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Genotoxicity *of Zizyphi Spinosi Semen* in Bacterial Reverse Mutation (Ames) Test, Chromosomal Aberration and Micronucleus Test in Mice

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ABSTRACT - *Zizyphi spinosi semen* (*Z. spinosi*) has been used in traditional Chinese medicine for the treatment of rheumatoid arthritis and wounds. However, toxicity in high doses was often observed due to the presence of alkaloids. This study was conducted to investigate the potential genotoxicity of *Z. spinosi* in vitro and in vivo. This was examined by the Bacterial reverse mutation (Ames) test using *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA, Chromosomal aberration was investigated using Chinese hamster lung cells and the micronucleus test using mice. *Z. Spinosi* did not induce mutagenicity in the Ames test, and it did not produce chromosomal aberration in Chinese hamster lung cells with and without metabolic activation, nor in the micronucleated polychromatic erythrocytes in the bone marrow cells in mice. Based on these results, it is concluded that *Z. spinosi* does not have mutagenic potential under the conditions examined in each study.

Key words: reverse mutation, chromosome aberration, micronucleus, mice, *Zizyphi spinosi semen*

Introduction

Traditional Chinese medicines have been used in the treatment of disease by the Chinese and other Asians for thousands of years. Although there is scientific evidence for numerous benefits arising from Chinese herbal medicines, their general use should be viewed with caution. Adverse effects can develop due to intrinsic toxicity, adulteration, substitution, contamination, and interaction with other drugs[1,2]. *Zizyphi spinosi semen* (*Z. spinosi*), called "SANJOINE domestically, is the semen of the fruit of *Zizyphi jujube Miller*. The fruit has been used as a traditional medicine in Chinese and Korea, where they are believed to alleviate stress, and are believed to have antifungal, antibacterial, antiulcer, anti-inflammatory, sedative, antispastic and antioxidant actions[3-8]. *Z. spinosi* is used for the treatment of insomnia, anxiety, and childhood convulsions in oriental countries. Many chemical components have been found in *Z. spinosi* over the years, and these include alkaloids, triterpenes and flavones[9-14]. The active constituent of *Z. spinosi* has protective effects on the cardiovascular system in hypertensive rats, as well as on the nerve system, especially against seizure[15-16]. Little information is available in the scientific literature on this compound considering the importance of scientific evaluation of commonly used herbal medicines, so the aim of this study was to determine the genotoxicity/mutagenicity of semen water extracts from *Z. spinosi* by using the Ames test, the chromosomal aberration test, and the micronucleus test.

The Ames test is a biological assay that is used to assess the mutagenic potential of medical compounds. The test is used worldwide as an initial screen to determine the mutagenic potential of new medicines because there is a high predictive value of rodent carcinogenicity when a mutagenic response is obtained[17-18].

The short-term in vitro mammalian cell chromosome aberration test is used to assess the potential genotoxic hazard of test substances, and guidance is provided by regulatory authorities. Mammalian cells are cultured in vitro, exposed to a test substance, harvested, then microscope slides of the cells are prepared and the frequency of asymmetrical structure chromosome aberration is measured[19].

Micronuclei were first used to quantify chromosomal damage by Heddle JA et al and is now recognized as one of the most successful and reliable assays for genotoxic carcinogens[20,21].

In this study, we investigated the potential genotoxicity of *Z. spinosi* using the Ames test, the chromosomal aberration test (CHL cell) and the micronucleus test in bone marrow.

Materials and Methods

Chemicals

The chemicals and solvents used throughout the experi-

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ments were of analytical grade. 2-nitrofluorene(2-NF), 2-aminoanthracene(2-AA) were purchased from Aldrich (MO, USA) and sodium azide (SA), 9-aminoacridine(9-AA), 4-nitroquinoline-1-oxide(4-NQO) Mitomycin C (MMC), Benzo-[a]pyrene (B[a]P) were purchased from Sigma Chemical Co. (MO, USA). Dimethylsulfoxide (DMSO) was purchased from Merck (Whitehouse station, NJ, USA).

