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Genotoxicity Study of Water Extract of *Anemarrhena asphodeloides* and *Phellodendron amurense* in Bacterial and Mammalian Cell Systems

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ABSTRACT. In order to investigate the safety of a water extract (ADP) of 1 : 1 mixture of *Anemarrhena* rhizoma and *Phellodendron* cortex for alleviating benign prostate hyperplasia, genotoxicity studies in bacterial and mammalian cell assay systems, namely, the Ames bacterial reverse mutation and chromosomal aberration assays were performed. As shown by the results of the Ames bacterial reversion assay, ADP in the range of 625~5000 μg/plate did not induce mutagenicity in *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 strains in the absence or in the presence of S9 (the microsomal fraction of rat liver homogenate) metabolic activation. The IC_{50} (50% cell growth inhibition concentration) values of ADP for the chromosomal aberration assay were determined; these were 2425 μg/ml in the absence and 8126 μg/ml in the presence of S9 metabolic activation in Chinese hamster lung (CHL) fibroblast cell culture. No chromosomal aberration was observed in CHL cells treated with ADP at 2425, 1212.5 and 606.25 μg/ml in the absence, or at 8126, 4063 and 2031.5 μg/ml in the presence of S9 metabolic activation. These results show that under the conditions used, ADP does not harmfully affect the bacterial or mammalian cell system at the gene level.

Keywords: Bacterial reverse mutation assay, Chromosomal aberration assay, *Anemarrhena asphodeloides, Phellodendron amurense.*

INTRODUCTION

A new pharmaceutical composition (ADP) contains a water extract obtained from the mixture of *Anemarrhena* rhizoma (*Anemarrhena asphodeloides*) and *Phellodendron* cortex (*Phellodendron amurense*). Both plants have been used in traditional medicine (Ahn, 1988); *Anemarrhena* rhizoma for inflammation, fever, diarrhea, edema, lumbago and instability, and *Phellodendron* cortex for bacterial infection, hypertension, activation of the central nervous system, inflammation and hepatoprotection. Saponins from *Anemarrhena* (Matsuda *et al.*, 2001) and alkaloids from *Phellodendron* (Ivanovska, 1996) are known to be the main active components. Since those components had been reported to block alpha adronoceptors (Oh *et al.*, 2002), to relax smooth muscle (Cao *et al.*, 2001; Chang *et al.*, 1999) and to inhibit the effects of testosterone 5-reductase (Matsuda *et al.*, 2001), we performed functional tests further into the anti-BPH (benign prostate hyperplasia) property of ADP.

BPH is a disease of elderly men over 50 years of age (McConnel, 1998) and is considered as a degenerative disease characterized by the enlargement of the prostate gland due to intact androgen supply as the man gets old (Partin *et al.*, 1991). In early stage BPH, nodules generate in the transition (grandula) and periurethral zones (stromal), and continuously grow into the major part of the main mass of the prostate, in which case the central and peripheral zones are compressed and fibromuscular tissue develops between the BPH tissues. This progresses over several years, and symptoms appear due to obstruction of the urethra with BPH and to inflammation or infection of the urinary track.

The major treatments for BPH target reducing bladder outlet obstruction by alpha-adrenoceptor blockade in order to relax the smooth muscle tone of the prostate

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(Nishimatsu *et al.*, 1999). In a rat prostate strip study, ADP showed a relaxation effect on the prostate smooth muscle of the rat contracted by phenylephrine, an agonist of alpha-adrenoceptor, or contracted by electric field stimulation. In an *in vivo* experiment with rabbit, urethral pressure lowered after ADP administration, which produced comparable results to tamsulosin, the most widely prescribed drug for BPH (unpublished results).

Therefore, in order to evaluate the safety of ADP for medicinal use, we performed these genotoxic assays. Ames bacterial reverse mutation test using *Salmonella strains* and chromosomal aberration assay using Chinese hamster lung (CHL) cells were conducted according to Good Laboratory Practice (GLP) standards.

MATERIALS AND METHODS

Materials

The root of *Anemarrhena asphodeloides* Bunge (Liliaceae family) and the bark of *Phellodendron amurense* Rupr. (Rutaceae family) were purchased in the Kyungdong Herbal market in Seoul, Korea. They were authenticated by Professor Ki-Whan Bae, Department of Pharmacy (Div. Pharmacognosy), College of Pharmacy, Chung-nam University, Daejeon, Korea. A voucher specimen (No. ADP 100) was deposited at the herbarium of the Medvill central research laboratory, Seoul, Korea.

