In Vitro Genotoxicity Assessment from the Glycyrrhiza New Variety Extract

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Abstract: The various species that comprise the genus Glycyrrhiza (Licorice) have long been used as oriental herbal medicines in Asian countries. Wongam (WG), which is a new variety of Glycyrrhiza, was developed in Korea to overcome the limitations of low productivity, environmental restrictions, and an insufficient presence of glycyrrhizic acid and liquiritigenin. In this study, we evaluated WG extract’s genotoxicity through an in vitro bacterial reverse mutation (AMES) test, an in vitro chromosome aberration test, and an in vivo mouse bone marrow micronucleus test. In the AMES test, WG extract at concentrations of up to 5000 µg/mL showed no genotoxicity regardless of S9 mix. No chromosome aberrations appeared after 6 h in 1400 µg/mL WG extract at concentrations of up to 5000 µg/mL WG extract regardless of S9 mix or in 1100 µg/mL WG extract after 24 h without S9 mix. Nor was there a significant increase in the number of micronucleated polychromatic erythrocytes to total erythrocytes up to 5000 mg/kg/day for 2 days detected in the micronucleus test. These results confirm that WG extract is safe for use as an herbal medicine, as it precipitates no detectable genotoxic effects.

Keywords: Glycyrrhiza; wongam; genotoxicity; bacterial reverse mutation test; chromosome aberration test; micronucleus test

1. Introduction

The plant of the Glycyrrhiza genus (Licorice), which belongs to the family Leguminosae, is being used as a sweetener and traditional herbal medicine with a long history. The plants are widely distributed across arid/semiarid desert steppes and loess of hilly regions across Russia, Iran, Europe, the Middle East, the Americas, and Central Asia [1,2]. Licorice roots and shoots have the ecological values for windbreak and sand fixation as well as the bioactive compounds with numerous pharmacological effects including anti-inflammatory, anti-allergic, antioxidant, anti-ulcer, anti-fungal and neuroprotective properties [3–5]. Licorice is comprised of triterpene saponins (glycyrrhizin, ursasaponin,
and licorice-saponin), flavones (liquiritigenin and liquiritin), chalcone, coumarin and alkaloids [6,7]. Of the 21 species that comprise the genus *Glycyrrhiza*, only three produce glycyrrhizin (*G. uralensis* Fisch., *G. glabra* L., and *G. inflata* Batal.), the primary bioactive compound [8,9]. Each of these species of licorice are listed in the Korean Pharmacopoeia (KP) (12th, MFDS 2020). Ironically, when these *Glycyrrhiza* species are cultivated in Korea, the active component is not present in sufficient quantities to satisfy the standards of the KP, which requires more than 2.5% of glycyrrhizin and 0.7% of liquiritigenin to be considered ‘adequate’. The challenges associated with growing licorice, including low productivity, disease, and early leaf fall, mean that the country has mostly depended on imports [10]. To better meet domestic demand, the Korea Rural Development Administration developed a new hybridized *Glycyrrhiza* variety that combines *G. glabra* × *G. uralensis*, called 'Wongam (WG, *G. korshinskyi*)', which is improved the problems of the previously existing species. While WG’s properties, homogeneity, and pharmacological effects, such as enhancing immune response, being anti-allergenic, anti-neuroinflammatory, and anti-ulcerative colitis have been reported elsewhere, to date no study of its genotoxicity has been undertaken [11–14].

Herbal medicine is generally considered safe in Korea as a consequence of the natural origin of the ingredients that comprise treatments as well as its long-established role in Korean society. However, some toxicological studies have revealed the potential for herbal medicines to induce harmful genetic damage [15–18]. In other cases, a dearth of toxicity information has occasionally resulted in the misapplication of herbal medicine [19,20]. Toxicological assessments are, therefore, extremely important to public health, and to the regulatory decisions that affect drug safety and drug development.

Genotoxicity testing assesses the carcinogenicity and mutagenicity of potential medicines, pesticides, and chemicals to determine druggability [21]. Genotoxicity tests determine whether compounds induce genetic damage directly or indirectly in vitro and in vivo. A single test is insufficient to determine genotoxicity, which may be the result of various mechanisms. Genotoxicity is therefore assessed through a battery of tests. A typical 3-battery test includes an in vitro bacterial reverse mutation (AMES) test, an in vitro chromosome aberration test, and an in vivo mouse bone marrow micronucleus test [22]. In this study we performed a nonclinical evaluation of genotoxicity of WG water extract using a 3-battery test.

2. Materials and Methods

2.1. Chemicals and Reagents

Glycyrrhizic acid, 2-Aminoanthracene (2-AA), Benzo[a]pyrene (B[a]P), Sodium azide (SA), 2-Nitrofluorene (2-NF), 4-Nitroquinoline-1-oxide (4NQO), Acridine Mutagen ICR 191 (ICR-191), Cyclophosphamide monohydrate (CPA), Colchicine, and Acridine orange were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Methanol was purchased from Burdick & Jackson (Muskegon, MI, USA). Acetic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Distilled sterile water was obtained from Young Lin (Gangnam, Seoul, Korea).

