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Evaluation of the Genotoxicity of a *Gelidium elegans* Extract in Vitro and in Vivo

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Abstract *Gelidium elegans* is an edible red seaweed that exhibits a broad range of biological activities. However, there is little known about its genotoxic effects. The aim of this study was to evaluate the genotoxicity of a *Gelidium elegans* extract in three independent genotoxic tests, including a bacterial reverse mutation test, a micronucleus test, and a chromosomal aberration test. For five different strains of bacteria, the bacterial reverse mutation showed no increased reverse mutation upon exposure to 5000 μg/plate of the *Gelidium elegans* extract. Moreover, the frequency of micronucleated bone marrow polychromatic erythrocytes (MNPCE) was not altered by the *Gelidium elegans* extract. Likewise, the chromosome aberration did not increase in response to the *Gelidium elegans* extract. Together, these genotoxicity assessment studies suggest that *Gelidium elegans* extract could be considered a safe dietary ingredient.

Keywords: *genotoxicity, safety, Gelidium elegans, red seaweed, food ingredient*

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1. Introduction

Seaweed has become an important functional food resource in the food industry [1,2]. Seaweed contains a large amount of carbohydrates, vitamins, and polyphenols [3,4]. Recently, a number of studies have demonstrated the beneficial effects of red seaweed on the gut microbiome, coagulation, hypertension, and obesity due to its composition of bioactive compounds [5,6,7,8].

Gelidium elegans is one of the edible red seaweeds. It is becoming increasingly clear that *Gelidium elegans* conveys a number of health benefits to humans through the exhibition of a broad range of biological activities [9,10]. Despite the fact that *Gelidium elegans* shows tremendous promise as a biomedical natural resource, detailed studies have not been performed on the genotoxicity of *Gelidium elegans*.

In the present study, we elucidated the genotoxic effect of the *Gelidium elegans* extract using three independent assays, including a bacterial reverse mutation test, a bone marrow micro nucleus assay in ICR mice, and a chromosomal aberration assay in the cultured Chinese hamster lung cell line (CHL/IU), and these tests were in compliance with the test guidelines from the Korea Food and Drug Administration (KFDA) under Good Laboratory Practice regulations for Nonclinical Laboratory Studies and were consistent with OECD guidelines [11].

2. Materials and Methods

2.1. Test Substance

Gelidium elegans extract (GE) was provided by Newtree Co., Ltd (Seongnam, South Korea). The composition analysis showed that GE is made up of 47.6% carbohydrates, 16.9 % protein, 5.1 % moisture, 24.1 % crude ash, and 8.79 mg in 1 g of *Gelidium elegans* extract (Table 1).

Table 1. The composition of *Gelidium elegans* extract

Component	Gelidium elegans extracts
Carbohydrate	47.6 %
Crude protein	16.9 %
Moisture	5.1 %
Crude ash	24.1 %
Total polyphenols	8.79 mg per 1 g

2.2. Bacterial Reverse Mutation Assay

The bacterial reverse mutation assay was conducted by the pre-incubation method in the presence and absence of S9 metabolic activation [12,13]. Tester strains included S. *typhimurium* TA98, TA100, TA1535, and TA1537 and *E. coli* WP2uvrA, with and without S9 and corresponding positive control agents (i.e., NaN3, 2-NF, ICR-191, 4NQO, 2-AA, and B[a]P). All test substances and positive controls were dissolved in DMSO as a vehicle. A preliminary range-finding study was conducted for all tester strains at *Gelidium elegans* extract concentrations of 5, 25, 50, 150, 500, 1000, and 5000 μg/plate, which resulted in the definitive study concentrations of 50, 150, Evaluations were made at 500, 1000, and 5000 μg/plate. For each treatment, 0.1 mL of the *Gelidium elegans* extract or a control preparation was introduced into a sterilized test tube to which 0.1 mL of bacterial suspension was added. For preparations with S9, 0.5 mL of the S9 mix was also added, and for preparations without S9, 0.5 mL of a 0.1 M sodium phosphate buffer solution was

added. The mixtures were then incubated with gentle shaking for 20 min at 37 °C. Following this pre-incubation step, 2.0 mL of a molten top-agar solution was added to the entire test tube contents and was poured into a minimal glucose agar plate.

After agar solidification, the plates were incubated for 48 h at 37°C. Following incubation, the plates were examined for precipitation with the unaided eye and for microbial toxicity with a stereoscope. The numbers of revertant colonies were counted with either an automatic colony analyzer or via manual counting. Test substances were judged to be positive for mutagenicity when (1) they induced a dose-dependent increase in the number of revertant colonies to a level > two-fold of the negative control value and (2) when the dose-dependent increase was reproducible.