ADP was prepared as follows; briefly, a 1 : 1 mixture of *Anemarrhena* rhizoma and *Phellodendron* cortex was ground into a fine powder, and then stirred in distilled water. The resulting suspension was saturated with vapour at pressure (121°C/20 p.s.i.), and then concentrated. Precipitates containing coagulated proteins or insoluble materials were removed by centrifugation. The supernatant was then freeze-dried to yield a powder, which is referred to as ADP, at an yield of approximately 15%. The quality of ADP preparation was estimated by TLC and HPLC analysis using standard chemicals.

Chemicals such as 9-aminoacridine, 2-nitrofluorene, sodium azide, 2-aminoanthracene, cyclophosphamide, mitomycin C and Colcemid were obtained from Sigma Co., Ltd. (USA). The S9 fraction (the microsomal supernatant of rat liver homogenate) was purchased from Moltox™ (Molecular Toxicology Inc., USA). Eagle's minimum essential medium (EMEM) and fetal bovine serum (FBS) were purchased from the Invitrogen Corporation (Gibco). All chemicals used were of analytical grade.

Cell Culture

Chinese hamster lung (CHL) fibroblast cells were obtained from the Korean Cell Line Bank (KCLB). The karyotype of the CHL cells consisted of 25 chromosomes, which was well defined for cytogenetic analysis. The cells were maintained for 3~4 days passages and grown in a monolayer with EMEM supplemented with 10% FBS, 50 units/penicillin and 50 μg/ml streptomycin. The cells were maintained at 37°C in humidified 5% CO_2 atmosphere.

Salmonella typhimurium Reverse Mutation Assay

In order to evaluate the mutagenic potential of ADP, a bacterial reverse mutation assay was performed, as described by Ames (Ames *et al.*, 1975; Maron *et al.*, 1983) and the OECD guideline (1997). Briefly, the ADP dosage range for the use of the mutagenicity assay was determined by performing a toxicity assay using the strain *Salmonella typhimurium* TA 100 and half-log dose intervals of the test substance up to 5000 μg/plate. Strain TA 100 was chosen as a representative test strain because of its high spontaneous reversion rate. From the above toxicity assay the maximal nontoxic dose was determined, and four different analyzable concentrations were chosen with half log intervals up to the maximal nontoxic dose.

For the mutagenicity the tester strains (*Salmonella typhimurium* TA 98, TA 100, TA 1535, TA 1537) were cultured overnight at 37°C in a shaking water bath. Each inoculated strain in 0.1 ml suspension was mixed with 0.1 ml of the test extract and 0.5 ml of S9 mixture (or sodium phosphate instead of S9 mixture in the absence of S9 activation system) and voltexed few seconds. Then, after incubating the mixture in a water bath for 30 mins at 37°C, the mixture was mixed with top agar containing a minimal amount of histidine and then poured onto the surface of a petri dish containing 25 ml of solidified bottom agar. The finished plates were incubated for 48 hours at 37°C, then the number of revertant colonies were counted later. Negative/vehicle control plates containing no added test chemical and positive control plates containing appropriate amounts of chemicals known to be active were incubated with each test strain (Table 1).

All platings were done in triplicate, and the results are expressed as means± standard deviation for each condition. A response was considered to be positive if there was a dose-dependent increase in revertants per plate resulting in (1) at least a doubling of the background reversion rate for strains TA 98 or TA 100, and (2) at least a tripling of the background reversion rate for strains TA1535 or TA 1537 (Kim and Margolin, 1999). Statistical anaylsis of data was performed using Students' *t*-test. Significant difference was assessed between negative control and ADP-treated group and $P<0.05$ was considered to be a significant difference.

Table 1. Mutagenicity of ADP in *Salmonella typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537 in the absence or presence of S9 metabolic activation

Compound	Dose (μg/plate)	S9 mix	His$^+$ revertants/plate (Mean± S.D.)			
			TA 98	TA 100	TA 1535	TA 1537
D.W		-	25 ± 7	112 ± 49	21 ± 3	13 ± 3
ADP	625	-	28 ± 7	116 ± 19	22 ± 3	14 ± 1
	1250	-	31 ± 3	86 ± 5	24 ± 6	13 ± 3
	2500	-	44 ± 5	62 ± 4	23 ± 7	14 ± 2
	5000	-	39 ± 8	62 ± 10	39 ± 6	13 ± 2
SA	1	-	-	612 ± 56*	1272 ± 30*	-
2-NF	1	-	142 ± 6*	-	-	-
9-AA	80	-	-	-	-	57 ± 4*
D.W		+	66 ± 5	195 ± 12	27 ± 7	14 ± 2
ADP	625	+	67 ± 2	215 ± 7	23 ± 6	19 ± 6
	1250	+	53 ± 2	224 ± 3	26 ± 7	19 ± 4
	2500	+	55 ± 6	220 ± 11	22 ± 1	13 ± 6
	5000	+	54 ± 7	230 ± 7	33 ± 5	15 ± 3
2-AA	0.5	+	741 ± 36*	-	-	-
2-AA	1	+	-	1633 ± 108*	-	-
2-AA	2	+	-	-	475 ± 70*	406 ± 103*

