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Genotoxicity and 28-day repeated dose oral toxicity study of ovatodiolide in rats

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ABSTRACT

Ovatodiolide is a bioactive cembrane-type diterpenoid isolated from *Anisomeles indica* (L.) Kuntze. It has been proven that ovatodiolide is anti-inflammatory, anti-tumorigenic, anti-melanogenic and attenuates asthma by regulating signaling pathways. The aim of this study was to evaluate the safety of ovatodiolide by conducting genotoxicity tests and 28-day oral toxicity tests in rats. Genotoxicity assays were conducted by using a bacterial reverse mutation test and mammalian chromosomal aberration test to assess whether ovatodiolide causes reverse mutations and mutagenicity with or without metabolism activation. For the *in vivo* mammalian erythrocyte micronucleus test, mice were administered a single dose of 0, 250, 500 or 1000 mg/kg b.w. ovatodiolide by single gavage. In the acute oral toxicity test, rats were given a single dose of ovatodiolide 1000 mg/kg b.w. by single gavage. In the 28-day oral toxicity test, groups were divided into a control, ovatodiolide 10, 25 and 50 mg/kg b.w. The results showed that there was no mutagenicity in the bacterial reverse mutation test or the mammalian chromosomal aberration test with or without S9 fraction. Ovatodiolide did not produce an increase in micronucleated reticulocytes in the micronucleus test. The results revealed that the acute oral toxicity of ovatodiolide is over 1000 mg/kg b.w. in rats. Moreover, 10, 25 and 50 mg/kg b.w. of ovatodiolide did not cause a significant effect in rats. According to the results of the genotoxicity and oral toxicity studies in rats, ovatodiolide did not produce any adverse effects, and the tested doses can serve as clinical references.

1. Introduction

Modern societies are not only becoming increasingly aged, but they are also producing lots of modern diseases. According to the Ministry of Health and Welfare statistics in Taiwan in 2019, the top three major causes of the death were malignant tumors, heart disease and pneumonia. The top ten mortality rates for cancer were (1) tracheal, bronchial and lung cancer, (2) liver cancer and intrahepatic cholangiocarcinoma, (3) colon cancer, rectal cancer and anal cancer, (4) breast cancer in females, (5) oral cancer, (6) prostate cancer, (7) stomach cancer, (8) pancreatic cancer, (9) esophageal cancer and (10) cervical and uterine cancer.

Nowadays, the use of herbs to purify compounds for medicinal use has become increasingly important. One such herb is *Anisomeles indica* (L.) Kuntze. *Anisomeles indica* (L.) Kuntze is found throughout the southern and tropical regions of Asia. It has been commonly used in herbal medicines for various illnesses such as gastrointestinal disorders, liver disease and inflammatory skin disease [1]. However, there is little reported research discussing the safety of *Anisomeles indica* (L.) Kuntze. In the present study, the safety assessment utilized a flavonoid fraction of *Anisomeles indica* (L.) Kuntze leaf. The data showed that the acute oral toxicity of the flavonoid fraction was over 5000 mg/kg b.w. in mice. The flavonoid fraction did not produce symptoms of toxicity in the subacute toxicity study at a dose of 1000 mg/kg b.w. in mice. Furthermore, in the chromosome aberration test, the flavonoid fraction was not mutagenic or clastogenic at a dose of 1000 μg/mL. Flavonoid fractions can strongly suppress KB human oral cancer cell lines [2]. Ovatodiolide is a bioactive cembrane-type diterpenoid isolated from *Anisomeles indica* (L.) Kuntze.

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It has been proven in recent studies that ovatodiolide is anti-inflammatory [3], anti-melanogenic [1] and can attenuate the discomfort of asthma [4]. Furthermore, it has the ability of being anti-tumorigenic in cases of colon cancer and oral carcinoma, which has been proven in mice models [5,6]. In the previous study, intraperitoneally administrate 5 mg/kg ovatodiolide five times a week for seven weeks in CT26 syngeneic mouse model showed significantly suppressed tumorigenesis [5]. In *in vitro* studies, ovatodiolide can inhibit the growth of tumor cells in lung cancer [7], breast cancer [8], pancreatic cancer [9] and cervical cancer [10] through regulating signaling pathways show the anti-cancer effects of ovatodiolide.

Ovatodiolide has the potential for being a new drug candidate for use in humans. To date, ovatodiolide has been thoroughly studied, but the toxicity of ovatodiolide has not been evaluated. Therefore, the present study conducted a bacterial reverse mutation test, mammalian chromosomal aberration test, mammalian erythrocyte micronucleus test and oral toxicity study to evaluate the safety of ovatodiolide for clinical use in the future.

2. Materials and methods

2.1. Test article

Ovatodiolide, which appeared as a light-yellow crystal in these studies, was supplied by professor Yew-Min Tzeng of National Taitung University. The chemical structure and ORTEP diagram of ovatodiolide is shown in Fig. 1 and Table 1. It was stored at 25℃. The purity of ovatodiolide was 99.95 % and it was dissolved in dimethyl sulfoxide (DMSO) for the bacterial reverse mutation test, mammalian chromosomal aberration test, erythrocyte micronucleus test and oral toxicity tests (acute oral toxicity and 28-day oral toxicity in rats).

2.2. Bacterial reverse mutation test

2.2.1. Tester strains

Two of the tester strains used were *Salmonella typhimurium* TA98 and TA100, which were obtained from Bioresources Collection and Research Center, BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan. The other tester strains, S. *typhimurium* TA102, TA1535 and TA1537, were obtained from Discovery Partners International (DPI, California, USA).

2.2.2. Bacterial toxicity test

Five tester strains at concentrations of 1.25, 2.5 or 5 mg ovatodiolide/plate were co-cultivated on nutrient agar plates for 24 h. The results showed that the maximum non-toxic concentration of ovatodiolide was 2.5 mg/plate in S. *typhimurium* TA98, TA100, TA1535 and TA1537. As for S. *typhimurium* TA102, the maximum non-toxic concentration of ovatodiolide was 5 mg/plate (data not shown).

Table 1
Crystal data and structure refinement.