2.2. Preparation of Wongam

The roots of WG were purchased from the Korean Rural Development Administration, and extracted by Wonkwang Herb Co. (Jinan-gun, Jeollabuk-do, Korea). Two years' worth of WG’s main roots were harvested in winter (November), followed by washing and drying (55–58 °C for 3 days). Ground WG main roots (100 g) were extracted with distilled water (1 L) at 100 °C for 4 h 30 min. The extract was concentrated by the rotary evaporator at 70 °C for 3 h. The decoction was filtered using Whatman filter paper No.1. Finally, it was lyophilized (Batch method) and stored at 4 °C. The yield of the dried extract from the starting crude WG was 8.8%. The WG extract was diluted with distilled sterile water before experiment.
2.3. Experimental Animals

SPF ICR mice (Hsd:ICR CD-1®, 7 weeks old, 29.31–32.50 g, 6 mice per group) were purchased from Koatech (Pyeongtaek, Gyeonggi-do, Korea), and acclimated for 1 week before experiments. The animals were housed in an environmentally controlled room (temperature, 23 ± 3 °C; relative humidity, 55 ± 15%) under a 12/12-h light/dark cycle. Animals were supplied with irradiation-sterilized chow ad libitum. The animal experiments were performed in accordance with the guidelines by the Korean Ministry of Food and Drug Safety (MFDS, 2018) and the Organization for Economic Cooperation and Development (OECD, 1997) under GLP Regulations. This study was approved by the Preclinical Research Center, Chemon Inc.’s Institutional Animal Care and Use Committee (IACUC) (Approval Number: 19-M516).

2.4. In Vitro AMES Test

WG extract’s mutagenic potential was assessed by bacterial reverse mutation assay consistent with OECD TG471 [23]. Four histidine auxotroph strains of Salmonella typhimurium (TA100, TA1535, TA98 and TA1537) and a tryptophan auxotroph strain of Escherichia coli (WP2 uvrA) were chosen according to the previous studies [24,25]. The strains were purchased from Molecular Toxicology Inc. (Boone, NC, USA). The mutagenic activity of WG water extract was evaluated with or without S9 mix, which is an external metabolic activation system, from the rat livers using direct plate incorporation. Dose ranges were established using the five test strains with or without S9 mix in three plates per dose, according to the results of a range-finding test performed on the WG water extract. The highest dose administered in this study was 5000 µg/plate for all test strains, and five-serial diluted concentrations (0, 50, 150, 500, 1500 and 5000 µg/plate) were tested during the main study. A negative control (distilled sterile water) and positive controls for each strain with or without S9 mix were included. Positive controls with S9 mix were as follows: SA (0.5 µg/plate) for TA100 and TA1535, 2-NF (2 µg/plate) for TA98, 4NQO (0.5 µg/plate) for WP2 uvrA, and ICR-191 (0.5 µg/plate) for TA1537. Positive controls without S9 mix were as follows: 2-AA (1 µg/plate) for TA100 and TA1537, (2 µg/plate) for TA1535 and (6 µg/plate) for WP2 uvrA, and B[a]P (1 µg/plate) for TA98. For the plating assay, 0.5 mL of S9 mix (or sodium-phosphate buffer, pH 7.4 for non-activation plates), 0.1 mL of bacterial culture (approximately 0.5 × 10⁸ cells), and 0.1 mL of WG water extract were mixed with 2.0 mL of overlay agar. The contents were well mixed, then poured into the surface on a plate, which was contained with minimal agar. Plates were inverted after the overlay agar was solidified, then incubated at 37 ± 2 ºC for 50 ± 2 h. The revertant colonies were counted by macrography. Results are presented as the number of revertant colonies, and the increase factor was calculated by dividing the number of colonies in the treated plates with the number of colonies in the negative control plates. Experiments were conducted using triplicate plates for each concentration.

2.5. In Vitro Chromosome Aberration Test

A chromosome aberration test evaluates the potential for a substance to induce structural and/or numerical chromosome aberrations in cultured Chinese Hamster Lung (CHL/IU) cells, which were purchased from the American Type Culture Collection (Manassas, VA, USA). An in vitro chromosome aberration test was conducted according to OECD Guideline 473 [26]. Structural abnormalities and polyploidy as chromosome aberrations were examined in cultured CHL cells with or without S9 mix from the rat liver treated with Aroclor 1254. A preliminary range finding test was conducted to establish WG extract’s solubility and toxicity in CHL/IU cell growth. Relative increase in cell count (RICC) was calculated by using the following formula:

$$\text{RICC} (%) = \frac{\text{Increase in number of cells in treated culture (final} - \text{starting)}}{\text{Increase in number of cells in negative control cultures (final} - \text{starting})} \times 100$$
Maximum concentration was determined based on turbidity, cytotoxicity, pH, and RICC. B[a]P (20 µg/mL) and 4NQO (0.4 µg/mL) were used as positive control with or without S9 mix, respectively. Sterile water was the vehicle and was used for the negative control whether with or without S9 mix. CHL cells were seeded (5 × 10^4 cells/T25 flask) and maintained for 3 days before treatment of the reagents. WG extract (350–1400 µg/mL) or negative/positive controls were treated for 6 h in CHL cells, and consequently 18 h recovery with (6 + 18) or without (6 − 18) S9 mix (final 2%, v/v). WG extract or negative/positive controls were treated for 24 h, and then 0 h recovery without S9 mix (24 − 0). After 22 h, colchicine (final 1 µM) was treated for 2 h. The cells at metaphase were harvested, treated with 75 mM KCl, and finally fixed by methanol:acetic acid (3:1, v/v). Samples were stained with 5% (w/v) Giemsa solution after being air dried. Two samples per flask were prepared. Chromosome aberrations were estimated in 150 metaphases per sample under the microscope at 1000 × magnification. Structural aberration of chromosome was categorized into chromosome breaks (csb) or exchanges (cse), chromatid breaks (ctb) or exchanges (cte) with or without gaps. More than 10 aberrations including gaps (multiple aberration) or chromosome fragmentation were calculated by 1 aberration, which is categorized into ‘others’. Numerical aberration of chromosome was categorized by the number of centromeres into diploid (23–36 centromere), polyploid (more than 36 centromere), and endoreduplication.

2.6. In Vivo Mouse Bone Marrow Micronucleus Test

The in vivo micronucleus test was conducted according to OECD TG474 [27]. Oral administration of WG extract (1250–5000 mg/kg/day) for 2 days didn’t induce any toxicity total 4 days including treatment periods regardless of gender in the preliminary test. Therefore, 5000 mg/kg/day was selected as a maximum dose of WG extract through the preliminary test. Sterile water (negative control) or WG extract (1250–5000 mg/kg/day) were orally administrated for 2 days in male ICR mice. CPA (70 mg/kg, positive control) was intraperitoneally administrated at the second day of experiment. Mice were euthanized with CO2 24 h after the last treatment of WG extract or negative/positive control. The femurs were harvested, and then bone marrows were isolated with FBS using 23 gauge needles. The bone marrow cells were centrifuged and dropped on slides. The slides were fixed with methanol (5 min) after being air-dried. Cells were stained with acridine orange and observed under a fluorescent microscope (Ni–U with B-2A fluorescence filter set, Nikon, Tokyo, Japan) at 400× magnification. Distinction of micronuclei morphology followed Hayashi’s method [28]. Red indicates polychromatic erythrocytes (PCEs), dark gray with little fluorescence indicates normochromatic erythrocytes (NCEs), and a green dot with a red background indicates micronuclei. The number of micronucleate PCEs (MNPCEs) were calculated in 4000 PCEs per animal. A cytotoxic index was expressed by the ratio of PCEs to RBCs after calculation of more than 500 RBCs and PCE + NCE regardless of the absence of the micronucleus.

2.7. Statistical Analyses

2.7.1. In Vitro AMES Test

Statistical analysis was not conducted. Results were considered positive when the mean number of colonies, with concentration of one or more, were significantly increased in at least one strain.

2.7.2. In Vitro Chromosome Aberration Test

Statistical analysis was conducted by SPSS Statistics 22 for Medical Science software (IBM, Armonk, NY, USA). The metaphase with at least one structural aberration was categorized into structural aberration and applied to statistical analysis. The metaphase only gap was excluded in the statistical analysis. Negative and treatment groups were compared with Fisher’s exact test, and the difference was considered significant when p < 0.05. The dose response analysis was performed by linear-by-linear association of the χ² test and was considered significant when p < 0.05. The numerical aberration was
calculated in the same way of structural aberration when the metaphase had more than 37 centromere and the total of metaphase with endoreduplication. Results were considered positive when the frequency of metaphases with chromosome aberration was significant compared with negative in any one dose, increased dose-dependent, and was out of the range of histological control data (HCD) in at least one test article regardless of S9 mix.

2.7.3. In Vivo Mouse Bone Marrow Micronucleus Test

Statistical analysis was conducted by SPSS Statistics 22 for Medical Science software (IBM, Armonk, NY, USA). The frequency of micronuclei was applied to the non-parametric Kruskal–Wallis H-test. A Mann–Whitney U-test was used to analyze the negative and positive control data. The dose response analysis was performed by linear-by-linear association of the \( \chi^2 \) test. The PCE:RBC ratios and body weights were applied to the One-way ANOVA. The homogeneity of variance was evaluated by Levene test. Student’s t-test was used to analyze the mean differences between the negative and positive controls. The cytotoxicity was considered that the PCE:RBC mean ratios were significantly decreased compared with negative control. Results were considered positive when the frequency of MN-PCE was significantly increased compared with negative control, increased dose-dependent, and was out of the range of HCD in at least any one dose.

