Genotoxicity and 28-day repeated dose oral toxicity study of garlic essential oil in mice

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Abstract

Background and aim: Garlic essential oil (GEO) isolated from Garlic (Allium sativum L.) exerts biological activities in disease prevention, particularly in metabolic and liver diseases, and is used for a dietary therapy for centuries. However, due to the side effects associated with the excessive consumption of GEO, there is a need to evaluate the safety of the GEO.

Experimental procedure: Ames test using five Salmonella typhimurium strains (TA98, TA100, TA102, TA1535, and TA1537) and Chinese hamster ovary (CHO-K1) cells with or without metabolic activation (S9 system), and mammalian erythrocyte micronucleus test were used to assess the genotoxicity and clastogenic effects of GEO. A repeated dose of GEO (15, 25, and 50 mg/kg body weight, p.o.) were administrated to ICR mice for 28 days to ascertain the subacute toxicity of GEO.

Results and conclusions: The results of the Ames test with or without S9 system indicated that GEO did not induce mutagenicity nor have clastogenic effects in CHO-K1 cells with or without S9 activation. Furthermore, GEO did not affect the ratio of immature to total erythrocytes or the number of micronuclei in immature erythrocytes of ICR mice after 24 and 48 h. In a 28-day oral toxicity assessment, GEO (15, 25, and 50 mg/kg body weight, p.o.)-fed ICR mice exhibited normal behaviors, mortality, body weight, daily intake, hematology, clinical biochemistry, and organ weight. GEO shows no genotoxicity, and the no-observed-adverse-effect level (NOAEL) for GEO is considered to be greater than 50 mg/kg bw/day orally for 28 days in mice.

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compounds. Among OSCs, DAS, DADS, and DATS have been identified as the three major components of GEO. Previous studies have revealed that GEO exerts beneficial effects on both ethanol-induced fatty liver and high-fat diet-induced liver injury by activating the antioxidant and detoxification system. Moreover, the administration of GEO (0.25 mg/kg body weight (bw) per day) for 6 months has been shown to downregulate serum cholesterol and triglyceride levels in patients diagnosed with coronary heart disease and hyperlipidemia. Based on these findings, GEO has great potential for use as a dietary supplement or functional food to mitigate the symptoms of metabolic and liver diseases.

However, despite both A. sativum and GEO being listed as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA), several adverse effects, including diarrhea, gastrointestinal abnormalities, skin rash, nausea, and anemia, have been reported when consumed excessively. A. sativum and GEO being listed as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA), several adverse effects, including diarrhea, gastrointestinal abnormalities, skin rash, nausea, and anemia, have been reported when consumed excessively. Furthermore, hepatic injury was also observed in rats treated with fresh garlic extract added to drinking water (200 g/L) for 10 days. Raw garlic juice (0.5 mL) has also been reported to cause damage to the epithelial mucosal membrane after 2 h in rats; ulcers, shrinkage, and bleeding in the epithelial mucosa were also detected after 24 h of exposure. The median lethal dose (LD50) of aqueous extract of garlic and allyl compounds has been found to be 173.78 mL/kg bw and 204.17 mL/kg bw, respectively. Despite these findings, the toxic effects of A. sativum, GEO, and related products have rarely been reported in clinical studies, even at high dosages. Although several toxicological studies have concluded that garlic extract is safe and nontoxic, a comprehensive study focusing on the safety evaluation of GEO is lacking.

Since GEO has great potential for use as a functional food, and most of the functional foods are typically applied in the long term for health promotion or disease prevention, there is a need to evaluate the safety of GEO and define its no-observed-adverse-effect level (NOAEL) for potential precluding side effects. In the present study, we aimed to assess the genotoxicity and subacute toxicity of GEO using in vitro and in vivo genotoxicity assays and a 28-day subacute oral toxicity study in mice.