Empirical formula	$C_{20}\ H_{24}\ O_4$
Formula weight	328.39
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a = 10.7714(3) Å α = 90 °. b = 12.8674(3) Å β = 90 °. c = 13.0829(3) Å γ = 90 °.
Volume	1813.29(8) $Å^3$
Z	4
Density (calculated)	1.203 Mg/m^3
Absorption coefficient	0.668 mm^{-1}
F(000)	704
Crystal size	0.270 × 0.207 × 0.188 mm^3
Theta range for data collection	4.820 to 74.968°
Index ranges	−13 < = h< = 13, -15 < = k< = 16, -16 < = l< = 14
Reflections collected	11084
Independent reflections	3712 [R(int) = 0.0211]
Completeness to theta = 67.679°	99.2 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7539 and 0.6477
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	3712 / 0 / 219
Goodness-of-fit on F2	1.056
Final R indices [I>2sigma(I)]	R1 = 0.0343, wR2 = 0.0936
R indices (all data)	R1 = 0.0347, wR2 = 0.0942
Absolute structure parameter	−0.01(3)
Extinction coefficient	n/a
Largest diff. peak and hole	0.255 and -0.232 e. $Å^{-3}$

2.2.3. Ames test

For the Ames test, the concentrations of ovatodiolide were 0.16, 0.31, 0.63, 1.25 and 2.5 mg/plate in S. *typhimurium* TA98, TA100, TA1535 and TA1537. For S. *typhimurium* TA102, the concentrations of ovatodiolide were 0.31, 0.63, 1.25, 2.5 and 5 mg/plate. The five concentrations listed above were used with or without S9 fraction. In sterilized test tubes with 2 mL soft agar, 100 μL of the ovatodiolide solution were added, along with the vehicle control (DMSO), or positive control, 200 μL Histidine/Biotin mixture, 100 μL of tester strain solution and 200 μL of S9 fraction (for cultures with metabolic activation). The solution was stirred and poured onto minimal glucose agar plates. When the soft agar solidified, the minimal glucose agar plates were placed upside down and incubated at 37℃ for 48 h. All colonies in each plate were counted. The test article was considered to be mutagenic if the number of bacteria in the test group was more than twice that of the control group,

2.3. Mammalian chromosomal aberration test

Chinese hamster ovary cells (CHO-K1 cells) [11] were obtained from

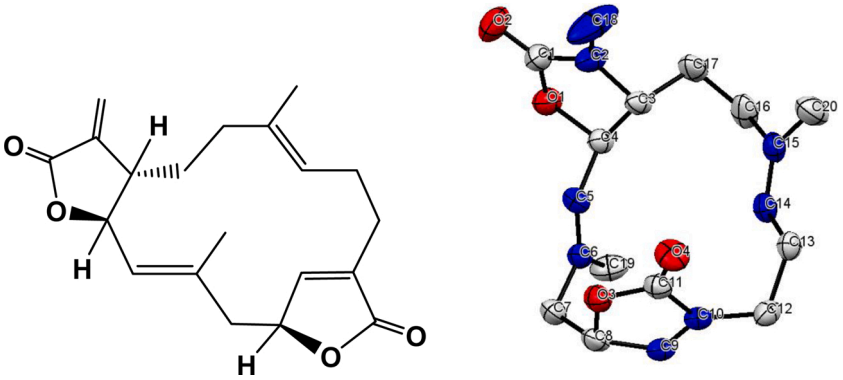

Fig. 1. The chemical structure and ORTEP diagram of ovatodiolide.

Bioresources Collection and Research Center, BCRC 60006, Food Industry Research and Development Institute, Hsinchu, Taiwan. CHO-K1 cells were cultured in Ham F-12 medium, 10 % Fetal bovine serum and 100 units/mL Penicillin-Streptomycin at 5% CO_2 and 37 °C in an incubator. To determine the half maximal inhibitory concentration (IC_{50}), MTT assays were conducted with or without metabolic activation. The results showed the values of IC_{50} were 77 μM and 20.8 μM with or without metabolic activation (data not shown). For the chromosomal aberration study, the CHO-K1 cells were seeded at a density of 2×10^5 cells/mL. Once the CHO-K1 cells attached, the cells were treated with a positive control (cyclophosphamide, 25 μg/mL) and different concentrations of ovatodiolide (55, 65 and 75 μM) in Ham F-12 medium for 3 h with metabolic activation. Then, the Ham F-12 medium was replaced and incubated for 18 h. CHO-K1 cells were also treated with a positive control (mitomycin C (2.5 μg/mL) and three different concentrations of ovatodiolide (12.5, 15.0 and 17.5 μM) in Ham F-12 medium for 18 h without metabolic activation. After 21 h (+S9) and 18 h (-S9), 100 μL colcemid (10 μg/mL) were added to accumulate metaphase cells until 24 h. The cells were then treated with 0.54 % KCl solution and fixing solution (acetic acid: methanol = 1:3). Diff-Quick-Kit stain was used for conventional staining of chromosomes. The metaphases were analyzed for the number and type of chromosomal aberrations and classified as chromatid (deletion, interchange, intrachange) or chromosome (gap, interchange, intrachange, ring, dicentric). All experiments were performed in triplicate with 300 mitotic cells calculated.

2.4. Mammalian erythrocyte micronucleus test

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (IACUC: 107−082). Six-week-old ICR male mice were purchased from BioLASCO Taiwan Co., Ltd (Yilan, Taiwan). The animal housing facility was maintained at 21 ± 2 °C with 50–65 % humidity under a 12 -h light/dark cycle. Laboratory Autoclavable Rodent Diet 5010 (LabDiet, St. Louis, MO) and water were supplied *ad libitum*. Twenty-five mice were divided into five groups (5 mice per group), including a negative control, positive control (cyclophosphamide 60 mg/kg, i.p.), and low, middle and high dose groups of ovatodiolide (250, 500, 1000 mg/kg). A volume of 10 mL/kg was administered once daily through oral gavage. Mice were weighed and monitored for clinical signs every day for three days after treatment. Blood was collected at 48 and 72 h after administration, smeared and stained with 0.1 % acridine orange. The number of reticulocytes in 1000 erythrocytes and micronuclei in 1000 reticulocytes were counted by fluorescence microscope (BX50, Olympus, Tokyo, Japan) at a length of 450−490 nm (blue excitation) and a 520 nm barrier filter.

2.5. Acute oral toxicity study

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (IACUC: 107−082) and followed M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals [12]. Five-week-old male and female Sprague-Dawley albino rats were purchased from BioLASCO Taiwan Co., Ltd (Yilan, Taiwan). In the animal housing facility, the temperature was maintained at 21 ± 2 °C with 50–65 % humidity under a 12 -h light/dark cycle. Laboratory Rodent Diet 5010 (LabDiet, St. Louis, MO) gamma-irradiated food and water were supplied *ad libitum*. Twenty rats were divided into two groups, with 5 males and 5 females designated to a control, and the other 5 males and 5 females designate to an ovatodiolide-treated group (1000 mg/kg). The dosing volume of 10 mL/kg was administered once a day through oral gavage. Clinical signs were observed and recorded every day for 14 days. The individual body weights of all animals were recorded weekly during the study. At the end of treatment, all animals were fasted and sacrificed under 2% isoflurane (Halocarbon Laboratories, USA) anesthesia in an inhalation chamber (MSS 003, Benchtop Small Animal Anesthesia Unit, U.K.).