3. Results

3.1. In Vitro AMES Test

Turbidity and precipitation were not observed up to 5000 \( \mu \)g/plate with or without the S9 mix. Precipitation was not apparent across any of the dose groups when mixing and colony counting were conducted with the preparation and top agar. A sterility check of WG extract and the S9 mix indicated that microbial colonization by the contamination was not present in any of the plates. In all doses of the WG extract groups with or without S9 mix, there was no increase of revertant and no increase in cytotoxicity in TA100, TA1535, TA98, TA1537, and WP2 \( \text{uvr}A \). The mean revertant of the positive controls were significantly increased compared with those of the negative control in all tested mutant bacterial strains. The number of viable cells were \( 0.65–4.38 \times 10^9 \) (TA strain) and \( 2.31 \times 10^9 \) (E. coli) CFU/mL in absorbance at 600 nm, and the treated viable cells per plate were more than \( 0.5 \times 10^8 \) CFU (Table 1).

3.2. In Vitro Chromosome Aberration Test

When WG extract was treated for 6 h with S9 mix, turbidity appeared at the 1400 \( \mu \)g/mL group. The frequency at which structural aberrations in the negative control group and the WG extract treatment groups appear were 0.33% (negative control group), 0.33% (350 \( \mu \)g/mL WG extract), 0.00% (700 \( \mu \)g/mL WG extract), and 0.00% (1400 \( \mu \)g/mL WG extract). No significant increase in the frequencies of structural aberrations was found in any of the dosed groups. However, the frequency of structural aberrations was significantly higher in the positive control group (17.67%). The frequency of numerical aberrations in the negative control group, the WG extract treatment groups, and the positive control group were 1.00% (negative control group), 0.33% (350 \( \mu \)g/mL WG extract), 1.00% (700 \( \mu \)g/mL WG extract), 1.67% (1400 \( \mu \)g/mL WG extract), and 0.00% (positive control group). We found no significant increase in the frequency of numerical aberrations in any of the dosed groups or in the positive control group compared against the negative control group.

When the WG extract was treated for 6 h in the absence of S9 mix, precipitation was observed in the 1400 \( \mu \)g/mL group. Structural aberrations in the negative control group and in the 350, 700, and 1400 \( \mu \)g/mL WG extract treatment groups were all 0.00%. No significant increase in the frequency of structural aberrations appeared in any of the dosed groups when compared against the negative control group. However, the frequency of structural aberrations was significantly higher in the positive control group (6.33%). Moreover, the frequency of numerical aberrations in the negative control group, the WG extract treatment groups and the positive control group were 1.00% (negative
control group), 0.67% (350 µg/mL WG extract), 0.33% (700 µg/mL WG extract), 0.33% (1400 µg/mL WG extract), and 0.33% (positive control group). No significant increase in the frequency of numerical aberrations was observed in any of the dosed groups or the positive control group when compared to the negative control group.

Table 1. In vitro Ames test of WG extract.

| Test Strain | Chemical Treated | Dose (µg/Plate) | Colonies/Plate [Factor] (a) |
|-------------|------------------|----------------|----------------------------|
|             |                  |                | With S9 Mix | Without S9 Mix |
| TA100       | WG               | 0              | 126 ± 8     | 113 ± 3     |
|             |                  | 50             | 136 ± 1 [1.1] | 108 ± 2 [1.0] |
|             |                  | 150            | 141 ± 2 [1.1] | 114 ± 1 [1.0] |
|             |                  | 500            | 130 ± 2 [1.0] | 115 ± 3 [1.0] |
|             |                  | 1500           | 122 ± 3 [1.0] | 107 ± 3 [1.0] |
|             |                  | 5000           | 151 ± 2 [1.2] | 103 ± 2 [0.9] |
| TA1535      | WG               | 0              | 31 ± 2      | 23 ± 1     |
|             |                  | 50             | 31 ± 2 [1.0] | 25 ± 3 [1.1] |
|             |                  | 150            | 32 ± 1 [1.0] | 24 ± 3 [1.1] |
|             |                  | 500            | 30 ± 1 [1.0] | 23 ± 2 [1.0] |
|             |                  | 1500           | 26 ± 1 [0.9] | 23 ± 3 [1.0] |
|             |                  | 5000           | 35 ± 2 [1.2] | 22 ± 2 [1.0] |
| TA98        | WG               | 0              | 30 ± 3      | 23 ± 2     |
|             |                  | 50             | 31 ± 1 [1.0] | 21 ± 1 [0.9] |
|             |                  | 150            | 28 ± 2 [0.9] | 22 ± 2 [0.9] |
|             |                  | 500            | 29 ± 1 [1.0] | 22 ± 2 [1.0] |
|             |                  | 1500           | 25 ± 1 [0.8] | 22 ± 2 [0.9] |
|             |                  | 5000           | 25 ± 1 [0.9] | 25 ± 1 [1.1] |
| TA1537      | WG               | 0              | 15 ± 2      | 12 ± 2     |
|             |                  | 50             | 15 ± 1 [1.0] | 9 ± 1 [0.8] |
|             |                  | 150            | 16 ± 2 [1.0] | 12 ± 1 [1.1] |
|             |                  | 500            | 16 ± 1 [1.1] | 12 ± 1 [1.0] |
|             |                  | 1500           | 17 ± 2 [1.1] | 11 ± 2 [1.0] |
|             |                  | 5000           | 17 ± 2 [1.2] | 14 ± 2 [1.2] |
| E. coli WP2 uvrA | WG | 0 | 23 ± 2 | 22 ± 1 |
|             |                  | 50             | 24 ± 1 [1.0] | 19 ± 1 [0.9] |
|             |                  | 150            | 25 ± 1 [1.1] | 19 ± 2 [0.9] |
|             |                  | 500            | 24 ± 1 [1.0] | 25 ± 2 [1.1] |
|             |                  | 1500           | 22 ± 2 [1.0] | 25 ± 2 [1.2] |
|             |                  | 5000           | 26 ± 3 [1.1] | 24 ± 2 [1.1] |

