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Micronucleus Test In Bone Marrow Cells And Oral Toxicity Study On Aloin-Quercetin In Rats

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Abstract:
The purpose of this study was to evaluate the toxicity of test substance, including aloin and quercetin. Toxicity was examined by micronucleus test using male ICR mouse and through oral administration toxicity test using male and female Sprangue-Dawley rats. Week 8 ICR mouse were treated with test substance through oral administration at 0, 1250, 2500, and 5000 mg/kg/day for two days. Then, the ratio of MNPCE(Micro nucleated polychromatic erythrocyte)to PCE(polychromatic erythrocyte) and the ratio of PCE to total erythrocytes were observed. The significant changes in these values were not observed. Sprague-Dawley rats were treated with test substance through oral administration at 0, 1250, 2500, and 5000 mg/kg and observed for 15 days. There were no deaths and no changes in clinical signs. The result suggested that test substance do not cause micronucleus in mouse bone marrow cells and the ALD(approximate lethal dose) of test substance is over 5000 mg/kg.

KeyWord*: Aloin, Quercetin, micronucleus test, toxicity test, oral administration, Approximate Lethal Dose*

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I. Introduction

With advances in medical technology leading to increased life expectancy, there is growing interest not just in longevity but also in health[1]. Particularly, there is increasing interest in aging as an inevitable natural process, driving continuous research into anti-aging strategies. Research on the effects of antioxidants on aging has been extensively conducted [2,3,4]. Consequently, there is a rising preference and development of high-performance cosmetics aimed at rejuvenating skin function impaired during aging. Moreover, interest and research into anti-aging through food intake have also been on the rise[5,6,7,8]. Research on plant extracts with anti-aging properties obtained from fruits, vegetables, and other plants has been actively pursued, with examples such as aloin from aloe vera and quercetin from onions[9,10,11,12,13,14,15,16,17,18].

Aloe vera, a perennial plant cultivated in tropical and subtropical regions worldwide, belongs to the family Liliaceae and comprises approximately 300-360 species[19,20,21]. The species commonly grown domestically include Aloe vera and Aloe arborescens. Aloe vera contains polysaccharides, amino acids, and minerals, phenolic compounds such as aloe emodine and aloesin, as well as bioactive compounds such as sterol and terpenoid.

As aloe vera is known to have antioxidant activity, antibacterial, anti-inflammatory, and tissue formation properties, it is used to treat and prevent diseases[22,23,24]. Particularly, the aloin found in aloe vera is recognized for its antioxidant effects[14.15].

Onions, plants belonging to the Allium genus in the Amaryllidaceae family, are found across a wide range of latitudes and altitudes. 90% of onions are made up of water, and onions also contain carbohydrates, proteins, vitamins, calcium, and iron. They are known for their potential in preventing hypertension, improving blood circulation, and preventing adult diseases such as heart disease. Specifically, quercetin exerts antioxidant effects and aids in cholesterol removal, thereby promoting smooth blood circulation[16,17,18,25].

Research has explored the effects of the mixture of aloe vera's aloin and onion's quercetin, hypothesizing various benefits including enhanced blood circulation and antioxidant properties[26,27,28,29]. However, it has been reported that while high concentrations of aloin exhibit antioxidant effects, lower concentrations may induce oxidative stress[11]. Therefore, experiments have been conducted to assess the genetic toxicity and oral toxicity of compounds such as aloe extract' containing aloin and quercetin, aiming to establish

their safety as natural extracts and to provide toxicity evaluation evidence required for approval. By using micronucleus test, we evaluate whether chromosomal abnormalities or mitotic mechanism abnormalities are caused in vivo in the bone marrow cells of ICR mice. The toxicity that appears when administered orally to Sprague-Dawley rat once was confirmed.