2. Materials and methods

2.1. Preparation of GEO

Garlic (A. sativum) was obtained from the Yunlin County Farmers' Association (Yunlin, Taiwan). GEO extraction was performed as described in our previous study. Briefly, garlic was stirred with 2 vol of distilled water and extracted by steam distillation for 4 h. Volatile extracts were then dehydrated to obtain GEO and stored at –20 °C until use. The yield of GEO from fresh garlic was 0.30 ± 0.05% (w/w), calculated according to the mean fresh weight of garlic used in each extraction procedure. The concentrations of three major components, namely DAS, DADS, and DATS in GEO were determined based on calibration curves constructed using the respective reference standards by a gas chromatograph (GC, Thermo Scientific Focus GC) equipped with an Al 3000 II autosampler, a flame ionization detector, and a Stabilwax (Crossbond Carbowax-PEG, Restek) capillary column (60 m × 0.32 mm, 1.0 μm thick film). The GC settings and analyses were performed as described in our previous work. GEO purity (97%), DADS (purity 80%) and DATS (purity 98%) were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Preparation of metabolic activation system (S9)

The procedure to obtain rat liver enzymes was performed as described previously, with some modifications. Briefly, five days before sacrifice, 3-methylcho-lanthrene was diluted in corn oil and i.p. injected into 8-weeks-old male Sprague-Dawley rats (20 mg/kg bw/day) for three consecutive days. Rats could consume

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**Abbreviations**

- ALB: albumin
- AST: aspartate aminotransferase
- ALT: alanine aminotransferase
- ALKP: alkaline phosphatase
- BASO: basophils
- BUN: blood urea nitrogen
- CHO: cholesterol
- CREA: creatinine
- Cl: chloride
- Ca: calcium
- DAS: diallyl sulfide
- DADS: diallyl disulfide
- DATS: diallyl trisulfide
- DMSO: dimethyl sulfoxide
- EO: eosinophils
- GC: gas chromatograph
- GEO: garlic essential oil
- GLU: glucose
- γ-GT: gamma-glutamyl transferase
- H&E: hematoxylin and eosin
- HGB: hemoglobin
- HCT: hematocrit
- IC50: half maximal inhibitory concentration
- IE: immature erythrocytes
- K: potassium
- LD50: median lethal dose
- LYMPH: lymphocytes
- MCV: mean corpuscular volume
- MCH: mean corpuscular hemoglobin
- MCHC: mean corpuscular hemoglobin concentration
- MNIE: micronucleated immature erythrocytes
- MONO: monocytes
- NEUT: neutrophils
- NOAEL: no-observed-adverse-effect level
- Na: sodium
- OECD: Organization for Economic Cooperation and Development
- P: phosphorus
- PLT: platelets
- RBC: red blood cells
- RDA: recommended dietary allowance
- SAC: S-allylcysteine
- SMC: S-methylcysteine
- SD: standard deviation
- SAC: S-allylcysteine
- TG: triglyceride
- TP: total protein
- T-BIL: total bilirubin
- WBC: white blood cell

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food and water ad libitum until 12 h before euthanasia, when food, but not water, was removed. On the fifth day of induction, the rats were euthanized with CO2 and immediately decapitated. The livers were isolated, washed with 0.15 M KCl, minced with sterile scissors, and homogenized using a Teflon pestle. Homogenates were centrifuged for 10 min at 9000 × g. The supernatant (S9 fraction) was collected and stored at −80 °C. An S9 mix containing the S9 fraction and cofactors (glucose-6-phosphate, β-nicotinamide adenine dinucleotide phosphate, MgCl2, KCl, and sodium phosphate buffer) was prepared immediately before performing the metabolic activation test.

2.3. Animal housing and dose design

Male and female ICR mice (five-week-old) were purchased from the Animal Center of the College of Medicine, National Taiwan University (Taipei, Taiwan). All mice were housed under controlled temperature (23 ± 2 °C), humidity (50–70% RH), and a 12 h/12 h light/dark cycle in the animal facility. Food and water were provided ad libitum. After one week of acclimatization, the mice were used for the in vivo mammalian erythrocyte micronucleus test and a 28-day oral toxicity study. In both studies, the dosages of GEO followed the required health food regulations from the Taiwan Food and Drug Administration (TFDA) based on the concept of margin of safety (MOS). According to a previous report, the recommended dietary allowance (RDA) in humans of GEO is 0.25 mg/kg bw/day. To determine the no-observed-adverse-effect level (NOAEL) of GEO, the 60-, 100-, and 200-fold exposures to human RDA were tested, corresponding to 15, 25, and 50 mg/kg bw, respectively, in mice. All animal procedures and dose design were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Taiwan University (IACUC approval no. NTU-101-EL-124).