Plant material

Z. spinosi, in this experiment, was purchased from a Korean traditional herbal medicine market and was identified by Prof. Yook of the Korean Medicine College of Gyeong-He University. The compound was extracted from the dried root bark of *Z. spinosi* using the standard hot water extraction method of Korean pharmacopeia and then freeze-dried.

Bacterial strains and CHL cells and S9

Salmonella typhimurium strain TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA were purchased from Molecular Toxicology Inc. (Boone, NC, USA).

Chinese hamster lung (CHL) cells were purchased from American type culture collection (Manasass, VA, USA)

A rat liver S9 fraction induced by aroclor in male Sprague-Dawley rats was purchased from Molecular Toxicology Inc. (Boone, NC, USA).

Bacterial reverse mutation (Ames) test

The test article was preincubated with the test strain and sterile buffer or the metabolic activation system for 20 min or more at 30~37°C prior to mixing with the overly agar and pouring onto the surface of a minimal agar plate. 0.05 or 0.1 ml of test article, 0.1 ml of bacteria, and 0.5 ml of S9-mix or sterile buffer were mixed with 2.0 ml of overlay agar. Tubes were aerated during pre-incubation by using a shaker. For an adequate estimate of variation, triplicate plating was used at each dose level. All plates in a given assay were incubated at 37 for 48 h. After the incubation period, the number of reverting colonies per plate was counted.

Chromosomal aberration test

The chromosome aberration test was performed under the following conditions: short time treatment (6h) with and without S9, and continuous treatment for 24 hours to examine the mutagenic potential of the test substance, *Z. spinosa,* using Chinese hamster lung (CHL) cells.

According to the MTT assay to determine the highest dose, there was no cytotoxicity regardless of the application of the S9 metabolic activation system. Therefore, to perform the test for the short time treatment (6h) with and without the S9 metabolic activation system, 1,250, 2,500 and 5,000 μg/mL concentrations were chosen for the tests, including the tests on the negative (ultra pure water) and positive control groups (MMC : 0.05 μg/mL, B[a]P : 20 μg/mL).

One hundred thousand cells (50,000 cells/ml × 5 ml) were seeded in a 60-mm plastic culture dish, incubated in a culture medium, and then replaced with test article suspended in culture medium. The cells were exposed to the test article for 24 h (or 6h and 24 h incubation with change of medium). For chromosome preparation, colcemid at a final concentration of 0.25 g/ml was added to the culture medium 2h before cell harvesting. Chromosomes were prepared by the air-drying method and stained with 2% Giemsa. The frequency of the cells with various types of structural aberrations including chromatid break, chromatid exchange, chromosome break, chromosome exchange and others (fragmentations except pulverization), for each dose in a 200 well-spread metaphase (100 metaphase/culture), as well as the cells with numerical aberration (polyploidy) were recorded.

Micronucleus test

The micronucleus test was performed on male ICR mice, using 5 mice per group and supplied by the Samtaco Co. Ltd. Gyeonggi, Korea. The animals were kept in a room with 12 h photo period at a temperature of 20~25°C and provided with animal feed and tap water. The animals were sacrificed at 24 h, after the test article had been administrated via gavage at a dose of 500, 1,000 and 2,000 mg/kg or MMC intraperitoneally at a dose of 2 mg/kg. The treatment concentration of the test article and collection time of bone marrow cell were determined on the basis of a preliminary dose-range finding test. The air dried slides with mice bone marrow micronuclei were stained with 5% Giemsa. A total of 2,000 polychromatic erythrocytes (PCEs) were scored per animal for determining the micronucleated polychromatic erythrocytes (PCEs/NCEs).

Results

Bacterial reverse mutation (Ames) test

Prior to the Ames test, the *Z. spinosi* extract was evaluated for inhibition of growth of the indicator strains used for the tests. The results showed that *Z. spinosi* did not inhibit growth with the dose used (data not shown). The results of the Ames test using the pre-incubation method shown in Table 1 revealed that the *Z. spinosi* extract was non-mutagenic towards *Salmonella typhimurium* and *Eshcherichia coli*.