II. Material And Methods

Material

Test material

The test material is provided by KimJeongMoon Aloe Ltd (Code No. C-1630). It consists of a compound including aloe extract, specifically a whitening powder containing quercetin (0.48 mg/g) and aloesin (0.06 mg/g). The material was used without adjustment for content and was administered in its original weight. Each dose of the test material was quantified according to the dosage for the day, homogenized with an excipient (sterile injection water) for uniformity.

Negative and positive control substance

For the negative control substance (excipient), sterile injection water (DAIHAN Pharm Co., Ltd., Lot No. 31N1F21) was used. The positive control substance used in the micronucleus test with ICR mice was Cyclophosphamide monohydrate (CPA) (CAS No. 6055-19-2, Lot No. SLBG4216V, Sigma-Aldrich, Co., USA). The CPA was dissolved in physiological saline solution (DAIHAN Pharm Co., Ltd., Lot No. 78N2K08) immediately before administration to achieve the appropriate dosage. CPA, used as the positive control substance, is an immunosuppressant. It damages the lymphatic system, leading to reductions in white blood cells and lymphocytes, thereby suppressing immune responses. CPA is also known as a DNA alkylating agent that induces oxidative stress[30]. Additionally, it has been used as a positive control substance in various micronucleus tests[31,32].

Raising environment and group designing

The experimental animals were housed in Room No. 8 of Building 2 at Chemon Nonclinical Research Institute, maintained at a temperature of 23±3℃, relative humidity of 55±15%, ventilation frequency of 10-20 air changes per hour, and a light cycle of 12 hours (lights on at 8 AM and off at 8 PM) with an intensity of 150-300 Lux. Animals had ad libitum access to water and feed. The diet supplied was TEKLAD CERTIFIED IRRADIATED GLOBAL 18% PROTEIN RODENT DIET 2918C (radiation-sterilized solid feed for experimental animals, Harlan Laboratories Inc., USA) from Dooyeol Biotech, and water was sterilized groundwater treated with ultraviolet disinfection and microfiltration. Specific pathogen-free (SPF) mice and rats used in the study were obtained at 7 weeks of age and administered the test materials at 8 weeks of age.

Animals were acclimated and ranked based on their body weights to ensure health during the acclimation period. Animals with weights close to the group average were randomly distributed to ensure uniform distribution of average weights within each group.

The experiment was conducted at Chemon Nonclinical Research Institute, which holds accreditation from AAALAC International (Association for Assessment and Accreditation of Laboratory Animal Care International, 2010). The study was approved by the Chemon Nonclinical Research Institute's Institutional Animal Care and Use Committee (IACUC) with approval numbers 14-M220 for mice and 14-R155 for rats.

Micronucleus test in male ICR mouse

The experiment was conducted based on the "Toxicity Test Standards for Pharmaceuticals and Others (Ministry of Food and Drug Safety Notice No. 2014-6, January 29, 2014)" and OECD Guideline for the Testing of Chemicals, TG 474 'Mammalian Erythrocyte Micronucleus Test'[33]. Furthermore, the study adhered to the "Nonclinical Test Management Standards" of the Ministry of Food and Drug Safety and OECD Principles of Good Laboratory Practice[34].

Raising environment

During the raising period, the temperature and relative humidity in the animal room were maintained at 21.0 ~ 22.0℃ and 53.9 ~ 58.6%, respectively. Bedding was supplied from a commercial source and sterilized by high-pressure steam before use. The mice were individually housed in polycarbonate cages measuring 170W×235L×125H (mm) throughout the quarantine, acclimation, dosing, and observation periods, with one mouse per cage. Cage bedding and water bottles were replaced weekly.

Male ICR mice were weighed upon arrival, acclimated for 7 days in the animal facility where the study was conducted, ensuring healthy individuals were used for the experiment. During the acclimation period, mice were identified using individual identification cards, and ear punch marking was used for identification during the dosing and observation periods.