2.3. Animal Husbandry and Maintenance

A total of 30 specific pathogen-free, 8-week-old male ICR mice, weighing between 29.98 and 34.08 g were purchased from Koatech (Pyeongtaek, Gyeonggi, South Korea) and were used for the micronucleus test after a week of quarantine and acclimatization. They were housed in a light-controlled room (light 07:00-19:00) that was maintained at 22 ± 3 ºC with a humidity of 55 ± 15 % and a light intensity of 150 to 300 lux (Chemon, Suwon, Gyeonggi, South Korea). The animals were allowed sterilized tap water and commercial rodent diet 2918C (Harlan, Madison, WI, USA) ad libitum. The genotoxicity tests were performed in compliance with Good Laboratory Practice Regulations and in accordance with standard guidelines for genotoxicity tests, including the OECD guidelines. The experimental protocol on mice was approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University accredited by AAALAC International (IACUC #: 150075).

2.4. Mouse Micronucleus Assay

The final concentration and volume of the *Gelidium elegans* extract was selected through the pre-acute toxicity test. The mice were administered 30 mL/kg/day volume of the *Gelidium elegans* extract at doses of 0, 1250, 2500, and 5000 mg/kg/day or a positive control of cyclophosphamide (CPA) at 70 mg/kg/day. The mice were administered with the proper concentration of the *Gelidium elegans* extract twice per day for 2 days. The positive control group received a single intraperitoneal dose of CPA on the last day. All of the animals were sacrificed one day after the final dose, and the bone marrow was harvested, smears were made, the slides were air-dried, fixed with methanol and stained with acridine orange solution (40 μg/mL) prior to a fluorescent microscopic examination (Nikon Eclipse Ni-U, Tokyo, Japan). From each animal, 4000 polychromatic and/or normochromatic erythrocytes (PCE and NCE, respectively) were examined and the PCE/NCE ratio was determined. An additional 500 PCE were examined per animal with the number of micronucleated polychromatic erythrocytes (MNPCE) recorded.

2.5. Chromosome Aberration Assay

This assay was conducted using the CHL/IU cell line, both in the presence and absence of S9 metabolic activation, which was prepared as described above [14]. All of the test substances and positive controls were dissolved in DMSO as a vehicle. The *Gelidium elegans* extract was used at concentrations of 37.5, 75, and 150 μg/mL for the chromosome aberration test with the presence of S9 metabolic activation, while 25, 50, and 100 μg/mL of *Gelidium elegans* extract was used for the chromosome aberration test in the absence of S9 metabolic activation. The definitive chromosome aberration test was assessed via two different procedures as follows: a 6 h exposure followed by an 18 h recovery treated in the absence and the presence of S9 metabolic activation and a 24 h continuous exposure.

2.6. Statistical Analysis

The results are presented as the means ± standard deviation. Statistical comparisons were performed using the Mann-Whitney test U test using SPSS (Chicago, IL) version 10. $p<0.05$ was considered significant.

3. Results

3.1. Bacterial Reverse Mutation Assay

Table 2. Results of the bacterial reverse mutation assay with the *Gelidium elegans* extract

Gelidium elegans extract (μg/plate)	Average number of revertants (number of colonies/plate)									
	Base pair substitution						Frame shift			
	TA100		TA1535		WP2uvrA/pKM101		TA98		TA1537	
	S9 mix		S9 mix		S9 mix		S9 mix		S9 mix	
	-	+	-	+	-	+	-	+	-	+
Negative controls	149 ± 7	192 ± 14	9 ± 2	10 ± 2	18 ± 2	23 ± 3	24 ± 4	28 ± 5	10 ± 1	10 ± 1
50	165 ± 13	181 ± 11	10 ± 1	12 ± 3	18 ± 1	21 ± 4	27 ± 6	33 ± 5	9 ± 2	10 ± 1
150	188 ± 15	186 ± 10	12 ± 2	10 ± 2	21 ± 4	28 ± 2	23 ± 2	32 ± 4	10 ± 2	10 ± 3
500	195 ± 4	199 ± 11	12 ± 1	8 ± 1	19 ± 2	27 ± 5	20 ± 2	37 ± 5	8 ± 2	9 ± 2
1500	166 ± 6	193 ± 8	10 ± 3	7 ± 1	22 ± 2	21 ± 2	20 ± 3	36 ± 2	7 ± 2	7 ± 1
5000	193 ± 9	178 ± 4	8 ± 0	8 ± 2	23 ± 3	27 ± 1	20 ± 2	30 ± 5	7 ± 1	8 ± 1
Positive controls										
NaN3	619 ± 46		339 ± 5							
2-NF							236 ± 32			
ICR-191									59 ± 9	
4NQO					143 ± 13					
2-AA		1704 ± 100		68 ± 6		119 ± 2				60 ± 3
B[a]P								114 ± 16		

Abbreviations: NaN3: Sodium azide, 2-NF: 2-Nitrofluorene, ICR-191: acridine mutagen ICR 191, 4NQO: 4-nitroquinoline N-oxide, B[a]P: benzo[a]pyrene, 2-AA: 2-Aminoanthracene.