2.4. Genotoxicity

The bacterial reverse mutation assay, in vitro mammalian chromosome aberration test, and in vivo micronucleus test were performed to evaluate the genotoxicity of GEO. These experiments followed the guidelines of the Organization for Economic Cooperation and Development (OECD) and the TFDA.

2.4.1. Bacterial reverse mutation assay (Ames test)

To identify substances that cause point mutations involving substitution, addition, or deletion of DNA base pair(s) in aminoacid-requireing strains of Salmonella typhimurium, a bacterial reverse mutation test was used. The histidine auxotroph mutant strains of S. Typhimurium, namely TA98, TA100, TA102, 1535 and TA1537, were obtained from the Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). To determine the mutagenicity of GEO, the plate incorporation method with the presence or absence of a metabolic activation system (S9) was performed as described previously and in accordance with the test guidelines of the OECD 471. The testing concentrations of GEO were 0.05, 0.1, 0.125, 0.25, 0.5, and 1 mg/plate, which followed the TFDA guidelines for the safety assessment of health food. Briefly, 0.1 mL of each test S. Typhimurium culture (10⁵ cells/mL), 2 mL of soft agar (0.75% agar and 0.5% NaCl, 48 ± 2 °C), 0.2 mL of 0.5 mM histidine/biotin, 0.5 mL of S9 mix (as appropriate), and the test substance were mixed in a tube and immediately poured into a minimal agar plate (1.5% agar, 2% glucose, and Vogel-Bonner medium E). Compounds with known mutagenic activity are used for positive control for each tester strain: 4-nitro-o-phenylenediamine (0.5 μg/plate) for TA100; 9-aminocaridine (0.5 μg/plate) for TA1537. For metabolic activation system (S9), Benzo[a]pyrene (1.0 μg/plate) is used for TA98 and TA102; 2-aminoanthracene (4 μg/plate) is used for TA100, TA1535 and TA1537. The Dimethyl sulfoxide (DMSO) is used for negative control. After the agar solidified, the plates were incubated for 48–72 h at 37 °C in the dark, and revertant colonies were counted using a microscope.

2.4.2. In vitro mammalian chromosomal aberrations test

To identify substances that cause structural chromosomal aberrations in cultured mammalian cells, we conducted an in vitro mammalian chromosomal aberration test. Chinese hamster ovary cells (CHO–K1) were obtained from the Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). Chromosomal abnormalities induced by GEO in the presence or absence of S9 were determined according to the OECD 473 guidelines. The concentrations of GEO to be tested adhered the TFDA guidelines for the safety assessment of health food. Since the half maximal inhibitory concentration (IC₅₀) of GEO in CHO–K1 cells was 15 μg/mL (unpublished results), the concentrations were set at 1, 1.5, 2.5, 5, and 10 μg/mL. Briefly, CHO–K1 cells (4 × 10⁵ cells/mL) were cultured in 6-cm dishes with complete medium of Ham F-12. The complete medium also served as a diluent for GEO and the negative control. Two of the known mutagens, mitomycin C (0.07 μg/mL) and cyclophosphamide (10 μg/mL), were used as positive controls. GEO (or appropriate controls) was introduced to the cells under the following conditions. For short-term treatment, GEO (or control) was incubated with or without S9 mix for 3 h and then changed to complete medium for a further 17 h. For continuous treatment, GEO (or control) was incubated without S9 mix for 20 h. After the aforementioned treatments, cells were treated with colchicine (0.2 μg/mL) for 2 h to induce metaphase arrest. Cells were harvested, spread onto glass slides, and stained with Giemsa solution (5%). Chromosome aberrations were observed under 1000 × magnification in 100 well-spread metaphases per concentration, with controls equally divided among the duplicates. The percentage of structural chromosomal abnormalities, including chromatid and chromosome types, was determined. Aberrations include gaps, breaks, dicentrics, rings, multiple aberrations, and acentric fragments.