Dose amount and group designing

Results from the preliminary micronucleus test using oral administration to ICR mouse bone marrow cells with 'compounds such as aloe extract' (Study No.: 14-MG-154P, conducted by ChemOn) showed that when the test material was administered at doses of 1250, 2500, and 5000 mg/kg/day to three animals per sex per dose for two days, no significant gross abnormalities were observed in any of the animals, and there were no notable differences in toxicity based on gender. Therefore, male ICR mice were used in this study, with 5000 mg/kg/day established as the highest dose based on body weight, and lower doses of 1250 and 2500 mg/kg/day were set at a ratio of 2. Additionally, a negative control group receiving only the vehicle and a positive control group receiving CPA were established. Ultimately, the test groups for the micronucleus test using oral administration to ICR mice were designed as shown in Table 1.

Table 1. Group designation of micronucleus test in male ICR mouse. G1 is negative control and G2-G4 is experimental groups. G5 is positive control(CPA administration). P.O. : Per Os(Oral administration). I.P. : Intraperitoneal Injection.

Group	Number of animals	Dose amount (mL/kg/day)	Dose (mg/kg/day)	Number of dose	Route
G1	6	10	0	2	P.O.
G2	6	10	1250	2	P.O.
G3	6	10	2500	2	P.O.
G4	6	10	5000	2	P.O.
G5	6	10(mL/kg)	70(mg/kg)	1	I.P.

The test substance, compounds such as aloe extract, was administered via an oral using a stomach tube equipped with a syringe at 24-hour intervals directly into the stomach twice. The dose administered was calculated based on the body weight measured on the day of administration (10 mL/kg/day). The positive control substance was administered via intraperitoneal injection, a common route, at a single dose of 10 mL/kg (equivalent to 70 mg/kg) on the second day of test substance administration. The administration site was sterilized using alcohol, and the substance was injected into the peritoneal cavity using a 26G needle. Similarly, the dose was calculated based on the body weight measured on the day of administration (10 mL/kg).

Clinical signs and changes in body weight observation

General clinical signs were observed once daily during the recovery period and three times daily during the dosing period (DAY 1, 2): before administration, immediately after administration, and one hour after administration. On necropsy day (DAY 3), observations were made once before necropsy. Body weights were measured for all animals on the administration day (prior to dosing) and on the day of sample collection.

Production and observation of bone marrow smears[37]

Bone marrow smears were prepared according to Schmid's method [35], typically around 24 hours after the final dose administration (second dose). Each mouse was euthanized using CO2 gas anesthesia, and one femur was extracted. The marrow was flushed using 2 mL of Fetal Bovine Serum through a 23 G needle to rinse and suspend the cells. The cell suspension was centrifuged at 1000 rpm for 5 minutes to remove the supernatant. The precipitated bone marrow cells were smeared onto glass slides, air-dried at room temperature, and fixed in methanol for 5 minutes. Two slides were prepared per animal, and after drying, the slides were stored in a slide box until staining and counting.

The fluorescent dye solution (Acridine orange solution, AO solution) was prepared by diluting acridine orange base (CAS No. 494-38-2) 0.05% solution with Sorensen buffer (pH 6.8) in a 1:4 (v/v) ratio, following an adaptation of Hayashi's method [36]. One well-prepared smear per animal, showing good morphology, was selected for staining immediately before counting. A suitable amount of AO solution was applied to the smear, covered with a coverslip, and a slide for observation was prepared. After 2 minutes, the staining condition was checked, and when appropriate staining was achieved, counting was performed using a fluorescence microscope (400x magnification, Nikon model Ni-U, B-2A fluorescence filter set).

The morphological identification of micronuclei was conducted according to Hayashi's method[36]. Polychromatic erythrocytes (PCEs) appeared as red fluorescent cells, whereas normochromatic erythrocytes (NCEs) exhibited minimal fluorescence and appeared as dark gray. Micronuclei, when present, appeared as green dots on a red background within PCEs. Observation involved counting 2000 PCEs per individual to determine the number of micronucleated polychromatic erythrocytes (MNPCEs). The frequency of micronuclei occurrence was expressed as the mean ± standard deviation of MNPCEs observed per 2000 PCEs per individual. Regardless of micronuclei presence, a total of 500 or more erythrocytes (PCEs + NCEs) were counted per

individual to calculate the ratio of PCEs to total erythrocytes (PCE:RBC(PCE+NCE)). This ratio served as an indicator of cytotoxicity.