SA: Sodium azide, 2-NF: 2-Nitrofluorene, 9-AA: 9-Aminoacridine,
2-AA: 2-Aminoanthracene.
*; $P<0.05$ comparison with negative control (D.W.) determined by Students' t-test.

Cell Growth Inhibition Assay

In order to determine the maximal concentration of the test extract for the *in vitro* chromosomal aberration assay, a cell growth inhibition assay was performed using a colorimetric MTT assay (Gerlier and Thomasset, 1986). The CHL cells were treated with five analyzable concentrations of the test extract at half log intervals up to 10 mg/ml for 6 or 24 hours in the absence or in the presence of S9 metabolic activation, respectively. From the results the IC_{50} (50% cell growth inhibition concentration) values of the test extract at each test condition were calculated and chosen as the maximal concentration for cytogenic assay.

In vitro Chromosomal Aberration Assay in CHL Cells

The *in vitro* cytogenic potential of the test extract was evaluated using a method described by Ishidate and Odashima (1977), OECD guideline (1997) and Ryu (Ryu *et al.*, 1993, 1994).

For the assay, three different doses, including the IC_{50} value as a maximum dose, were prepared, and separately added to 3-day-old cultures. Duplicated cultures were used at each concentration. Negative/vehicle cultures and appropriate positive control substances were employed; cyclophosphamide (CP, 10 μg/ml) was used for metabolic activation system and mitomycin C (MMC, 1 μg/ml) for inactivation. In the absence of metabolic activation, the cells were treated for 24 hours with the test extract or for 6 hours with the test extract followed by 18 hours incubation with fresh media. In the presence of metabolic activation, the CHL cells were treated for only 6 hours due to the toxicity of the S9 mixture. The cells were then maintained for 18 hours in fresh media to adjust a time equivalent to about 1.5 normal cell cycle lengths. Colcemid, a metaphase-arresting agent, was added to the cultures 2 hours before incubation was finished. The cells were harvested, swollen in hypotonic solution (0.075 M KCl), washed in ice-cold fixative (methanol : glacial acetic acid=3 : 1), and fixed on the slide by dropping. For microscopic examination, the cells were stained with 5% Giemsa. The chromosomal aberrations were counted on 200 well-spread meta-phase cells at a magnification of 1,000 under a microscope, according to the classification of aberration described in JEMS-MMS (1998). Breaks with a width of less than a chromatid were defined as gaps for the evaluation and were not treated as chromosomal aberrations. Polyploid and endoreduplicated cells were observed and counted.

Aberration frequencies, defined as the observed aberrations divided by number of cells counted, were analyzed using Fisher's exact test (Altman, 1993) with Dunnett's adjustment and compared with the results of the negative/vehicle control.

RESULTS AND DISCUSSION

Mutagenicity of ADP in the Bacterial Reverse Mutation Assay

The Ames bacterial reverse mutagenicity assay was

developed to detect materials that cause specific point mutations such as base-pair substitutions and frame-shift mutations in a bacterial model. This assay is generally performed in the absence and in the presence of an S9 metabolic activation in order to overcome the limitation of the *in vitro* single cell system containing no metabolic capacity of promutagen.

In the present study, to determine the dose range with respect to its toxicity, *Salmonella typhimurium* strain TA 100 in the absence and in the presence of S9 mixture, was treated with five different ADP concentrations, including the maximum dose of 5000 μg/plate but no significant increase in the histidine revertant was observed versus the negative/vehicle control. Thus, 5000 μg/plate was chosen as an optimal maximum concentration for the mutagenicity assay.

