of micronucleated erythrocytes among PCEs of bone marrow in all AMA-0-treated groups for 24 and 48 hr were not significantly different, compared to the negative control and they were not dose-dependent. Also, the AMA-0 did not induce the inhibition of erythrocyte-growth. Therefore, under this test condition it was confirmed that the AMA-0 did not induce micronuclei on the bone marrow cells of mouse.

DISCUSSION

Even if *A. membranaceus* has shown a variety of pharmacological effect and has been used, botanic sources for pharmacological use are limited to its root. Recently, the aerial parts such as leaves and stems of *A. membranaceus* showed the chemical components sharing partly with them on the root (15,16). Especially, the polysaccharides were isolated from the aerial parts of *A. membranaceus* and showed important pharmacological roles in immune-enhancing in our previous work (8). Thus, the aerial parts of *A. membranaceus* have also pharmacological properties with potential application in form of functional foods. In general, Astragalus is safe. The safety study shows that doses as high as 100 g/kg of raw herb have been given by lavage to rats with no adverse effects (17). And the LD_{50} of Astragalus in mice was determined to be approximately 40 g/kg when administered by intraperitoneal injection (18). Also, the aerial parts of *A. membranaceus* was proved to be as safe as the root through a 90-day oral toxicity study in rats (9). In a series of safety evaluation for the aerial parts of *A. membranaceus*, a genotoxicity test was carried out in this study to check the mutagenisity. For genotoxicity test, the leaf and stem polysaccharides fraction of *A. membranaceus* was extracted with hot water and then fractionated by ultrafiltration. In conclusion, the three genotoxicity assays provided strong overall support that AMA-0 lacks mutagenic and/or clastogenic potential. AMA-0 did not exhibit mutagenicity under the conditions of the bacterial reverse mutation assay in S. typhimurium or E. coli tester strains with or without metabolic activation at levels up to the test limit doses 5 μg/plate and 2000 mg/kg in mice. This indicates the aerial parts of *A. membranaceus* would be safe enough for a food material and a dietary supplement.

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