The validity criteria for the test were as follows: 1) At least 5 animals survived to necropsy in all groups. 2) The mean PCE:RBC ratio in both the test substance-treated group and the positive control group was at least 20% of the negative control group. 3) The frequency of micronucleated polychromatic erythrocytes (MNPCEs) among 2000 PCEs was less than or equal to a mean of 10.0 (0.5%) in the negative control group and at least a mean of 50 (2.5%) in the positive control group. If all three conditions were met, the test was considered valid, and the results were interpreted accordingly.

Statistical analysis and judgement

The test results were statistically analyzed using SPSS (ver. 10.1K) software with a significance level set at $p < 0.05$. For the frequency of micronuclei induction, non-parametric Kruskal-Wallis H-test was conducted using ranked data, while comparisons between the negative control and positive control groups were analyzed using the Mann-Whitney U-test. A positive response was determined when the frequency of MNPCEs in the test substance-treated group showed statistically significant and dose-dependent increases or consistent positive reactions at one or more doses.

For the PCE:RBC ratio and body weight data, assuming normality of the data, parametric one-way analysis of variance (ANOVA) was applied. Homogeneity of variance was tested using the Levene test, and differences in means between the negative control and positive control groups were analyzed using independent samples t-tests. Cytotoxicity was determined when the mean PCE:RBC ratio in the test group showed statistically significant decreases compared to the negative control group.

Toxicity test in Sprague-Dawley Rats

The experiment was conducted based on the "Toxicity Test Standards for Pharmaceuticals and Others (Ministry of Food and Drug Safety Notice No. 2013-121, April 5, 2013)". Furthermore, the study adhered to the "Nonclinical Test Management Standards(No. 2013-40, April 5, 2013)" of the Ministry of Food and Drug Safety and OECD Principles of Good Laboratory Practice[34].

Raising environment

During the toxicity experiment using Sprague-Dawley rats, the temperature and relative humidity in the animal room ranged from 21.7 to 22.5°C and 55.4 to 59.6%, respectively. Rats were housed in stainless steel mesh cages with no more than 3 animals of both sexes per cage throughout quarantine, acclimatization, dosing, and observation periods. Water quality was monitored daily, and water bottles were replaced weekly. Upon receipt, rats were visually inspected, weighed, and acclimated for 7 days in the animal facility where general observations were conducted at least once daily.

During the acclimatization period, red meteoric tags were used for identification, while black meteoric tags were used during the dosing and observation periods. Individual identification cards indicating the dose color were attached to the cages, and each cage was assigned a unique identification number.

Dose amount and group designing

Considering that the test substance is a natural extract intended for clinical use at a planned dose of 2000 mg per 60 kg (= 33.33 mg/kg/day), a high dose of 5000 mg/kg was selected, which is approximately 150 times the clinical planned dose. Lower doses of 1250 and 2500 mg/kg/day were then set at a 2:1 ratio. A negative control group receiving only the excipient (excipient control group) was also included. Ultimately, the test groups for the single-dose oral toxicity study using SD rats were structured as shown in Table 2.

Table 2. Group designation of toxicity test in Sprague-Dawley Rats. G1 is negative control and G2-G4 is experimental groups.

Group	Sex	Number of animals	Dose amount (mL/kg)	Dose (mg/kg)
G1	M / F	5 / 5	10	0
G2	M / F	5 / 5	10	1250
G3	M / F	5 / 5	10	2500
G4	M / F	5 / 5	10	5000

The test substance, compounds such as aloe extract, was administered once daily via oral, using a dose volume calculated based on the body weight measured on the day of administration (10 mL/kg). Additionally, rats underwent an overnight fasting period (approximately 16-20 hours) to empty the stomach contents before direct administration into the stomach using a stomach tube equipped with a syringe for oral administration. Feed was replenished 3-4 hours after dosing.