2.4.3. In vivo mammalian erythrocyte micronucleus test

The in vivo mammalian micronucleus test was used to screen for genotoxic substances by determining micronuclei formation, that is, lagging chromosome fragments or whole chromosomes. To ascertain the genotoxicity of GEO, we performed a micronucleus test using mice in accordance with the OECD 474 guidelines. Briefly, 50 male ICR mice (six weeks old) were divided into five groups (n = 10): negative control (olive oil, 0.1 mL by oral administration), positive control (mitomycin C, 2 mg/kg bw by i.p. injection), and GEO (single oral dose at 15, 25, or 50 mg/kg bw) groups. GEO was mixed and diluted with 0.1 mL olive oil before oral administration to the mice. At 24 h and 48 h post-treatment, blood samples (4 μL) were collected from the orbital sinus, smeared onto slides, and stained with acridine orange (1 mg/mL). Micronucleated reticulocytes were observed using fluorescence microscopy. The number of micronucleated immature erythrocytes (MNIE, based on 2000 erythrocytes/animal) and immature erythrocytes (IE, based on 1000 erythrocytes/animal) were recorded.

2.5. 28-day oral toxicity study

To assess the subacute toxicity of GEO in animals, a repeated dose 28-day oral toxicity study was conducted in mice in accordance with OECD 407 guidelines. Briefly, male and female ICR
mice (six weeks old) were randomly divided into four groups (n = 10/sex/group). The treatment groups were orally administered a low-, medium-, or high-dose GEO (15, 25, and 50 mg/kg bw/day, respectively) for 28 consecutive days. GEO was mixed and diluted with 0.1 mL of olive oil before administration. Mice in the negative control group were orally administered 0.1 mL of olive oil. The mice were observed once a day during the experimental period for their general appearance, such as behavioral changes, daily activities, rough hair coat, porphyrin discharges in eyes and nose. Food and water intake and body weight were recorded twice per week. At the end of the experiment (day 29), the mice underwent blood collection and laparotomy under anesthesia. Then, the major organs, such as the brain, heart, liver, spleen, lung, kidney, adrenal, thymus, testis (male), epididymis (male), ovary (female), and uterus (female), were isolated, weighed, and recorded. The relative organ weight was determined based on the terminal body weight.

2.5.1. Haematological and serum biochemical analysis

Blood samples were collected from the abdominal aorta and transferred to ethylenediaminetetraacetic acid tubes for haematological analysis. Hematological parameters, including red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), white blood cell (WBC), neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), and basophils (BASO), were measured. Blood samples were centrifuged at 3000 g for 30 min to obtain serum for biochemical analysis. Biochemical parameters, including cholesterol (CHO), triglyceride (TG), urea nitrogen (BUN), creatinine (CREA), total protein (TP), total bilirubin (T-BIL), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), glucose (GLU), γ-glutamyltransferase (γ-GT), calcium (Ca), phosphorus (P), sodium (Na), potassium (K), and chloride (Cl), were analyzed.

2.5.2. Histopathological analysis

Organs were fixed in 10% neutral buffered formalin for one week, embedded in paraffin, and sliced into 5-μm sections for histopathological examination. The sectioned samples were stained with hematoxylin and eosin (H&E). A blinded histological assessment of the sectioned samples under a microscope with a digital camera was performed by a veterinary pathologist at the Graduate Institute of Veterinary Pathobiology of the National Chung Hsing University, Taiwan. The histopathological images were enlarged 400 times and the scale bars were included by ImageJ.

2.6. Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical comparisons among experimental groups were analyzed by one-way analysis of variance and Tukey’s multiple comparison test (genotoxic and clastogenic evaluations) or Dunnett’s post-hoc test (28-day subacute toxicity study) using the SPSS software program. Differences were considered significant at P < 0.05.

3. Results and discussion

3.1. Chemical composition of GEO

The three major components of GEO, DAS, DADS, and DATS were analyzed with GC. As shown in the GEO chromatogram (Supplementary Fig. 1), the concentrations of DAS, DADS, and DATS were 6.7%, 40.7%, and 21.6%, respectively, which accounted for 69.0% of the total oil. The chemical composition of GEO was similar to our previous work. Also, the result of GEO profile is in consistent with previous studies, demonstrating that DADS is the major organosulfur compound in GEO.