2.6. 28-day oral toxicity study

2.6.1. Animals and experimental design

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (IACUC: 107−082) and followed the Organization for Economic Co-operation and Development (OECD) guidelines 407 [13]. Four-week-old male and female Sprague-Dawley albino rats were purchased from BioLASCO Taiwan Co., Ltd (Yilan, Taiwan). The animals were housed in a facility that was maintained at 21 ± 2 °C with 50–65 % humidity under a 12 -h light/dark cycle. Laboratory Autoclavable Rodent Diet 5001 (LabDiet, St. Louis, MO) and water were supplied *ad libitum*. The groups were divided as follows: control (10 % DMSO), and ovatodiolide 10, 25 and 50 mg/kg b.w. It has been proven that intraperitoneal administration of ovatodiolide (5 mg/kg) five days a week, for seven weeks, significantly suppressed the growth of colon tumorigenesis in mice. Since the absorption rate of the compound through intraperitoneal injection and the metabolic rate of mice is about 10 times faster than oral administration and humans, respectively, the dosage in the previous study is equivalent to the oral therapeutic dose of 5 mg/kg in humans. In this study, the selected doses were 10, 25 and 50 mg/kg, which is approximately 2, 5 and 10 times the therapeutic dose. The rats were administered a single daily dose of 10 mL/kg for 28 days via gavage. The clinical signs of individual rats were observed and recorded every day for 28 days, and the individual body weights and feed consumption of all animals were recorded weekly. At the end of treatment, all animals were fasted and sacrificed under 2% isoflurane (Halocarbon Laboratories, USA) anesthesia in an inhalation chamber (MSS 003, Benchtop Small Animal Anesthesia Unit, U.K.).

2.6.2. Urinalysis

On the first and last day of the treatment period, all animals from each group were subjected to urinalysis. Urine from all animals was collected overnight, and the volume and color were recorded. Analysis of urine was performed using an automatic urine analyzer (Clinitex 100 Urine Chemistry Analyzer, Miles Inc. Diagnostic Division Ellchart, IN, USA) and test strips (AMES Reagent Strips).

2.6.3. Hematology and biochemistry

Blood samples were collected and kept in sterile tubes containing anticoagulant and analyzed using an automatic hematological analyzer (Sysmex XE2100, Kobe, Japan) and biochemistry analyzer (ADVIA 1800, Siemens, NY, USA).

2.6.4. Gross examination, organ weight and histopathology

Necropsy, weighing and gross examination of the brain, heart, thymus, liver, spleen, kidneys, adrenal glands, testes (males) and ovaries (females) were performed. All organ samples were fixed in 10 % neutral buffered formalin, trimmed, embedded, and stained with hematoxylin-eosin (HE) for histopathological examination. The adrenal glands, aorta, brain, bone, bone marrow (femur, sternum), esophagus, eyes, Harderian gland, heart, kidneys, large intestine, liver, lungs, lymph nodes, mammary glands, optic nerve, ovaries, oviduct, pancreas, pituitary gland, parathyroid gland, salivary gland, sciatic nerve, skeletal muscle, skin, small intestine, spinal cord, spleen, stomach, thymus, thyroid gland, tongue, trachea, urinary bladder, male reproductive organs (epididymis, prostate gland, seminal vesicle, testes) or female reproductive organs (cervix, ovaries, uterus, vagina) of the control and high dose groups of both genders were trimmed for histopathological examination.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation, and the difference between the control and treatment groups was determined by the Student's *t*-test. MicroSoft Excel software was used for all statistical analyses and the statistical significance levels were determined by a 2-tailed test ($p < 0.05$).

3. Results

3.1. Bacterial reverse mutation test

There was no inhibition at any concentration of the ovatodiolide group or the control group in any tester strain with or without rat liver S9 fraction. However, a greater than 2-fold increase in the number of revertants per plate was only observed in the positive control group in the five *Salmonella typhimurium* strains. The data shows ovatodiolide did not cause reverse mutations in *Salmonella typhimurium* TA98, TA100, TA102, TA1535 or TA1537 with or without S9 fraction. The results of each colony are summarized in Table 2.

3.2. Mammalian chromosomal aberration test

Without S9 fraction, the frequencies of aberrant cells were 2.7 ± 1.5 % and 17.3 ± 3.8 % in the negative and positive control groups, respectively. With S9 fraction, the frequencies of aberrant cells were 2.0 ± 1.7 % and 15.3 ± 3.8 % in the negative and positive control groups, respectively. Accordingly, significant differences were found between the positive and negative controls for the frequencies of aberrant cells with or without S9 fraction. On the other hand, without S9, the frequencies of aberrant cells were 5.0 ± 1.0 %, 8.0 ± 3.6 % and 6.7 ± 2.5 % in the 12.5, 15.0 and 17.5 μM ovatodiolide-treated groups, respectively. With S9, the frequencies of aberrant cells were 3.3 ± 0.6 %, 7.3 ± 3.5 % and 6.0 ± 3.5 % in the 55, 65 and 75 μM ovatodiolide-treated groups, respectively. Treatment with ovatodiolide did not produce any significant difference when compared to the negative control, with or without S9 fraction. All the data for chromosomal aberration is summarized in Table 3.

3.3. Mammalian erythrocyte micronucleus test

There were no abnormal changes in clinical signs or body weight in any of the treated groups. The permillage of reticulocytes were 11.6 ± 1.8‰ and 8.8 ± 2.3‰ in the positive control group at 48 and 72 h post administration, which was significantly decreased compared to the negative group. Moreover, in terms of micronuclei, the permillage of micronuclei was 27.2 ± 5.1‰ and 16.4 ± 4.4‰ in the positive control group at 48 and 72 h post administration, which was significantly increased compared to the negative group. In contrast, there were no statistically significant differences in the permillage of reticulocytes or micronuclei in any of the ovatodiolide-treated groups compared to the negative control after 48 or 72 h of treatment. Data are shown in Table 4.