No dose-dependent increases in revertant colonies or bacterial toxicity were observed with the *Gelidium elegans* extract up to 5000 µg/plate, regardless of metabolic activation (Table 2). Moreover, the *Gelidium elegans* extract did not induce more than a two-fold increase in the mean number of revertant colonies per plate compared to the negative control.

The positive controls, for each strain, resulted in the expected increase in the number of revertant colonies. Our results indicate no evidence of gene mutagenic potential under the conditions used in this test for the *Gelidium elegans* extract.

3.2. Effect of *Gelidium elegans* Extracts on Bone Marrow Micronucleus Tests in ICR Mice

Based on the MNPCE and PCE/NCE ratio frequencies, the results of the genotoxicity in the bone marrow of the ICR mice treated with the *Gelidium elegans* extract are presented in Table 3. The negative control group (DMSO) exhibited a 6.67 MCPCE frequency, whereas the positive control, with CPA at 70 mg/kg/day, significantly increased approximately 26-fold in MNPCE frequencies compared to the negative control group ($p<0.01$). In the genotoxicity test, the groups that were administered 1250, 2500, and 5000 mg/kg/day of the *Gelidium elegans* extract exhibited approximately 7.5, 6.67, and 7.4 MNPCE frequencies, respectively.

At all of the tested doses, the *Gelidium elegans* extract did not cause an increase in the frequency of MNPCE compared to the negative control. Our results reveal that the *Gelidium elegans* extract has no genotoxic effect in vivo.

The ratio of PCE/NCE can be used to evaluate the cytotoxic effect of bioactive compounds [15,16]. We, therefore, calculated the ratio of PCE/NCE to evaluate the cytotoxicity of the *Gelidium elegans* extract. In the groups administered 1250, 2500, and 5000 mg/kg/day of the *Gelidium elegans* extract, the PCE/NCE ratios were 0.55, 0.54, and 0.55, respectively. The negative control group (DMSO) showed a 0.55 PCE/NCE ratio, whereas the positive control, treated with CPA 70 mg/kg/day, had a 0.4 PCE/NCE ratio.

Table 3. Bone marrow micronucleus tests and mortality in male ICR mice

Gelidium elegans extract	Dose (mg/kg/day)	Body weights		MNPCE/4000PCE	PCE/NCE Ratio	Mortality (dead / total)
		Administration	Sacrifice			
	0	35.55 ± 1.14	35.01 ± 1.37	6.67 ± 1.86	0.55 ± 0.02	0 % (0 /6)
	1250	35.05 ± 1.00	34.67 ± 1.30	7.50 ± 2.07	0.55 ± 0.02	0 % (0 /6)
	2500	35.13 ± 1.47	35.08 ± 1.83	6.67 ± 1.51	0.54 ± 0.01	0 % (0 /6)
	5000	35.67 ± 1.22	35.13 ± 1.04	7.40 ± 2.61	0.55 ± 0.01	16.6 % (1 /6)
CPA	70	35.85 ± 1.08	35.40 ± 0.63	175.00 ± 29.18**	0.40 ± 0.01**	0 % (0 /6)

Values presented are the mean ± standard deviation. ** Significantly different from the negative control group at $P<0.01$.
Abbreviations: MNPCE: PCE with one or more micronuclei, PCE: Polychromatic erythrocyte, CPA: Cyclophosphamide monohydrate.

These results indicate that CPA caused a significant decrease in the PCE/NCE ratio compared to the negative control group ($p<0.01$). However, for all of the groups administered the *Gelidium elegans* extract, there was not a statistically significant decrease in the PCE/NCE ratio compared to the negative control. These results suggest that the *Gelidium elegans* extract has no cytotoxic effect at the 5000 mg/kg/day dose.

3.3. Chromosome Aberration Assay

The in vitro chromosomal aberration test using CHL/IU cells was performed to evaluate the effect of the *Gelidium elegans* extract on chromosomal aberration in vitro.