3.2. GEO did not cause genotoxicity or clastogenicity in vitro or in vivo

To evaluate the mutagenesis of GEO, we conducted a bacterial reverse mutation test using five S. typhimurium strains (TA98, TA100, TA102, TA1535, and TA1537) in the presence or absence of a metabolically activated condition (S9 system). According to the initial antibacterial test, we found significant toxicity to all strains when the concentration was set at 1 mg/plate (unpublished data). Hence, we tested GEO at concentrations of 0.05, 0.1, 0.125, 0.25, and 0.5 mg/plate. This finding is consistent with previous studies, which also demonstrate that garlic and its pure compound, allicin act as bactericides. As shown in Table 1, we found that GEO at all concentrations did not significantly increase the number of revertant colonies for any strain employed either in the presence or absence of metabolic activation (S9) as compared to the negative control group. However, the average number of revertants in all five strains showed a significant increase in the positive control group.

The average number of the revertants in the positive group were 3- to 4-fold greater than those that found in the negative control group, which could be indicated as genotoxicity. This indicates that all the data obtained from this test are valid. Also, GEO at all concentrations decrease the number of revertant colonies for any strain employed either in the presence or absence of metabolic activation (S9) as compared to the positive control group. Collectively, these results indicated that GEO did not cause a reverse mutation in S. typhimurium.

We further investigated the clastogenic effect of GEO using a chromosomal aberration test in mammalian CHO–K1 cells in the presence or absence of S9. According to our initial toxicity assessment, the IC50 of GEO in CHO–K1 cells was 15 μg/mL (unpublished data), and we then tested GEO at concentrations of 1, 1.5, 2.5, and 10 μg/mL in CHO–K1 cells. As shown in Table 2, chromosomal aberrations were observed in approximately 4% of the cells in the negative control group. We found the frequencies of chromosomal aberrations in our negative control group and GEO treatments were in consistence to a previous report, which were around 2–5% and were considered as no clastogenic effect. Further, incidences of aberrant cells in the negative control group are normally similar between treatment regimens. GEO at concentrations of 1, 1.5, 2.5, and 10 μg/mL did not induce any obvious aberrations in chromosomes in any of the treatment groups (without S9 for 3 h or 20 h; with S9 for 3 h) when compared to the negative control group. In our case, the overall percentage of aberrations in GEO was 3–5%, which was similar to that in the negative control group. Conversely, approximately 15% of cells in the positive control group showed noticeable aberrations in chromosomes and chromatids, including gaps, breaks, dicentrics, rings, multiple aberrations, andacentric fragments. This increase in the percentage of chromosomal aberrations in the positive group was markedly higher than that in the negative control group, confirming the clastogenicity of compounds used in this test. In summary, these results suggested that GEO did not induce chromosomal aberrations in cultured mammalian CHO–K1 cells.

In 1999, Spielmann et al. found that IC50 in vitro cytotoxicity was able to predict the LD50 in vivo oral toxicity study, and suggested to use the predicted LD50 as the starting dose. A recent toxicological study further claimed that in vitro genotoxicity test may also exhibited a similar impact and applicability. We calculated the predicted LD50 of GEO via the IC50 (15 μg/mL) obtained from CHO–K1 cells, and the result was 13.7 mg/kg bw. In addition to the theoretical calculation, the RDA in humans of GEO is 0.25 mg/
Table 1
Mutagenicity assay of GEO on Salmonella typhimurium TA98, TA100, TA102, TA1535, and TA1537 in the presence or absence of metabolic activation.