Clinical signs, changes in body weight and necropsy observation

General clinical signs were observed at least once daily throughout the experiment period. On the day of dosing (Day 1), observations were conducted continuously for up to 1 hour post-dosing and then hourly until 6 hours post-dosing. The study continued observations until Day 15 after dosing, with Day 1 being the administration day.

Regarding body weight, measurements were taken for all animals on Day 1 (pre-dose), and subsequently on Days 2, 4, 8, and 15.

At the conclusion of the study on Day 15, all surviving animals were euthanized using CO2 anesthesia. They were then dissected by opening the abdominal cavity, cutting the inferior vena cava and aorta, and thoroughly examining all organs visually. This procedure was conducted to assess any potential gross abnormalities or effects of the test substance on internal organs.

Statistical analysis and judgement

The results were statistically analyzed using the widely used statistical package SPSS (ver. 10.1K), with a significance level set at $p < 0.05$. For the statistical analysis of body weight, assuming normality of the data, parametric one-way analysis of variance (ANOVA) was applied. Since no deaths occurred during the study, the median lethal dose (LD50) was not calculated.

III. Result And Discussion

Micronucleus test in male ICR mouse

Among 2,000 PCEs (polychromatic erythrocytes) per individual, the frequency of PCE (MPCE) with micronuclei averaged 0.33, 0.67, 1.17, and 1.50 in the order of the negative control group (0), the test substance 1250, 2500, and the 5000 mg/kg/day administration group. As a result of performing a nonparametric Kruskal-Wallis' H-test using the ranked micronuclei-induced frequency results, the test substance administration group did not show a statistically significant difference from the negative control group. On the other hand, the micronuclei frequency in the positive control group was 57.83, showing a statistically significant increase compared to the negative control group ($p<0.01$) (Figure 1).

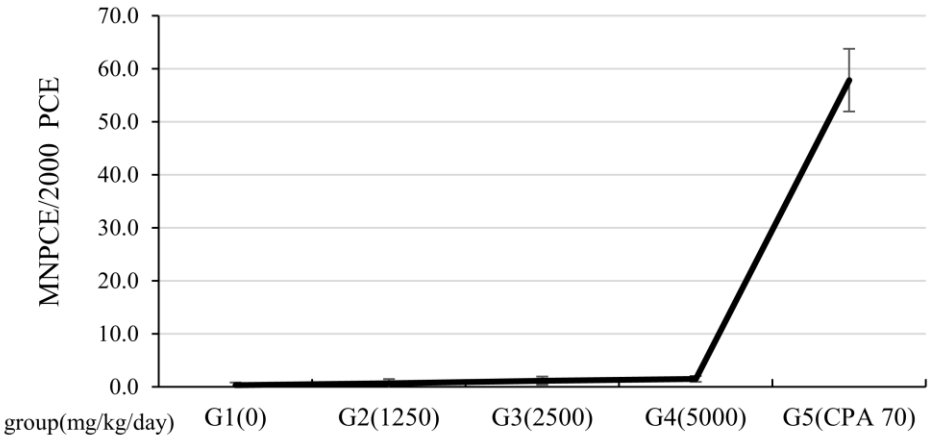

Figure 1. The ratio of MNPCE to PCE in male ICR mouse in the in vivo micronucleus test. G1 is negative control and G2-G4 is experimental groups(experimental material administration for each amount for 2 days). G5 is positive control(CPA 70mg/kg administration).

The PCE:RBC ratio, which is an indicator of cytotoxicity, is average 0.56, 0.54, 0.54, and 0.56 in the order of the negative control group (0), the test substance 1250, 2500, and the 5000 mg/kg/day administration group. As a result of applying one-way analysis of variance to the PCE:RBC ratio data, no statistically significant change was observed in all test substance administration groups compared to the negative control group. Meanwhile, the PCE:RBC ratio was 0.42, showing a statistically significant decrease compared to the negative control group ($p<0.01$) (Figure 2).