3.4. Acute oral toxicity study

None of the rats showed any obvious morbidity or clinical signs of toxicity after administration of ovatodiolide at the single dose of 1000 mg/kg b.w. Furthermore, body weights, gross findings in organs, absolute organ weights, and relative organ weights exhibited no significant differences in the ovatodiolide-treated group when compared to the control group. The data for body weights and absolute organ weights are shown in Fig. 2 and Table 5, respectively. Hematology showed statistical differences between the ovatodiolide-treated and control groups (Table 6). However, these data were within the normal reference range in SD rats, indicating the changes were not related to ovatodiolide. No significant difference was found between the ovatodiolide-treated and control groups in biochemistry analysis (Table 7).

According to histopathological examination, no significant treatment-related lesions were found in the ovatodiolide-treated group. Only non-specific focal tubular cysts in the kidneys were found. There were no treatment-related changes associated with ovatodiolide in the acute oral toxicity study.

Table 2
Revertant changes of ovatodiolide in *Salmonella* TA98 and TA100 mutagenicity test.

Group	Number of revertant (colony/plate)				
	TA98	TA100	TA102	TA1535	TA1537
Without S9 metabolic activation					
Negative[1]	32.3 ± 3.3[3]	192.3 ± 5.7	295.0 ± 5.7	12.7 ± 1.2	8.3 ± 1.7
Positive[2]	430.3 ± 36.8*	2716.0 ± 246.4*	1805.0 ± 127.4*	1877.3 ± 185.9*	994.0 ± 142.6*
Ovatodiolide					
0.16	33.0 ± 4.9	194.7 ± 4.2	–	13.7 ± 2.5	9.3 ± 1.2
0.31	29.0 ± 4.1	195.7 ± 4.0	294.0 ± 3.6	11.7 ± 5.2	9.7 ± 4.6
0.63	29.3 ± 1.7	193.7 ± 7.5	294.3 ± 3.3	13.0 ± 4.2	6.0 ± 1.4
1.25	28.3 ± 5.4	188.3 ± 13.0	294.7 ± 4.8	16.3 ± 2.9	7.7 ± 1.2
2.5	33.0 ± 3.7	186.7 ± 10.2	292.3 ± 5.0	11.3 ± 2.4	6.7 ± 1.7
5.0	–	–	289.3 ± 1.7	–	–
With S9 metabolic activation					
Negative	43.0 ± 6.2	170.7 ± 6.2	341.3 ± 7.4	12.0 ± 2.2	5.7 ± 0.9
Positive	4547.0 ± 244.6*	772.0 ± 72.5*	700.3 ± 18.7*	185.7 ± 55.4*	181.0 ± 28.6*
Ovatodiolide					
0.16	33.7 ± 4.5	175.0 ± 5.1	–	12.0 ± 4.3	5.7 ± 0.9
0.31	37.7 ± 3.9	171.0 ± 5.0	330.7 ± 7.4	14.0 ± 4.5	8.7 ± 2.1
0.63	36.7 ± 2.5	176.0 ± 6.2	331.3 ± 6.8	12.7 ± 1.7	8.0 ± 2.2
1.25	39.7 ± 3.4	164.7 ± 2.4	339.0 ± 2.4	10.0 ± 0.8	8.7 ± 1.7
2.5	45.7 ± 2.5	174.7 ± 10.9	340.0 ± 4.5	10.3 ± 2.5	5.7 ± 0.9
5.0	–	–	342.0 ± 5.9	–	–

- Not done.
[1] Negative control for ovatodiolide was added with DMSO.
[2] Positive reagents without S-9 mix reactions were 2.5 μg/plate 4-nitroquinoline-*N*-oxide for TA98, 5 μg/plate sodium azide for TA100; Positive reagents without S-9 mix reactions were 5 μg/plate sodium azide for TA1535, 0.5 μg/plate mitomycin C for TA102, and 50 μg/plate 9-aminoacridine for TA1537; positive reagent with S-9 mix was 5 μg/plate 2-aminoanthracene for all *Salmonella* strains.
[3] Data are presented as mean ± SD ($n = 3$).
* Significant difference of colonies more than two folds of negative control and treated groups at $p < 0.05$.

3.5. 28-day oral toxicity study

3.5.1. Clinic signs, body weight and feed consumption

There were no obvious signs of morbidity or clinical signs of toxicity after administration of different doses of ovatodiolide. After weeks three and four, the body weight changes of male rats were significantly decreased in the high dose ovatodiolide group when compared to the control group (Fig. 3). At the same time, body weight gain and its percentage of male rats decreased at week 3 in the high-dose ovatodiolide group compared to the control group. The male rats' body weights decreased at week 4 in the middle-dose ovatodiolide group when

Table 3
Percentages of chromosomal aberration test after incubation with ovatodiolide in the cultured CHO-K1 cells with or without S9 for 24 h.

Group	Total aberrations	Frequency of chromosomal aberration (%)[2]
Without S9		
Negative control[1]	8/300	2.7 ± 1.5[3]
Mitomycin C (2.5 μg/mL)	52/300	17.3 ± 3.8*
Ovatodiolide (μM)		
12.5	15/300	5.0 ± 1.0
15.0	24/300	8.0 ± 3.6
17.5	20/300	6.7 ± 2.5
With S9		
Negative control	6/300	2.0 ± 1.7
Cyclophosphamide (25 μg/mL)	46/300	15.3 ± 3.8*
Ovatodiolide (μM)		
55	10/300	3.3 ± 0.6
65	22/300	7.3 ± 3.5
75	18/300	6.0 ± 3.5

[1] Negative control:1% DMSO.
[2] Two slides were prepared and stained with Diff Quik Kit for 3 steps and a total number of 300 metaphases were counted for each dosage. All results were expressed in number of aberration per plate.
[3] The number of cells with damage chromosomes was recorded from which the rate of mutation was calculated. Aberration rate (%) = (number of cells with damage chromosomes/100) × 100. Data are expressed as mean ± SD, n = 3.
* Significant difference between the negative control and treated groups at $p < 0.05$.

Table 4
Changes of reticulocytes with micronuclei in the peripheral blood of male mice after orally treatment with ovatodiolide.