| Treatment | Revertant colonies |
|-----------|--------------------|
|           | TA98   | TA100  | TA102  | TA1535 | TA1537 |
| Without S9 metabolic activation |
| Negative control† | 51 ± 5.3 | 216 ± 5.0 | 299 ± 7.2 | 29 ± 2.6 | 13 ± 2.6 |
| Positive control‡  | 132 ± 5.6** | 606 ± 7.0** | 761 ± 7.2** | 133 ± 5.1** | 53 ± 4.5** |
| GEO (mg/plate)         |
| 0.05     | 56 ± 5.3** | 219 ± 3.5** | 294 ± 6.7** | 28 ± 1.9** | 12 ± 2.5## |
| 0.1      | 47 ± 2.5** | 211 ± 8.5** | 310 ± 5.0## | 30 ± 1.9## | 13 ± 1.5## |
| 0.125    | 55 ± 4.0## | 220 ± 5.5## | 280 ± 12.1## | 29 ± 3.2## | 12 ± 1.2## |
| 0.25     | 51 ± 7.2## | 206 ± 7.6## | 290 ± 2.5## | 28 ± 2.1## | 11 ± 3.1## |
| 0.5      | 58 ± 4.5## | 205 ± 5.1## | 301 ± 4.7## | 30 ± 1.0## | 12 ± 1.7## |
| With S9 metabolic activation |
| Negative control†  | 57 ± 2.5 | 224 ± 8.7 | 293 ± 12.2 | 30 ± 1.0 | 10 ± 2.1 |
| Positive control‡  | 165 ± 3.2** | 715 ± 4.5** | 935 ± 4.7** | 105 ± 5.1** | 45 ± 4.5** |
| GEO (mg/plate)         |
| 0.05     | 52 ± 3.0## | 209 ± 13.4## | 292 ± 4.5## | 29 ± 1.5## | 7 ± 0.6## |
| 0.1      | 51 ± 5.5## | 226 ± 6.1## | 306 ± 5.0## | 32 ± 2.1## | 9 ± 0.6## |
| 0.125    | 54 ± 4.6## | 229 ± 5.0## | 294 ± 4.0## | 33 ± 2.1## | 11 ± 1.2## |
| 0.25     | 52 ± 2.3 | 213 ± 10.1## | 293 ± 5.7## | 31 ± 2.1## | 9 ± 0.6## |
| 0.5      | 49 ± 5.3## | 218 ± 11.0## | 302 ± 3.1## | 31 ± 2.3## | 11 ± 2.1## |

Data are presented as the mean ± SD (n = 3 plates/group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc multiple comparison test. ** P < 0.01 denotes significant differences compared to negative control group. ## P < 0.01 denotes significant differences compared to positive control group.

† Dimethyl sulfoxide (DMSO).
‡ Cyclophosphamide (10 µg/mL).
§ G, chromosome gap; B, chromosome break; D, dicentric chromosome; R, ring; g, chromatid gap; b, chromatid break; MA, multiple aberration; AF, acentric fragment.

Table 2
Chromosomal aberrations test of GEO-treated Chinese hamster ovary (CHO–K1) cells.

| Treatment | No. of chromosome aberrations in 100 cells† |
|-----------|-----------------------------------------------|
|            | G     | B     | D     | R     | g     | b     | MA    | AF    |
| 3 h without S9 metabolic activation |
| Negative control† | 1 0 0 0 2 2 0 0 5 |
| Positive control‡ | 1 4 0 0 3 4 2 1 15 |
| GEO (µg/mL) | 1   | 0 0 0 0 2 1 0 0 3 |
| 1.5    | 1 0 0 0 2 2 0 0 4 |
| 2.5   | 1 1 0 0 1 1 1 0 5 |
| 5     | 0 1 0 0 2 1 0 0 4 |
| 10    | 1 1 0 0 2 1 0 0 5 |
| 20 h without S9 metabolic activation |
| Negative control† | 0 1 0 0 2 1 0 0 4 |
| Positive control‡ | 1 5 0 1 5 3 1 0 17 |
| GEO (µg/mL) | 1   | 1 0 0 0 1 0 1 0 3 |
| 1.5    | 1 0 0 0 1 2 0 0 4 |
| 2.5   | 0 1 0 0 2 1 1 0 5 |
| 5     | 0 0 0 0 2 0 1 0 3 |
| 10    | 0 0 1 0 1 0 2 0 4 |
| 3 h with S9 metabolic activation |
| Negative control† | 0 0 0 0 2 2 0 0 4 |
| Positive control‡ | 1 4 0 1 2 2 3 1 14 |
| GEO (µg/mL) | 1   | 1 1 0 0 1 2 0 0 5 |
| 1.5    | 1 1 0 0 1 1 1 0 5 |
| 2.5   | 0 0 1 1 2 0 0 4 |
| 5     | 1 0 0 0 1 2 0 0 4 |
| 10    | 0 0 0 0 1 2 0 0 3 |

† Dimethyl sulfoxide (DMSO).
‡ Mitomycin C (0.07 µg/mL).
§ G, chromosome gap; B, chromosome break; D, dicentric chromosome; R, ring; g, chromatid gap; b, chromatid break; MA, multiple aberration; AF, acentric fragment.

kg bw/day based on a previous report.10 In order to determine the NOAEL of GEO in animals, we set 60-, 100-, and 200-fold exposures to human RDA, which correspond to 15, 25, and 50 mg/kg bw, respectively, in mice. Interestingly, these two different methods both support that 15 mg/kg bw can be applied to the in vivo oral toxicity study as a starting dose. We therefore used 15, 25, and 50 mg/kg bw GEO to conduct safety evaluation in the following in vivo toxicological studies.