Positive controls

| Test Strain | Chemical Treated | Dose (µg/Plate) | Colonies/Plate |
|-------------|------------------|----------------|---------------|
| TA100       | 2-AA             | 1.0            | 2896 ± 150 [23] |
| TA1535      | 2-AA             | 2.0            | 340 ± 34 [11.1] |
| TA98        | 8[a]P            | 1.0            | 276 ± 10 [9.3] |
| TA1537      | 2-AA             | 1.0            | 197 ± 22 [13.1] |
| WP2 uvrA    | 2-AA             | 6.0            | 162 ± 13 [7.1] |
| TA100       | SA               | 0.5            | 358 ± 29 [3.2] |
| TA1535      | SA               | 0.5            | 358 ± 25 [15.8] |
| TA98        | 2-NF             | 2.0            | 360 ± 9 [15.4] |
| TA1537      | ICR-191          | 0.5            | 126 ± 10 [10.8] |
| WP2 uvrA    | 4NQO             | 0.5            | 189 ± 7 [8.7] |

(a) Three plates/dose were used. No. of colonies of treated plate/No. of colonies of negative control plate. 2-AA, 2-Aminoanthracene; 8[a]P, Benzo[a]pyrene; SA, Sodium azide; 2-NF, 2-Nitrofluorene; ICR-191, Acridine Mutagen ICR 191; 4NQO, 4-Nitroquinoline-1-oxide.

When the WG extract was treated for 24 h without the metabolic activation, there was no turbidity or precipitation apparent in any of the dosed group. Structural aberrations in the negative control group and the WG extract treatment groups appeared at a rate of 0.00% (negative control group), 0.00% (250 µg/mL WG extract), 0.00% (500 µg/mL WG extract).
extract), 0.00% (1000 µg/mL WG extract), and 0.33% (1100 µg/mL WG extract). Structural aberrations did not appear in any of the dosed groups at a statistically significant higher rate than the negative control group. The frequency of structural aberrations was significantly increased in the positive control group (6.33%). The percentage of numerical aberrations in the negative control group, the WG extract treatment groups, and the positive control group was 0.67% (negative control group), 0.00% (250 µg/mL WG extract), 0.33% (500 µg/mL WG extract), 0.33% (1000 µg/mL WG extract), 1.00% (1100 µg/mL WG extract), and 1.00% (positive control group). In summary, no statistically significant increase in the frequency of numerical aberrations was observed in any of the dosed groups or in the positive control group (Table 2).