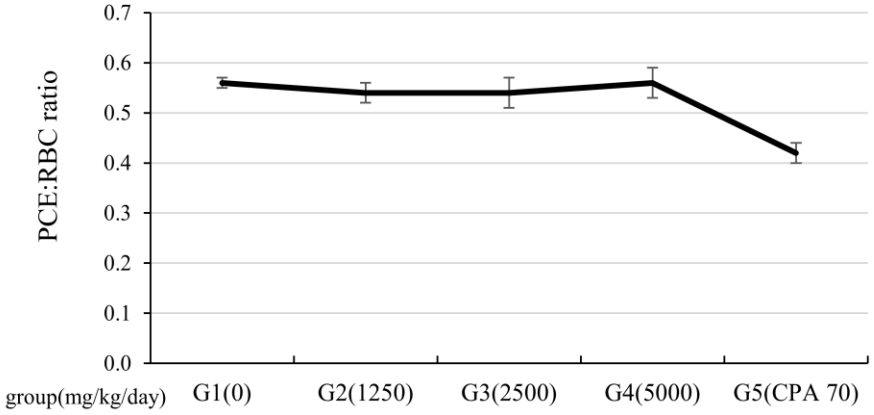

Figure 2. The ratio of polychromatic erythrocytes (PCE) to total erythrocytes in male ICR mouse in the in vivo micronucleus test. G1 is negative control and G2-G4 is experimental groups(experimental material administration for each amount for 2 days). G5 is positive control(CPA 70mg/kg administration).

As a result of comparing the weights of each group, there was no statistically significant difference in all test substance administration groups (Figure 3).

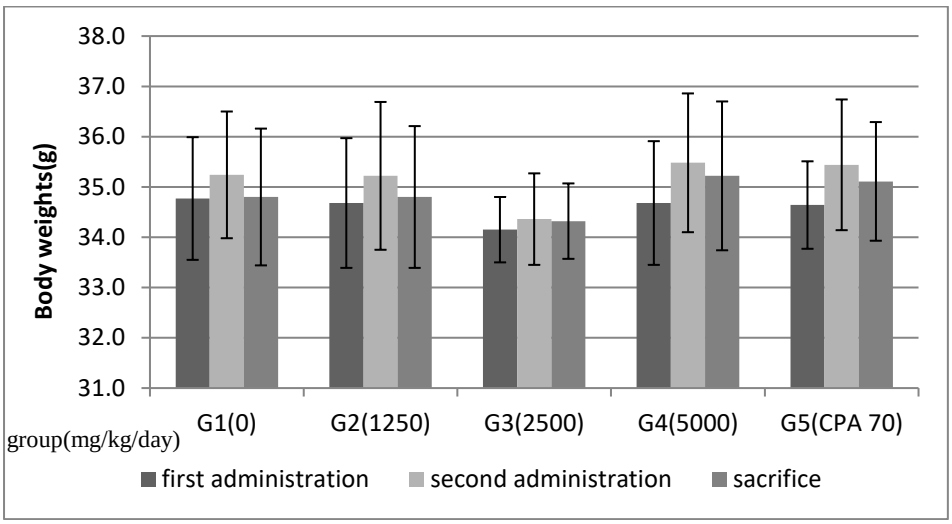

Figure 3. Changes in body weight of male ICR mouse in the in vivo micronucleus test. G1 is negative control and G2-G4 is experimental groups(experimental material administration for each amount for 2 days). G5 is positive control(CPA 70mg/kg administration).

In addition, no specific visual abnormalities were observed due to the administration of the test substance in all surviving animals.

Toxicity test in Sprague-Dawley Rats

No dead animals were observed in all groups. Soft stools and soiled perineal region were observed at 2 hours after administration in one male in the 5000 mg/kg administration group, and no specific changes were observed for the remaining animals. As a result of checking the weight and autopsy results, no changes related to the test substance were observed (Figure 4).