Chromosomal aberration test

Z. spinosi did not induce numerical and structural chromosome aberration regardless of the application of the S9 metabolic activation system when compared with the negative control group (Table 2).

Table 1. Mutagenicity assays for *Z. spinosi* extract based on non-metabolic activation using *Salmonella typhimurium* and *Escherichia coli* strain

With/without S9 mix		Conc. of test material (μg/plate)	Average of revertant colonies (Mean SD)				
			Base-pair substitution type			Frameshift type	
			TA100	TA1535	WP2uvrA	TA98	TA1537
S9 mix(-)		0	158 ± 4	12 ± 2	117 ± 4	38 ± 3	6 ± 1
		312.5	171 ± 8	11 ± 1	112 ± 1	33 ± 2	7 ± 1
		625	158 ± 11	11 ± 1	120 ± 5	41 ± 4	7 ± 1
		1,250	159 ± 17	10 ± 2	120 ± 6	34 ± 3	8 ± 2
		2,500	159 ± 19	11 ± 1	115 ± 4	38 ± 1	8 ± 1
		5,000	164 ± 6	11 ± 1	116 ± 4	38 ± 2	7 ± 0
S9 mix(+)		0	153 ± 9	15 ± 2	119 ± 5	37 ± 3	8 ± 2
		312.5	159 ± 13	14 ± 2	117 ± 3	42 ± 7	8 ± 1
		625	153 ± 9	14 ± 1	114 ± 13	41 ± 1	9 ± 2
		1,250	157 ± 7	15 ± 1	118 ± 7	38 ± 4	8 ± 2
		2,500	167 ± 12	15 ± 1	119 ± 5	35 ± 3	10 ± 1
		5,000	161 ± 12	15 ± 2	115 ± 4	37 ± 2	10 ± 2
+control	Without S9 mix	Compound	SA	SA	4NQO	2-NF	9-AA
		Conc. (μg/plate)	1.5	1.5	5.0	5.0	80
		Colony No.	431 ± 22	420 ± 13	747 ± 17	533 ± 40	765 ± 59
	With S9 mix	Compound	2-AA	2-AA	2-AA	2-AA	2-AA
		Conc. (μg/plate)	1.0	2.0	2.0	1.0	2.0
		Colony No.	573 ± 54	180 ± 13	337 ± 50	588 ± 8	184 ± 1

Expressed as mean ± SD
SA (sodium azide), 4-NQO (4-nitroquinoline-1-oxide), 2-NF (2-nitrofluorene), 9-AA (9-aminoacridine), 2-AA (2-aminoanthracene)

Table 2. Structural and numerical chromosome aberration mutagenicity of *Z. spinosi* extract based on metabolic and non-metabolic activation (S9)

Time(h)	With/without S9 mix	Treatment	Conc. (μg/ml)	Percent(%) of cells showing structural chromosome aberrations							
				Cell No.	ctb	csb	cte	cse	other	total	Poly ploidy
6h	S9 mix (-)	*Z. spinosi*	0	200	0	1	0	0	0.5	1.5	1.5
			1,250	200	0.5	0	0	0.5	1.5	2.5	1
			2,500	200	0.5	0.5	0.5	0.5	1	3	0.5
			5,000	200	1	0.5	2	0	0	3.5	2
		MMC	0.05	200	2	2	4	5	8.5	22.5	13.5
	S9 mix (+)	*Z. spinosi*	0	200	0.5	0	0.5	0	0	1	0.5
			1,250	200	0.5	0	0.5	0	0	1	1.0
			2,500	200	0.5	0	0	0	0	0.5	2.0
			5,000	200	0.5	0.5	0	0.5	0.5	2	2.0
		B[a]P	20	200	3.5	1.5	2.5	4.5	1.5	10	13.0
24h	S9 mix (-)	*Z. spinosi*	0	200	0.5	0	0.5	0.5	0	1.5	1.5
			1,250	200	1	1	0	0	0	2	2.0
			2,500	200	1	0	0	0.5	0	1.5	1.5
			5,000	200	0.5	0	0.5	1	0.5	2.5	2.5
		MMC	0.05	200	1.5	4	3	4	1	21	13.5

MMC (Mitomycin C), B[a]P (Benzo[a]pyrene)

Micronucleus test

There were no significant differences in the rate of appearance of micronucleated polychromatic erythrocytes among the polychromatic erythrocytes between the treatment and control groups (Table 3). In addition, the PCE/(PCE+NCE) ratio did not show cytotoxicity.