We evaluated the potential toxicological effects of GEO on peripheral blood micronuclei using an in vivo mouse model. As shown in Table 3, mice administered a single dose of GEO at 15, 25, or 50 mg/kg bw exhibited no significant differences in the amount of MNIE and IE compared to the negative control group at either 24 or 48 h. A significant difference in the amount of MNIE and IE was observed in the positive control group (mitomycin C) at either 24 or 48 h post-treatment (P < 0.01). This is consistent with a previous study, which revealed a higher incidence of micronuclei in peripheral blood erythrocytes 48 h after mitomycin C treatment in rodents.40 In addition, the amount of immature erythrocytes in the total erythrocyte population is regarded as an indicator of cytotoxicity or erythropoiesis in evaluating a given substance.41 We found a significant increase in MNIE in the positive groups at both 24 and 48 h, demonstrating the validity of mitomycin C. In contrast, the proportion of immature erythrocytes and the incidence of micronuclei in the peripheral blood cells of mice were not changed by GEO administration as compared to the negative control group. Further, significant differences in the amount of MNIE and IE in all GEO treatments were observed when compared to the positive control group. These results indicated that GEO did not affect micronuclei formation or erythropoiesis in an in vivo mammalian erythrocyte micronucleus test. Overall, our results suggested that GEO did not cause genotoxicity or clastogenicity, which are consistent to previous reports.42,43

3.3. GEO did not induce toxic effects in a 28-day oral toxicity study

Next, we conducted an oral toxicity study to assess the potential
### Table 3
Micronucleus assay for the effect of GEO on peripheral blood cells of mice.

| Treatment               | 24 h         | 48 h         |
|------------------------|--------------|--------------|
|                        | MNIE         | IE           | MNIE         | IE           |
| Negative control       | 4.30 ± 0.95  | 25.28 ± 5.67 | 6.20 ± 1.14  | 20.88 ± 5.49 |
| Positive controlb      | 15.10 ± 1.29**| 14.2 ± 4.70**| 25.60 ± 2.37**| 11.32 ± 3.15**|
| 15 mg/kg               | 5.20 ± 1.48**| 26.9 ± 6.02**| 7.20 ± 1.14**| 19.5 ± 6.30**  |
| 25 mg/kg               | 5.10 ± 1.52**| 26.4 ± 8.67**| 6.70 ± 1.34**| 18.36 ± 5.85** |
| 50 mg/kg               | 5.40 ± 1.43**| 24.58 ± 8.99**| 6.90 ± 0.99**| 19.68 ± 5.99** |

Data are presented as the mean ± SD (n = 10 mice/group). Statistical analysis was performed using one-way ANOVA followed by Tukey’s post-hoc multiple comparison test. **, \( P < 0.01 \) denotes significant differences compared to negative control group. *, \( P < 0.05 \), **, \( P < 0.01 \) denote significant differences compared to positive control group.

a Olive oil.
b Mitomycin C (2 mg/kg bw, i.p.).
c Number of micronucleated immature erythrocytes (MNIE, based on 2000 erythrocytes/animal).
d Number of immature erythrocytes (IE, based on 1000 erythrocytes/animal).

Fig. 1. Effects of GEO on body weight, water intake and food intake of ICR mice treated in a 28-day subacute toxicity study. Body weight, daily water intake (g/day) and daily food intake (g/day) at the end day of each week in male (A, C, E) and female (B, D, F) mice orally administered sterile distilled water (control) or GEO (15, 25, and 50 mg/kg bw) daily for 28 days was presented. Data are presented as the mean ± SD (n = 10 mice/group), followed by one-way analysis of variance (ANOVA) and Dunnett’s post-hoc test. *, \( P < 0.05 \) denotes significant differences compared to control group.
toxic effects in a subacute manner (28-day). No ophthalmological abnormalities or deaths were observed during the course of the study. Changes in body weight were not significantly different between all treatment groups and controls in either sex or time, except for day 14 (Fig. 1A and B). We noticed a significant increase in body weight of female mice in the high dose (50 mg/kg bw) of GEO group as compared to those in the control group on day 14 (Fig. 1B); however, this higher body weight was not observed after 14 days. A significant increase in water intake was observed in male mice belonging to the medium dose (25 mg/kg) of the GEO group 14 days. A significant increase in water intake was observed in male mice belonging to the medium dose (25 mg/kg) of the GEO group on day 7 (Fig. 1C). Similarly, this increase was transient and appeared not to be related to GEO treatment since it no longer existed after day 7 (Fig. 1C and D). There was no obvious change in food intake in either male or female mice during the course of the study (Fig. 1E and F). Collectively, the overall results suggested that oral GEO treatment for 28 days did not cause significant changes in body weight or daily intake in mice.