Group/ Intervals	Dose (mg/kg)	RETs/1000RBCs (‰)	Mn-RETs/1000RETs (‰)
Male			
48 h			
NC[1]	0	44.8 ± 6.2	1.6 ± 0.5
PC	60	11.6 ± 1.8*	27.2 ± 5.1*
Ovatodiolide			
	250	38.4 ± 3.0	1.2 ± 1.1
	500	43.6 ± 6.8	1.4 ± 1.1
	1000	38.0 ± 5.8	1.0 ± 1.2
72 h			
NC	0	50.2 ± 5.6	1.4 ± 1.1
PC	60	8.8 ± 2.3*	16.4 ± 4.4*
Ovatodiolide			
	250	47.4 ± 5.4	0.8 ± 1.1
	500	49.8 ± 5.5	0.8 ± 1.3
	1000	48.2 ± 5.8	0.6 ± 0.9

NC: negative control (6.7 % DMSO); RETs: reticulocytes; RBCs: erythrocytes; Mn-RETs: micronucleated reticulocytes; PC: positive control (Cyclophosphamide 60 mg/kg bw. ip).
[1] Data are expressed as the mean ± SD ($n = 5$).
* Significant difference in compared with the negative control and treated groups at $p < 0.05$.

compared to the control group. For the female rats, body weight gain decreased at week 1 in the low-dose ovatodiolide group when compared to the control group (40.2 ± 6.3 g). Body weight gain and its percentage decreased at week 2 (19.1 ± 5.7 g; 10.1 ± 2.3 %) in the middle-dose ovatodiolide group compared to the control group (25.0 ± 6.4 g; 13.0 ± 2.7 %) (data not shown). For weekly food consumption changes in rats, there were no significant differences in the ovatodiolide-treated groups during week 1 to week 3. The data is missing due to a computer error in week 4. Feed efficiency in the female rats was 17.8 ± 2.7 %, which was lower in the low-dose ovatodiolide group when compared to the control group (20.4 ± 2.6 %) (data not shown).

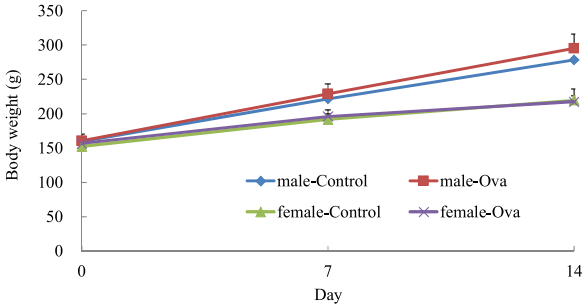

Fig. 2. Body weight changes of rats in the ovatodiolide single oral toxicity study.

Table 5
Absolute organ weight changes of rats in the ovatodiolide acute oral toxicity study.

Group	Brain (g)[1]	Heart (g)	Thymus (g)	Liver (g)
Male				
Control	1.9 ± 0.0[2]	0.9 ± 0.1	0.4 ± 0.1	8.2 ± 0.5
Ovatodiolide	2.0 ± 0.1	1.0 ± 0.2	0.5 ± 0.1	9.6 ± 2.0
Female				
Control	1.9 ± 0.1	0.8 ± 0.0	0.4 ± 0.1	6.7 ± 0.3
Ovatodiolide	1.9 ± 0.1	0.8 ± 0.1	0.4 ± 0.0	6.8 ± 0.4

Group	Kidney (g)	Adrenal (g)	Spleen (g)	Testis /Ovary (g)
Male				
Control	2.2 ± 0.2	0.04 ± 0.01	0.4 ± 0.1	2.8 ± 0.1
Ovatodiolide	2.3 ± 0.4	0.04 ± 0.01	0.5 ± 0.1	2.7 ± 0.2
Female				
Control	1.6 ± 0.1	0.05 ± 0.01	0.4 ± 0.1	0.07 ± 0.01
Ovatodiolide	1.7 ± 0.1	0.05 ± 0.01	0.4 ± 0.1	0.08 ± 0.02

Control: 10 % DMSO; ovatodiolide (1000 mg/kg bw).
No significant difference of parameters was found between the control and treated groups at $p > 0.05$.
[1] Organ weight (g).
[2] Data are expressed as the mean ± SD (n = 5).

3.5.2. *Urinalysis*

Urinalysis of female rats on day 28 revealed that the volume of urine (2.1 ± 0.7; 2.0 ± 1.0 mL) was significantly decreased in the middle and high-dose ovatodiolide groups when compared to the control group (3.8 ± 1.4 mL). Urobilinogen in the female rats (2.6 ± 1.8 mg/dL) on day 28 was significantly decreased in the high dose ovatodiolide group when compared to the control group (1.0 ± 1.3 mg/dL) (data not shown).

3.5.3. *Hematology and biochemistry*

Hematology and analysis of biochemical parameters showed statistical differences between the ovatodiolide-treated and control groups (Tables 8 and Table 9). However, these data were within the normal reference range in SD rats, indicating the changes were not related to ovatodiolide.

3.5.4. *Gross examination, organ weight and histopathology*

Gross examination did not reveal any significant differences in any group. However, one male rat in the middle-dose ovatodiolide group was diagnosed with focal hydrocephalus in the brain. This is randomly found in SD rats and was not considered to be related to treatment with ovatodiolide.

The absolute organ weights of the heart and thymus were significantly decreased in the high-dose ovatodiolide male rats when compared to the control group (Table 10). Relative organ weight changes of the liver were increased in the high-dose ovatodiolide male rats when compared to the control group. Relative organ weight changes

Table 6
Changes of hematological parameters of rats in the ovatodiolide acute oral toxicity study.

Group		Control	Ovatodiolide	Control	Ovatodiolide
Sex		Male		Female	
RBC[1]	10^6/μL	7.9 ± 0.2[2]	8.0 ± 0.6	8.2 ± 0.4	8.3 ± 0.4
HGB	g/dL	15.4 ± 0.5	16.0 ± 0.6	16.2 ± 0.5	16.2 ± 0.6
HCT	%	49.1 ± 2.0	51.2 ± 1.6	49.3 ± 1.7	50.2 ± 2.0
MCV	fL	62.3 ± 2.6	64.3 ± 3.8	60.1 ± 1.6	60.7 ± 1.5
MCH	pg	19.6 ± 0.8	20.1 ± 1.2	19.8 ± 0.9	19.6 ± 0.7
MCHC	g/dL	31.5 ± 0.8	31.2 ± 0.7	32.9 ± 0.7	32.3 ± 0.7
PLT	10^3/μL	1302.6 ± 79.7	1411.2 ± 64.2*	886.0 ± 433.4	1197.5 ± 122.0
WBC	10^3/μL	6.6 ± 2.2	6.6 ± 4.0	7.7 ± 2.5	6.2 ± 3.2
Lymphocyte	%	85.2 ± 6.8	72.4 ± 10.5	82.8 ± 4.9	85.9 ± 1.3
Neutrophil	%	11.7 ± 4.7	20.3 ± 6.4*	11.4 ± 2.0	10.8 ± 1.2
Monocyte	%	2.7 ± 2.4	6.5 ± 5.4	3.3 ± 2.1	2.2 ± 1.9
Eosinophil	%	0.2 ± 0.2	0.6 ± 0.4*	2.4 ± 1.6	1.1 ± 0.1
Basophil	%	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1

Control: 10 % DMSO; ovatodiolide (1000 mg/kg bw).
[1] RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets; WBC, white blood count.
[2] Data are expressed as the mean ± SD (n = 5).
* Significant difference between the control and treated groups at $p < 0.05$.