Table 3. Micronucleus assay of *Z. spinosi* extract on mice bone marrow cells

Sample	Dose (mg/kg)	Incubation Time (h)	No. of MNPCEs/1000 PCEs (mean ± SD)	PCEs/NCEs (Mean ± SD)
Z. spinosi	0	24	1.3 ± 0.3	0.500 ± 0.022
	500	24	1.2 ± 0.3	0.487 ± 0.014
	1,000	24	1.0 ± 0.4	0.501 ± 0.010
	2,000	24	1.3 ± 0.8	0.498 ± 0.018
Mitomycin C	2	24	76.16.1	0.491 ± 0.015

Expressed as mean ± SD, n = 5, 2000 PCEs scored per animal, mean ± SD, n = 5 1,000 ethyrocytes (PCEs and NCEs) scored per animal.

Discussion

Interest in Chinese herbal medicines is generally due to its traditional use in Asian countries and the natural origin of the medicines. Although many beneficial biological activities have been scientifically confirmed, caution is called for in the public use of these herbal medicines. Although most herbal products are considered safe if used at the recommended doses, unfavorable effects can still occur[22]. Therefore, genotoxicity tests are important in evaluating the safety and efficacy of natural products[23]. Mutagenicity testing using the Ames test, the chromosomal aberration test and the micronucleus test (the three battery test) is the most frequently used method and is recommended by regulatory agencies for determining genetic risk (KFDA, FDA, OECD). Also they have a high predictive value for carcinogenicity in rodents when a mutagenic response is obtained[24]. In the present study, treatment of the test article did not induce mutagencity in *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and in *Escherichia coli* WP2uvrA, with and without metabolic activation. All Salmonella strains are histidine-dependent, and the *Escherichia coli* strain used in the tests is typtophan-dependent. Revertants are identified as colonies that grow in low levels of histidine or tryptophan. Frameshift and base-pair substitution defects are represented to identify both types. In this study, *Z. spinosi* showed a cytotoxic effect and the doses that were decided on were over 50% greater than live cell concentration. This is similar to the results of a previous report which showed that the crude methanolic extract of Zizyphus jujube was highly cytotoxic, and had antifungal and insecticidal activities[25]. *Z. spinosi* did not induce chromosomal aberration in the short term and the continuous treatment of Chinese hamster lung cells. The chromosomal aberration test is used to identify agents that cause structural chromosomal aberrations in cultured mammalian cells. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. Chromosome mutations are the cause of many human genetic diseases, and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour suppressor genes of somatic cells are involved in cancer induction in humans. Zizyphus jujube extracts protect against hydroquinone-induced clastogenicity[26]. They inhibit the chromosomal aberration of bone marrow cells in hydroquinone treated mice. In this study, *Z. spinosi* also did not show any induction of the micronucleus in mice bone marrow cells. Formation of micronuclei is an indication of induced chromosome damage. Some toxic metabolites cannot reach the bone marrow cell if their lives are short. Therefore the micronucleus test is a very useful tool for risk assessment of the short-life toxic metabolites, which can affect blood lymphocytes. In this study, we extracted the test article from *Z. spinosi* using hot water because it is the same method used in clinical treatment.That means the clinical treatment of *Z. spinosi* may have no mutagenicity to humans.

In conclusion, through the use of the three-core test system for genetic damage, we found that the root bark extracts from *Z. spinosi* lack mutagenicity. Future studies are still required for a better understanding of the chronic toxicity of the extracts of *Z. spinosi*.

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