At the end of the experiment, all animals were euthanized and subjected to hematology, serum biochemistry, and histopathology. As shown in Supplementary Table 1, treatment with GEO at any dose for 28 days did not alter hematological parameters. The serum biochemistry of GEO in mice is summarized in Supplementary Table 2. We found the significant increases in the blood glucose levels in male mice treated with medium and high doses (25 and 50 mg/kg bw) of GEO compared to those in the control group ($P < 0.05$). The changes by medium and high doses of GEO were +15.8% and +14.7%, respectively. Hyperglycemia induces several physiological and pathological alterations, such as elevation in oxidative stress, formation of advanced glycation end-products, damages on pancreatic beta cells, and insulin resistance, which all exacerbate toxic effects on cells. Therefore, the blood glucose is often monitored in toxicological studies. The baseline levels and the fluctuation of blood glucose in mice were similar to those in a previous report. The increase in blood glucose in GEO-treated mice may be attributed to the nature of glucose sensitivity in the ICR strain, which was observed in a previous study. Since the garlic product has been reported to have beneficial effects on blood glucose, GEO and its active components are less likely to cause hyperglycemia. The higher blood glucose levels found in GEO-treated mice may also come from the strong odor of the GEO. The odor either activates the alarm system to release corticosterone which antagonizes the insulin action, or triggers the endocrine system to increase the circulating glucose by different metabolism between animals and humans. Nonetheless, no changes in serum biochemistry were observed in a dose-response manner. Hence, we concluded that there is insufficient evidence of toxicity in hematology or serum biochemistry found after oral treatment with GEO for 28 days.

3.4. GEO did not induce macropathological or histopathological lesions

The relative organ weights isolated from the animals are shown in Supplementary Table 3. No significant differences in any relative organ weights were found in either male or female mice treated with GEO for 28 days. Representative histological images of vital organs of mice in the control and high dose (50 mg/kg bw) of GEO are shown in Fig. 2. No obvious histopathological findings were detected in the brain, heart, liver, spleen, lung, kidney, stomach, or intestine in either male or female mice after a 28-day consecutive treatment with a high dose (50 mg/kg bw) of GEO. These assessments concluded that there were no substance-induced pathological lesions in mice treated with GEO for 28 days.

4. Conclusion

In the present study, we demonstrated for the first time that GEO is non-genotoxic, based on findings from a bacterial reverse mutation test, chromosome aberration test, and micronucleus test. No obvious oral toxicity was found in mice treated with 15, 25, and 50 mg/kg bw/day GEO for 28 days. Our findings suggest that GEO is safe at a dose of 50 mg/kg bw/day in mice, corresponding to a 200-fold exposure of the human equivalent dose. We also concluded that the NOAEL of GEO can be considered to be greater than 50 mg/kg bw/day for both male and female mice. These findings support
the prospective applications of GEO for the development functional foods or dietary supplements in human subjects in future clinical research.

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**Author contributions**

**Yu-En Lin**: Writing-original draft, Writing-review & editing, Data curation, Validation, Visualization. **Meng-Hsuan Lin**: Formal analysis, Investigation. **Ti-Yen Yeh**: Methodology, Formal analysis, Investigation. **Yi-Syuan Huang**: Writing-review & editing. **Fu-Chuo Peng**: Methodology, Supervision, Writing-review & editing. **Shing-Hwa Liu**: Supervision, Writing-review & editing. **Lee-Yan Sheen**: Supervision, Funding acquisition.

**Declaration of competing interest**

The authors declare that they have no competing interests.

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**Appendix A: Supplementary data**

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