For the mutagenicity assay, four different concentrations (5000, 2500, 1250, 625 μg/plate) and four strains of *Salmonella typhimuriums*, namely, TA98, TA100, TA1535, and TA1537 were used. The results were represented as the number of histidine revertant colonies on the histidine minimal agar plate, as shown in Table 1. Negative/vehicle controls of each strain showed the background ranges in terms of histidine revertant counts, while the positive controls as known mutagens caused the expected increases in revertant colonies. ADP treatment at four different doses did not significantly increase the counts of the histidine revertants in all four strains of bacteria in absence or in the presence of S9 metabolic activation ($P>0.05$), and no dose-related mutagenic responses were observed. These results suggest that ADP does not have genotoxic activity in prokaryotic cells.

Cytotoxicity of ADP in CHL Cells

Generally, the IC_{50} value is evaluated for use as an optimal maximum dose for cytogenetic assays, since excessively high doses may lead to artifactual positive responses and some cytotoxic doses should be tested. From the present study using a MTT assay, the IC_{50} values of ADP against CHL fibroblast cells in the absence and in the presence of S9 metabolic activation were determined to be 2425 μg/ml and 8126 μg/ml, respectively, which suggests that some metabolites of the ADP components are less cytotoxic than the parent components.

Clastogenicity of ADP in CHL Cells

The chromosomal aberration assay is a popular method of evaluating clastogenicity in *in vitro* systems. Mammalian cells used for this cytogenetic assay should have a stable, well-defined karyotype, a short generation time, a low chromosome number, and large chromosomes. For this reason, Chinese hamster cells (CHL or CHO) and human lymphocytes have been extensively used. CHL cells were employed in the present

Table 2. Chromosomal aberration assay for ADP in Chinese hamster lung (CHL) cells in the absence or presence of S9 metabolic activation

Treatment				Chromosome aberrations/200 cells									
			S9 mix	Chromatid Type		Chromosome Type		Total aberration (%)	Extra aberration				
Compound	Con. (μg/ml)	h		Br	Ex	Br	Ex		ctg	csg	poly	endo	nor
D.W.	-	6	-	1	0	0	0	0.5	0	0	1	0	198
ADP	2425	6	-	3	1	0	0	2.0	2	0	0	0	194
	1212.5	6	-	3	2	0	0	2.5	1	0	0	0	197
	606.25	6	-	1	0	0	0	0.5	1	1	0	0	198
MMC	1	6	-	31	24	3	1	29.5	2	1	0	0	162*
D.W.	-	6	+	1	2	0	0	1.5	0	0	0	0	197
ADP	8126	6	+	5	2	0	0	3.5	0	0	0	0	193
	4063	6	+	2	1	0	0	1.5	1	0	0	0	196
	2031.5	6	+	5	0	0	0	2.5	0	0	0	0	195
CP	10	6	+	31	25	11	3	35	8	3	0	0	153*
D.W.	-	24	-	2	0	0	0	1.0	0	0	0	0	198
ADP	2425	24	-	2	1	0	0	1.5	2	0	0	0	195
	1212.5	24	-	3	1	0	0	2.0	3	1	0	0	193
	606.25	24	-	2	1	0	0	1.5	2	0	0	0	195
MMC	1	24	-	42	33	6	2	41.5	3	1	0	0	139*

Con. : concentration, Br : breakage, Ex : exchange, ctg : chromatid gap, csg : chromosome gap, poly : polyploid, endo : endoreduplicate, nor : normal, MMC : mitomycin C, CP : cyclophosphamide.
*; $P<0.05$ comparison with negative control (D.W.) determined by Fisher's exact test.

study, and the treatment times used were 6 or 24 hours in the absence and 6 hours in the presence of S9 metabolic activation. Clastogenicities were recorded for the specific classes of aberrations and aberration frequencies, and shown in Table 2. The negative/vehicle controls in the absence or in the presence of the S9 mixture revealed only 0.5~1.5% spontaneous chromosomal aberrations in 200 metaphase cells. In the case of the positive controls, cyclophosphamide (10 μg/ml) an indirectly-acting mutagen that requires metabolic activation and mitomycin C (1 μg/ml) a directly-acting mutagen, induced remarkable chromosomal aberrations in about 29.5~41.5% of CHL cells. ADP treatments resulted in mean percentages of aberrant cells ranging from 0.5 to 3.5% in the absence or in the presence of S9 metabolic activation and were not significantly different from those of the negative/vehicle controls ($P>0.05$).

The results of all above studies indicated that ADP in the absence or in the presence of S9 metabolic activation did not induce mutagenicity in the *Sallmonella* bacterial system or clastogenicity in a mammalian cell system. We suggest that ADP representing a water extract of 1 : 1 mixture of *Anemarrhena asphodeloides* and *Phellodendron amurense* provides a basis for further development as a potent anti-BPH material due to its inability to cause mutation or chromosomal aberration.

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