Table 4. Chromosome aberration test with the *Gelidium elegans* extract in CHL/IU cells

Gelidium elegans extract (µg/mL)	Time (h)	S9 mix	Percentages of cells showing aberrations						Numerical aberrations (%)	Structural aberrations (%)
			Observed cells	Chromosome type		Chromatid type		Others		
				csb	ces	ctb	cte			
Negative control	6 -18	+	150	0	0	0.5	0	0	0	0.33
25		+	150	0	0	0.5	1	0	0	1.00
50		+	150	0	0	0.5	0.5	0	0	0.67
100		+	150	0	0	1	0.5	0	0	1.33
Positive control B[a]P (20 µg/mL)		+	150	0	0.5	8.5	25.5	0.5	0	16.67**
Negative control	6 -18	-	150	0	0	0	0.5	0	0	0.33
25		-	150	0	0	1.5	0.5	0	0	1.33
50		-	150	0	0	1	1	0	0	1.33
100		-	150	0	0	1.5	0	0	0	1
Positive control 4NQO (0.4 µg/mL)		-	150	0	1	6	11	5	0	9.67**
Negative control	24 - 0	-	150	0	0	0	0	0	0	0
25		-	150	0	0	0	0	0	0	0
50		-	150	0	0	1.5	0	0	0	1
100		-	150	0	0	0	0	0	0	0
Positive control 4NQO (0.4 µg/mL)		-	150	0	0	3.5	14	6	0	11.33**

a: Treatment + recovery time ** *Significantly different from the negative control group at P*<0.01., ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange, B[a]P: benzo[a]pyrene, 4NQO: 4-nitroquinoline N-oxide.

As shown in Table 4, the *Gelidium elegans* extract was tested at concentrations of 25, 50, and 100 μg/mL in the CHL/IU cells, and an incidence of numerical or structural chromosomal aberrations of the *Gelidium elegans* extract was not observed after a short term treatment of 6 h followed by an 18-h recovery time in the presence or absence of the S9 metabolic activation.

Furthermore, the *Gelidium elegans* extract did not cause a significant increase in the incidence of numerical or structural chromosomal aberrations after continuous treatment for 24 h at concentrations of 25, 50, and 100 μg/mL in the absence of the S9 metabolic activation. As expected, both the positive controls, containing either B[a]P for treatment with S9 metabolic activation or 4NQO for treatment with and without S9 metabolic activation, resulted in an increase in the number of chromosome aberrations in the CHL/IU cells. These data indicate that the *Gelidium elegans* extract exhibited no genotoxic activity and, thus, supports the safety of the *Gelidium elegans* extract at the tested dose.

4. Discussion

The aim of this study was to determine the genotoxicity of a *Gelidium elegans* extract using three different genotoxicity tests in vitro and in vivo. This is the first report, to our knowledge, to provide the genotoxic potential of a *Gelidium elegans* extract using in vitro and in vivo assays. The bacterial reverse mutation assay has been used to determine the mutagenic activity of many compounds in prokaryotic cells with a presence or absence of metabolic activation by mammalian enzymes [17]. In this study, the bacterial reverse mutagenic activity of the *Gelidium elegans* extract was assessed in the TA98, TA100, TA1535, and TA1537 *S. typhimurium* tester strains and *E. coli* WP2 uvrA/pKM101 in the presence or absence of S9 metabolic activation. Based on a repression in the number of revertant colonies, the mutagenic activity was evaluated. The *Gelidium elegans* extract did not exhibit mutagenic activity in the presence or absence of S9 metabolic activation. The *Gelidium elegans* extract, up to 5000 μg/plate, was insufficient at exhibiting mutagenicity against the bacteria tester strains TA100, TA1535, TA98, and TA1537 compared to the negative control.

The micronucleus assay has been used extensively to test the cytoxicity of compounds [16,18]. In the micronucleus assay, there were no increases in the frequency of MNPCE from the *Gelidium elegans* extract at the doses of 1250, 2500, and 5000 mg/kg/day compared to the control group. While CPA showed cytotoxicity due to the decrease in the PCE/NCE ratio in the bone marrow micronucleus test in mice. These data agree with the results that the *Gelidium elegans* extract did not induce cytotoxicity in cultured 3T3-L1 cells [8]. Although the morality rate was observed as one out of six (16.6%) from the *Gelidium elegans* extract at a concentration of 5000 mg/kg/day compared to the negative control group, this did not correlate with the frequency of MNPCE.

The chromosome aberration test has also been used to determine the potential of a chemical to induce numerical and structural chromosomal aberrations in CHL/IU cells [14]. In the current study, the results of the chromosome aberration assay revealed that there were no significant increases in numerical and structural chromosomal aberrations in the absence or presence of the S9 mix for either the short (6 h treatment followed by an 18 h recovery) or continuous (24 h treatment) exposure periods at doses of 25, 50, and 100 μg/mL *Gelidium elegans* extract compared to the negative control group.

In conclusion, the results of three independent genotoxicity tests confirmed that the *Gelidium elegans* extract was devoid of genotoxic effects under our experimental conditions. Therefore, we suggest that the *Gelidium elegans* extract could be considered a safe dietary ingredient.

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Conflict of Interest

The authors declare no conflicts of interest.

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