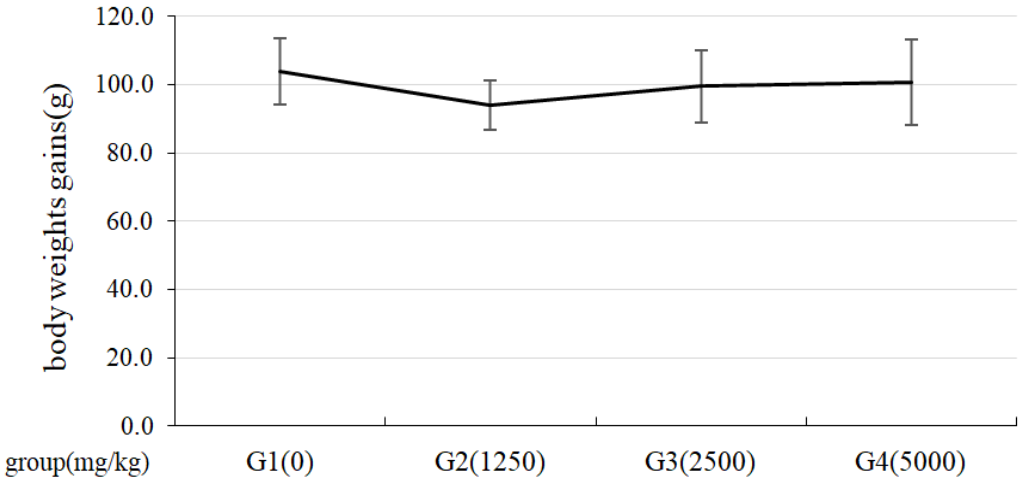

Figure 4. Changes in body weight of Sprague-Dawley Rats in the in vivo toxicity test. G1 is negative control and G2-G4 is experimental groups(experimental material administration for each amount).

As a result of counting PCEs with micronuclei in 2000 PCEs per individual within the dose range (less than 5000 mg/kg/day) applied in this test, there was no statistically significant increase in all groups administered with the test substance compared to the negative control group, and the positive test criteria were not satisfied. Therefore, it is judged that test substance 'compounds such as aloe extract' do not cause micronuclei in mouse bone marrow cells under this test condition. In addition, as a result of testing to determine the toxicity caused by single oral administration of test substance to Sprague-Dawley rat, no dead animals were observed, and there were no changes related to the test substance in body weight and autopsy findings. Soft stool and soiled perineal region in one animal that temporarily appeared at 2 hours after administration in the 5000 mg/kg administration group is a symptom that can be observed when the test substance is administered after fasting, so it is not considered to be affected by the test substance. Therefore, when 'compounds such as aloe extract' is administered orally to Sprague-Dawley rat once, the approximate lethal dose (ALD) under this test condition is judged to exceed 5000 mg/kg.

In addition, according to the results of the Chemeon commissioned test, a return mutation test using bacteria (group 1 E. coli, group 4 Salonella bacteria) and a chromosomal abnormality test using Chinese Hamster Lung (CHL) cells have been conducted using 'compounds such as aloe extract', a test substance used in this test (Chemon Study No.:14-VG-150P, 14-VG-151, 14-VG-152P, and 14-VG-153). As a result, the compounds did not cause a return mutation in the test strain used and did not cause a chromosomal abnormality in CHL cells. Therefore, finally, it is judged that 'compounds such as aloe extract' are not genetically toxic.

IV. Conclusion

Based on the above results, ALD (Approximate Lethal Dose) of a test substance 'compounds such as aloe extract' exceeds 5000 mg/kg and does not induce micronuclei in mouse bone marrow cells under the test conditions. Therefore, it is determined that a complex such as aloe extract may be evaluated as a safe natural product extract that does not cause chromosomal abnormalities.

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