Table 7
Serum biochemistry changes in the liver and renal function of rats in the ovatodiolide acute oral toxicity study.

Group	AST (U/L)[1]	ALT (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
Male				
Control	78.0 ± 8.9[2]	26.8 ± 2.6	18.6 ± 2.4	0.3 ± 0.0
Ovatodiolide	79.4 ± 8.0	30.2 ± 5.0	17.8 ± 2.2	0.3 ± 0.1
Female				
Control	71.8 ± 7.1	23.6 ± 2.5	16.6 ± 2.6	0.3 ± 0.0
Ovatodiolide	78.0 ± 33.9	26.6 ± 6.5	13.8 ± 1.3	0.3 ± 0.1

Control: 10 % DMSO; ovatodiolide (1000 mg/kg bw).
[1] AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.
[2] Data are expressed as the mean ± SD (n = 5).

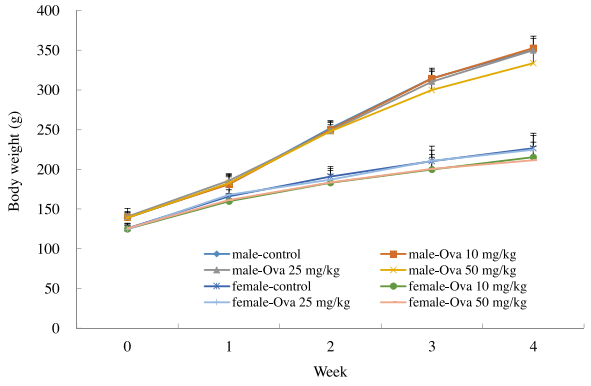

Fig. 3. Body weight changes of rats in the 28-day oral toxicity study of ovatodiolide.

of the liver were significantly increased in the low and high-dose ovatodiolide female rats when compared to the control group. Relative organ weight changes of the kidneys were significantly increased in the high-dose ovatodiolide female rats when compared to the control group. Relative organ weight changes of the ovaries were significantly increased in the middle-dose ovatodiolide female rats when compared to the control group (Table 11.). The final body weights in the high-dose ovatodiolide male and female rats were slightly decreased compared to the control group, which caused an increase in the relative organ weight changes. For the relative brain weight changes in male rats, the ratio of the thymus was significantly decreased in the high-dose ovatodiolide group when compared to the control group. For the relative brain organ weight changes of female rats, the ratio of ovary significantly increased in the middle-dose ovatodiolide group when compared to the control group. The results are summarized in Table 12.

According to histopathological examination, no significant treatment-related lesions were found in the ovatodiolide-treated group. However, one male rat in the middle-dose ovatodiolide group was diagnosed with focal hydrocephalus in the brain. This randomly occurs in SD rats and was not considered a toxic effect related to ovatodiolide. Some non-specific lesions, including focal spermatic granuloma in the epididymis; multifocal mononuclear cell infiltration in the Harderian gland, heart, and prostate gland; focal tubular casts, focal tubular cysts, focal tubular infarct, and multifocal tubular mineralization in the kidneys; and fatty change in the liver were observed in some of the control and ovatodiolide-treated rats (data not shown). All of the lesions mentioned above occurred in both the control and high-dose ovatodiolide-treated rats, and the degree of lesions and incidence showed no correlation.

4. Discussion

Although *Anisomeles indica* (L.) Kuntze has been used to treat various kinds of diseases in the past, previous studies have not addressed the relative safety of ovatodiolide.

Poorly soluble drugs are a real problem in pharmaceutical studies. There are many organic solvents like DMSO, ethanol, acetone, acetonitrile and so on, which can be used as a vehicle in an Ames assay [14]. Ovatodiolide is difficult to dissolve in water, but has a high solubility in DMSO. Accordingly, we chose DMSO as a solvent in the bacterial reverse mutation test. There was no toxicity observed in S. *typhimurium* TA102. It is speculated that there is only the *uvrB* gene which can strengthen the accurate excision repair mechanism in strain TA102 [15].

Propranolol, a cardiac medication, can be used to treat hypertension. In mammalian chromosomal aberration tests, the data shows that propranolol can cause chromosomal aberrations without S9 fraction. However, the toxicity of propranolol can be reduced with the presence of S9 fraction. Accordingly, rat liver extracts play an important role for reducing the toxicity of certain medicine [16]. In our study, the results showed the half maximal inhibitory concentration (IC_{50}) without S9 fraction is 20.8 μM, but the half maximal inhibitory concentration with S9 fraction is 77 μM. It is evident that the toxicity of ovatodiolide might decline in the presence of S9 metabolism. In previous studies, the IC_{50} was 4.4 μM in Ca9−22 oral squamous cell carcinoma [17] and 9.2 and 4.5 μM in MDA-MB-231 and HS578 T breast cancer [18]. It is clear from previously published data that ovatodiolide is more toxic to cancer cells than normal cell lines, which shows that ovatodiolide has a great potential for development. In the results of the chromosomal aberration test, ovatodiolide showed no signs of mutagenicity.

Aneugens can be detected through peripheral blood or bone marrow in mice and bone marrow in rats with a single dose treatment. Additionally, with exposure to clastogens, the data shows that the number of micronuclei increased in the peripheral blood of rats and mice. The differences between aneugens and clastogens in the peripheral blood of rats and mice suggests that the removal of micronuclei is probably due to the size of the micronuclei [19]. After administration for 48 h, the peak

Table 8
Hematological parameter changes of rats treated with ovatodiolide in the 28-day oral toxicity study.