### Table 2. In vitro chromosome aberration test of WG extract.

| Dose (µg/mL) | Time (h) | S9 Mix | Observed Cells | Percentage of Cells Showing Aberrations | Chromosome Type | Chromatid Type | Others Gaps | No. Aberrant Metaphase (a) | PP + ER | RICC(%) |
|-------------|----------|--------|----------------|----------------------------------------|----------------|----------------|-------------|--------------------------|---------|---------|
|             |          |        |                | Ch chromosome type                     |                |                | +Gaps       | −Gaps                    |         |         |
|             |          |        |                | csb, cse, ctb, cte                      |                |                |             |                          |         |         |
| 0           | 6 + 18   | +      | 150            | 0.0, 0.0, 1.5, 0.0                      | 0.0            | 0.0            | 0.33        | 0.33                     | 1       | 100     |
| 350         | 6 + 18   | +      | 150            | 0.0, 0.0, 0.0, 0.5                      | 0.0            | 0.0            | 0.33        | 0.33                     | 0.33    | 91      |
| 700         | 6 + 18   | +      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0            | 0.0            | 0           | 0                        | 1       | 85      |
| 1400 T      | 6 + 18   | +      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0            | 0.0            | 1.67        | 0                        | 0       | 85      |
| Positive Control |    |        |                |                                        |                |                |             |                          |         |         |
| B[a]P 20    | 6 + 18   | +      | 150            | 0.0, 1.0, 8.0, 33.5                     | 0.0            | 1.5            | 17.67       | 17.67 **                  | 0       | 69      |
| 0           | 6 − 18   | −      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0            | 0.0            | 0           | 0                        | 1       | 101     |
| 350         | 6 − 18   | −      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0            | 0.0            | 0           | 0                        | 0.67    | 101     |
| 700         | 6 − 18   | −      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0            | 0.0            | 0           | 0                        | 0.33    | 99      |
| 1400 T      | 6 − 18   | −      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0            | 0.0            | 0           | 0                        | 0.33    | 95      |
| Positive Control |    |        |                |                                        |                |                |             |                          |         |         |
| 4NQO 0.4    | 6 − 18   | −      | 150            | 0.0, 0.0, 1.5, 12.5                     | 2.0            | 0.5            | 6.33        | 6.33 **                  | 0.33    | 77      |
| 0           | 24 − 0   | −      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0            | 0.0            | 0           | 0                        | 0.67    | 100     |
| 250         | 24 − 0   | −      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0            | 0.0            | 0           | 0                        | 0       | 92      |
| 500         | 24 − 0   | −      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0            | 0.0            | 0           | 0                        | 0.33    | 79      |
| 1000        | 24 − 0   | −      | 150            | 0.0, 0.0, 0.0, 0.0                      | 0.0, 0.5       | 0.33           | 0           | 0.33                     | 0.33    | 50      |
| 1100        | 24 − 0   | −      | 150            | 0.0, 0.0, 0.5, 0.0                      | 0.0, 0.5       | 0.67           | 0.33        | 1                        | 47      |         |
| Positive control |    |        |                |                                        |                |                |             |                          |         |         |
| 4NQO 0.4    | 24 − 0   | −      | 150            | 0.0, 0.5, 3.0, 10.0                     | 2.0            | 0.0            | 6.33        | 6.33 **                  | 1       | 64      |

(a) Inclusive/exclusive gaps. 150 metaphases were examined per culture. ** Significantly different from the negative control at p < 0.01. T Turbidity at the end of the treatment. Gaps, Chromosome type + Chromatid type gaps; csb, Chromosome type break; cse, Chromosome type exchange; ctb, Chromatid type break; cte, Chromatid type exchange; Other: Metaphases with more than 10 aberrations (including gaps) or with chromosome fragmentation; PP, Polyploid; ER, Endoreduplication; B[a]P, Benzo[a]pyrene; 4NQO, 4-Nitroquinoline-1-oxide.

### 3.3. In Vivo Mouse Bone Marrow Micronucleus Test

There were no unscheduled deaths, clinical signs, or significant changes in body weight detected in the WG extract administration groups.

The MNPCEs frequencies in the negative control group and the WG extract groups were 3.83 (negative control group), 3.33 (1250 µg/mL WG extract), 4.17 (2500 µg/mL WG extract), and 4.00 (5000 µg/mL WG extract) in the 4000 PCEs per subject. There was no significant increase in MNPCEs frequencies in any of the dosed groups compared against the negative control group. However, MNPCE was significantly increased in the positive control group (62.17).

PCE:RBC ratios, which is a cytotoxicity index, in the negative control group and the WG extract groups were 0.52 (negative control group), 0.52 (1250 µg/mL WG extract), 0.52 (2500 µg/mL WG extract), and 0.53 (5000 µg/mL WG extract) in the 500 RBCs per subject.
The PCE:RBC ratio did not vary significantly across any of the dosed groups compared to the negative control group. However, the ration was significantly lower in the positive control group (0.41) (Table 3).

Table 3. In vivo bone marrow micronucleus test of WG extract.

| Dose (mg/kg/Day) | Animals per Dose | Body Weight (g) at the Time of Sacrifice | MNPCE/4000PCE (Mean ± SD) | PCE:RBC Ratio (Mean ± SD) | (% Control) |
|-----------------|------------------|-----------------------------------------|---------------------------|---------------------------|------------|
|                 |                  | Day 0                                   | Day 1                     |                            |            |
| 0               | 6                | 35.34 ± 0.96                            | 35.34 ± 1.61              | 3.83 ± 1.47               | 0.52 ± 0.02 | 100        |
| 1250            | 6                | 35.09 ± 1.21                            | 34.35 ± 1.32              | 3.33 ± 2.16               | 0.52 ± 0.03 | 100        |
| 2500            | 6                | 34.98 ± 1.16                            | 34.93 ± 1.25              | 4.17 ± 1.47               | 0.52 ± 0.03 | 100        |
| 5000            | 6                | 34.75 ± 1.36                            | 34.99 ± 1.41              | 4.00 ± 1.79               | 0.53 ± 0.02 | 102        |
| Positive control | CPA 70           | 6                                        | 34.63 ± 1.23              | 35.08 ± 1.21              | 62.17 ± 8.80** | 0.41 ± 0.03** | 78         |

** Significantly different from the negative control group at \( p < 0.01 \). Vehicle, Sterile distilled water for injection. Vehicle and Test article were orally administered to mice for two consecutive days. CPA was intraperitoneally administered to mice once on the day of the 2nd admin. Mice were euthanized approximately 24 h after the final administration. Bone marrow smears were prepared approximately 24 h after the final administration. MNPCE, Micronucleated polychromatic erythrocyte; PCE, Polychromatic erythrocyte; RBC, Red blood cells (polychromatic erythrocyte + normochromatic erythrocyte); CPA, Cyclophosphamide monohydrate (positive control article).

4. Discussion

The purpose of this study is to assess the potential genotoxicity of WG. WG is a Glycyrrhiza new variety, and various pharmacological effects including enhancing immune response and being anti-allergenic, anti-neuroinflammatory, and anti-ulcerative colitis are investigated [11–14]. Therefore, the safety information of WG should be required to develop a therapeutic agent. In our previous report, we revealed the general toxicity of WG to estimate the potential risk to organs and the relationship between exposure and response [29,30]. As a result of the general toxicity test, target organs and no-observed-adverse-effect level (NOAEL) were not observed in up to 5000 mg/kg/day of WG extract [31]. Further, we conducted the genotoxicity of WG in this study to estimate carcinogenicity, mutagenicity by damage to DNA or chromosome [21]. As a result of the genotoxicity test, WG extracts did not induce genotoxic or mutagenic activities. Therefore, our studies could contribute to providing safety information to clinical as well as to register the WG as a new species of Glycyrrhiza in the KP.

According to previous reports [32], licorice is composed of more than 400 compounds, including saponins (glycyrrhizin, uralsonapin, licorice-saponin, actoxyglycyrrhizin, apio-glycyrrhizin, and araboglycyrrhizin), flavonoids (liquiritigenin, liquiritin, rhamnolliuiritin, licohalcone, liocflavone, and licoisoflavanone), phenols (liquiritin, liquiritin apioside, chromenes, coumarins, and dihydrostilbenes), essential oils (\( \alpha / \beta \)-pinene, octanol, and estragole) and other compounds (fatty acids, glucose, sucrose, starch, polysaccharides, and sterols). We also identified the numerous ingredient profiling of WG by TOF-MS analysis. Approximately 3000 chemicals including glycyrrhizic acid and liquiritigenin were detected in the WG water extract (Figure S1).

Glycyrrhizic acid (glycyrrhin) is the major triterpenoid saponin present in licorice, which has various pharmacological effects such as being anti-inflammatory, anti-ulcerogenic, anti-allergic, antioxidant, anti-hepatotoxic, anti-tumor, and anti-viral [24,33,34]. Accordingly, a composition of at least 2.5% glycyrrhizic acid is an indicator component of licorice that must be present for its medicinal use according to the KP. According to a previous report, WG ethanol extract contains 3.9% glycyrrhizic acid [10]. In this study, we determined through HPLC analysis that water extract of WG contains 25 mg glycyrrhizic acid per g (2.5%) (data not shown). Accordingly, water and ethanol extract of WG both contain glycyrrhizic acid in sufficient quantities for medicinal use.

The AMES test is the most widely employed initial screen for mutagenic potential for its convenience and low-cost. The AMES test uses the histidine auxotroph strains.
of *S. typhimurium* and the tryptophan auxotroph strain of *E. coli* to detect frameshift mutations or point mutations, including the addition, deletion, or substitution of one or more DNA base pairs [35]. The *S. typhimurium* strains used in the original assay have a mutation in the histidine synthesis gene and are auxotrophic to histidine. When the bacteria are exposed to a potential mutagen and plated in histidine-limited media, colony forming is developed because bacteria mutated and reverted to being prototrophic to histidine. A similar process is followed with respect to the tryptophan auxotroph strains of *E. coli*. Accordingly, counting colony units is a means of assessing mutagenic potential. Test substances are evaluated with or without S9 mix to investigate promutagens and direct mutagen activity [36,37]. Compared with a negative control group, we observed no cytotoxicity and an increase of the number of bacterial revertants in *S. typhimurium* (TA100, TA1535, TA98, and TA1537) and *E. coli* (WP2 *uvrA*) up to 5000 µg/plate, regardless of the presence of S9 mix (Table 1), suggesting that WG extract didn’t induce the base pair substitution mutations (TA100, TA1535 and WP2 *uvrA*) and frameshift mutations (TA98 and TA1537) under in vitro conditions of this study.

Also, the potential mutagenic properties of WG extract were evaluated using a chromosome aberration test. Chromosome aberrations correlate with genetic disorders like cancer, which causes structural and numerical chromosome aberrations [38]. Abnormal replication or repair of DNA lesions can directly or indirectly induce double-stranded DNA damage, which leads to breakage of the double-strand structural. This is the primary cause of structural chromosome aberrations [39]. The disruption of cell cycle checkpoints or the inhibition of topoisomerase damage to DNA, which is the cause of the numerical chromosome aberrations [40]. A chromosome aberration test is able to identify structural and numerical chromosome aberrations using cultured mammalian cells, and is accordingly used to assess the genotoxicity of candidate compounds. CHL cells are commonly used in chromosome aberration tests due to their sensitivity to mutagens and their low chromosome number, which facilitates scoring [41]. Moreover, because it is widely used, a broad database of CHL cells already exists [42]. When WG extract was treated for 6 h with S9 mix, the frequency of numerical aberrations in the 1400 µg/mL WG extract treatment group was beyond the range of HCD. However, the result was not statistically significantly increased and not a dose-related response. At that time, we observed no significant increase in the frequency of structural aberrations in any of the WG-dosed groups. Nor did we observe a significant increase the frequency of structural and numerical aberrations in any of the WG-dosed groups after the WG extract was treated for 6 h and 24 h without S9 mix (Table 2). These results suggest that WG extract didn’t damage DNA under the in vitro conditions of this study.