Sex		Male				Female			
Group		Control	Ovatodiolide			Control	Ovatodiolide		
			10 mg/kg	25 mg/kg	50 mg/kg		10 mg/kg	25 mg/kg	50 mg/kg
RBC[1]	10^6/μL	7.7 ± 0.4[2]	7.9 ± 0.4	8.0 ± 0.3	7.9 ± 0.2	8.1 ± 0.5	8.1 ± 0.3	8.2 ± 0.3	8.3 ± 0.6
HGB	g/dL	15.9 ± 0.5	16.0 ± 0.4	16.4 ± 0.4*	16.2 ± 0.4	15.6 ± 1.0	15.8 ± 0.3	15.8 ± 0.5	16.0 ± 1.0
HCT	%	48.3 ± 1.7	48.3 ± 1.6	49.6 ± 1.3	49.3 ± 1.6	48.1 ± 3.2	47.9 ± 0.9	48.3 ± 1.7	48.9 ± 3.0
MCV	fL	62.7 ± 2.0	61.4 ± 1.5	62.3 ± 1.7	62.1 ± 1.6	59.6 ± 1.3	59.6 ± 2.1	59.0 ± 1.2	59.0 ± 1.1
MCH	pg	20.6 ± 0.7	20.4 ± 0.7	20.6 ± 0.8	20.5 ± 0.5	19.4 ± 0.5	19.7 ± 0.7	19.2 ± 0.5	19.3 ± 0.4
MCHC	g/dL	32.9 ± 0.6	33.2 ± 0.5	33.1 ± 0.5	32.9 ± 0.4	32.5 ± 0.4	33.1 ± 0.3*	32.6 ± 0.4	32.7 ± 0.2
PLT	10^3/μL	1054.2 ± 326.2	1256.2 ± 129.0	1246.5 ± 115.4	1215.2 ± 162.1	1196.0 ± 222.3	1305.2 ± 217.5	1112.8 ± 400.2	1298.8 ± 94.2
WBC	10^3/μL	5.9 ± 1.9	7.6 ± 3.6	7.7 ± 2.6	7.4 ± 1.3	4.7 ± 1.4	6.3 ± 1.9	4.6 ± 1.0	5.0 ± 2.0
Lymphocyte	%	81.5 ± 12.0	84.0 ± 5.1	85.4 ± 5.8	81.0 ± 11.6	85.0 ± 4.2[3]	85.0 ± 5.0[3]	86.4 ± 5.2	84.9 ± 7.0
Neutrophil	%	17.1 ± 11.9	14.5 ± 5.4	13.2 ± 5.6	17.4 ± 11.6	12.2 ± 3.9[3]	12.0 ± 3.9[3]	10.3 ± 4.6	12.2 ± 6.5
Monocyte	%	0.6 ± 0.1	0.5 ± 0.2	0.7 ± 0.3	0.8 ± 0.4	0.6 ± 0.33	0.6 ± 0.33	0.8 ± 0.3	0.8 ± 0.3
Eosinophil	%	0.8 ± 0.5	0.9 ± 0.4	0.7 ± 0.4	0.7 ± 0.3	2.1 ± 0.93	2.2 ± 1.13	2.4 ± 0.8	2.1 ± 0.9
Basophil	%	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.13	0.2 ± 0.23	0.1 ± 0.1	0.1 ± 0.1

Control: 5%DMSO.
[1] RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC: white blood cell.
[2] Data are expressed as the mean ± SD (n = 10).
[3] Data are expressed as the mean ± SD (n = 9).
* Significant difference between the control and treated groups at $p < 0.05$.

Table 9
Serum biochemistry changes of rats treated with ovatodiolide in the 28-day oral toxicity study.

Sex		Male				Female			
Group		Control	Ovatodiolide			Control	Ovatodiolide		
			10 mg/kg	25 mg/kg	50 mg/kg		10 mg/kg	25 mg/kg	50 mg/kg
AST[1]	U/L	91.1 ± 46.2[2]	82.7 ± 10.8	76.9 ± 8.3	131.0 ± 107.5	68.7 ± 6.8	95.1 ± 51.2	68.5 ± 9.6	70.0 ± 9.8
ALT	U/L	40.1 ± 28.2	33.4 ± 6.9	29.8 ± 6.0	59.4 ± 52.1	28.2 ± 3.5	45.1 ± 45.2	22.7 ± 3.7*	27.9 ± 4.4
ALP	U/L	186.7 ± 47.2	180.8 ± 29.5	172.7 ± 47.9	169.1 ± 35.5	120.0 ± 22.7	112.3 ± 27.0	108.9 ± 30.9	103.0 ± 19.9
BUN	mg/dL	17.6 ± 2.3	16.3 ± 3.8	16.2 ± 4.1	18.1 ± 6.8	15.6 ± 2.0	17.2 ± 1.4	16.2 ± 2.5	18.3 ± 1.8*
Creatinine	mg/dL	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
CK	U/L	198.4 ± 79.5	180.9 ± 53.8	193.7 ± 78.4	227.9 ± 163.4	118.9 ± 29.9	155.0 ± 53.7	104.6 ± 21.1	118.0 ± 25.1
Cholesterol	(mg/dL	39.1 ± 6.5	41.2 ± 8.	46.4 ± 7.6*	45.8 ± 11.6	54.3 ± 7.9	57.7 ± 12.0	52.8 ± 8.7	57.9 ± 9.6
Amylase	U/L	1760.9 ± 229.2	1694.1 ± 176.1	1682.4 ± 258.8	1633.8 ± 314.6	1189.3 ± 175.3	1078.8 ± 61.8	965.8 ± 161.7*	1131.8 ± 106.6
Glucose	mg/dL	177.4 ± 31.3	158.7 ± 19.2	167.2 ± 24.2	166.4 ± 13.8	134.0 ± 18.5	130.3 ± 17.7	119.5 ± 17.8	129.7 ± 12.3
GGT	U/L	1.7 ± 1.2	<1	2.0 ± 0.0	2.3 ± 1.5	2.7 ± 0.6	1.8 ± 1.1	1.3 ± 0.5*	2.6 ± 1.8
LDH	U/L	241.5 ± 176.9	203.1 ± 135.8	213.8 ± 173.6	169.0 ± 71.8	95.7 ± 36.8	135.1 ± 89.6	84.0 ± 31.1	120.0 ± 47.1
TB	mg/dL	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
UA	mg/dL	1.9 ± 1.3	1.6 ± 0.3	1.5 ± 0.2	1.7 ± 0.5	1.4 ± 0.3	1.4 ± 0.3	1.2 ± 0.2	1.3 ± 0.2
Globulin	g/dL	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.2	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1*
Albumin	g/dL	3.7 ± 0.2	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.8 ± 0.1*	3.8 ± 0.2	3.9 ± 0.2*
A/G		2.2 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	2.2 ± 0.2	2.2 ± 0.1	2.2 ± 0.2	2.3 ± 0.2	2.2 ± 0.1
HDL-C	mg/dL	10.2 ± 1.8	11.0 ± 2.8	11.8 ± 2.7	11.0 ± 2.5	14.9 ± 2.5	16.2 ± 3.2	14.3 ± 3.3	15.7 ± 3.2
TP	g/dL	5.4 ± 0.3	5.4 ± 0.2	5.4 ± 0.2	5.5 ± 0.3	5.3 ± 0.2	5.5 ± 0.1*	5.4 ± 0.3	5.7 ± 0.3*
TG	mg/dL	30.1 ± 7.8	26.7 ± 7.4	32.1 ± 7.7	26.1 ± 6.5	32.7 ± 7.4	31.6 ± 8.7	29.1 ± 7.8	26.7 ± 3.4*
Ca^{2+}	mg/dL	9.5 ± 0.5	9.6 ± 0.3	9.6 ± 0.3	9.6 ± 0.5	9.5 ± 0.2	9.7 ± 0.3	9.6 ± 0.2	9.8 ± 0.2*
Cl^-	mEq/dL	104.0 ± 2.7	105.3 ± 1.3	104.9 ± 1.7	105.1 ± 1.9	106.4 ± 1.8	106.5 ± 1.6	106.7 ± 1.5	107.0 ± 0.9
K^+	mEq/dL	5.4 ± 1.7	4.9 ± 0.4	4.8 ± 0.5	4.8 ± 0.7	5.7 ± 0.6	5.2 ± 0.4	5.3 ± 0.4	5.3 ± 0.6
Mg^{2+}	mg/L	2.0 ± 0.3	1.9 ± 0.1	1.8 ± 0.2	2.0 ± 0.3	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.2	2.3 ± 0.1*
Na^+	mEq/dL	142.1 ± 1.8	141.7 ± 1.3	141.6 ± 1.4	141.8 ± 1.0	139.8 ± 1.2	141.0 ± 1.9	140.2 ± 1.1	140.3 ± 1.4
Phosphates	mg/dL	9.0 ± 1.8	8.6 ± 0.9	8.4 ± 0.6	8.9 ± 1.3	7.8 ± 0.8	7.5 ± 0.7	7.8 ± 1.3	8.1 ± 0.5