A micronucleus test is a useful means of assessing the mutagenicity of test articles that affect the equitable distribution of chromosomes under the cell division [43]. This test is typically performed using bone marrow from experimental animals, as the tested substance can conveniently be orally administrated to the animals and then digested, metabolized, and spread to the target organs (including bone marrow). The micronucleus test is especially well suited to an evaluation of mutagenicity as it permits consideration of multiple processes and reflects the cytogenic effects of active forms of test substance [44,45]. Chromosomal damage, such as double strand chromosome breakage (clastogenesis) or impaired spindle (aneugenicity), induces the creation of micronuclei; the number of micronuclei, therefore, is an indicator of chromosomal damage [46]. We observed no statistically significant increase in the frequency of MNPCEs after administration of up to 5000 mg/kg/day of WG compared to the negative control group. Moreover, the PCE:RBC ratio, which is an index of cytotoxicity, was not significantly decreased by administration of up to 5000 mg/kg/day of WG compared to the negative control group. These results indicate that WG extracts did not induce the chromosomal damage under the conditions of this study.

A number of previous studies have reported on the genotoxicity of licorice extracts and compounds. *G. glabra* water extract induced mutagenicity at 2500 µg/mL in the
AMES test (TA100), but chloroform and methanol extracts did not [47]. In a study of chromosome aberration and micronucleus tests in human lymphocytes, \textit{G. glabra} methanolic extract did not occur genotoxicity after up to 20 µg/mL was administered per day [48]. Licorice flavonoid oil (LFO) induced structural chromosome aberrations when given at concentrations higher than 600 µg/mL with S9 mix in CHL cells. Pursuant to the AMES test, LFO did not increase the number of revertant colonies up to 5000 µg/plate in any tester strains (TA100, TA1535, TA98, TA1537, and WP2 uvrA), and nor did LFO increase the frequency of MNPCeEs when up to 5000 mg/kg/day was provided, but cytotoxicity was observed when 5000 mg/kg/day was administered in micronucleus tests of F344 rats [49]. Standardized de-glycyrrhizinated \textit{G. glabra} extract did not induce genotoxicity when up to 501 µg/mL was provided (according to the AMES II™ test using TA98 and TAMix strains), or when between 40 µg/mL with 4 h treatment and 14.6 µg/mL with 18 h treatment (according to the chromosome aberration test using CHO-K1 cells), or when 40 µg/mL was administered (according to the micronucleus test using the CHO-K1 cells). However, cytotoxicity was observed when 40 µg/mL was administered in the chromosome aberration test as well as the micronucleus test [50]. Disodium glycyrrhizinate (up to 5000 µg/plate) didn’t increase the number of revertant colonies in the AMES test (TA92, TA1535, TA100, TA1537, TA94, and TA98), but did induce structural chromosome aberrations at 4000 µg/mL in the chromosome aberration test on the CHL cells. Trisodium glycyrrhizinate (up to 10,000 µg/plate) didn’t increase the number of revertant colonies in the AMES test (TA92, TA1535, TA100, TA1537, TA94, and TA98), but induced structural chromosome aberrations when administered at 4000 µg/mL in the chromosome aberration test on the CHL cells [51]. Monoammonium glycyrrhizinate (MGL) (up to 5000 µg/plate) didn’t induce genotoxicity in the AMES test (TA98, TA100, TA102, TA1535, and TA1537), up to 1500 µg/mL in the chromosome aberration test using human lymphocytes, and up to 250 mg/kg (i.v.) in the micronucleus test using mouse bone marrow. Cytotoxicity was observed when 1500 µg/mL was administered in the chromosome aberration test [51]. In sum, we suppose that WG water extract shows lower genotoxicity than the other extracts from other licorice species.

WG extract didn’t induce the genotoxic or the mutagenic activities in any of the genotoxic assessments, including the in vitro AMES test, the in vitro chromosome aberration test, and the in vivo mouse bone marrow micronucleus test. This result suggests that WG extract is a low potential risk of carcinogenesis, which is better than other licorice species. In addition, our results could support the safety information of WG to patients or healthcare providers for clinical purposes and contribute to registering ‘Wongam’ as new variety of \textit{Glycyrrhiza} in the KP.

**Supplementary Materials:** The following is available online at https://www.mdpi.com/article/10.3390/app112110257/s1, Figure S1: MALDI-TOF MS spectrum from the Wongam water extract.

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