Control: 5% DMSO.
[1] AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; BUN: blood urea nitrogen; CK: creatine kinase; LDH: lactate dehydrogenase; TB: total bilirubin; GGT: gamma glutamyl-transferase; UA: uric acid; HDL-C: high density lipids - cholesterol; TG: triglyceride; TP: total protein; Ca^{2+}: calcium; Cl^-: chloride; K^+: potassium; Na^+: sodium; phosphates$^-$: inorganic phosphates.
[2] Data are expressed as the mean ± SD (n = 10).
* Significant difference between the control and treated groups at $p < 0.05$.

Table 10
Absolute organ weight changes of rats treated with ovatodiolide in the 28-day oral toxicity study.

Sex/Group	Brain (g)	Heart (g)	Thymus (g)	Liver (g)	Kidney (g)	Adrenal (g)	Spleen (g)	Testis/Ovary (g)
Male								
Control	2.0 ± 0.1[1]	1.18 ± 0.11	0.6 ± 0.1	9.8 ± 0.6	2.6 ± 0.5	0.05 ± 0.01	0.5 ± 0.1	2.9 ± 0.3
Ovatodiolide								
10 mg/kg	2.0 ± 0.1	1.20 ± 0.10	0.6 ± 0.1	10.0 ± 0.7	2.7 ± 0.3	0.05 ± 0.01	0.6 ± 0.1	2.8 ± 0.3
25 mg/kg	2.0 ± 0.1	1.14 ± 0.06	0.6 ± 0.1	10.3 ± 0.8	2.7 ± 0.2	0.05 ± 0.01	0.5 ± 0.1	3.0 ± 0.2
50 mg/kg	2.0 ± 0.1	1.08 ± 0.07*	0.5 ± 0.1*	10.1 ± 1.0	2.6 ± 0.3	0.05 ± 0.01	0.5 ± 0.1	2.8 ± 0.2
Female								
Control	1.9 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	6.0 ± 0.5	1.6 ± 0.2	0.05 ± 0.01	0.4 ± 0.1	0.06 ± 0.02
Ovatodiolide								
10 mg/kg	1.8 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	6.1 ± 0.7	1.6 ± 0.2	0.06 ± 0.01	0.4 ± 0.1	0.06 ± 0.01
25 mg/kg	1.9 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	6.2 ± 0.6	1.7 ± 0.1	0.05 ± 0.01	0.4 ± 0.1	0.07 ± 0.02
50 mg/kg	1.8 ± 0.1	0.8 ± 0.0	0.4 ± 0.1	6.2 ± 0.5	1.7 ± 0.1	0.06 ± 0.01	0.3 ± 0.0	0.06 ± 0.01

Control: 5% DMSO.
[1] Data are expressed as the mean ± SD (n = 10).
* Significant difference between the control and treated groups at $p < 0.05$.

Table 11
Relative organ weight changes of rats treated with ovatodiolide in the 28-day oral toxicity study.

Sex/Group	Brain (%)	Heart (%)	Thymus (%)	Liver (%)	Kidney (%)	Adrenal (%)	Spleen (%)	Testis/Ovary (%)
Male								
Control	0.6 ± 0.0[1]	0.3 ± 0.0	0.2 ± 0.0	2.8 ± 0.1	0.7 ± 0.1	0.01 ± 0.00	0.2 ± 0.0	0.8 ± 0.1
Ovatodiolide								
10 mg/kg	0.6 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	2.8 ± 0.1	0.8 ± 0.1	0.01 ± 0.00	0.2 ± 0.0	0.8 ± 0.1
25 mg/kg	0.6 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	2.9 ± 0.2	0.8 ± 0.0	0.01 ± 0.00	0.2 ± 0.0	0.9 ± 0.1
50 mg/kg	0.6 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	3.0 ± 0.2*	0.8 ± 0.1	0.01 ± 0.00	0.1 ± 0.0	0.8 ± 0.1
Female								
Control	0.8 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	2.6 ± 0.1	0.72 ± 0.06	0.02 ± 0.01	0.2 ± 0.0	0.025 ± 0.007
Ovatodiolide								
10 mg/kg	0.9 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	2.8 ± 0.2*	0.76 ± 0.08	0.03 ± 0.00	0.2 ± 0.1	0.030 ± 0.007
25 mg/kg	0.8 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	2.7 ± 0.1	0.77 ± 0.07	0.02 ± 0.00	0.2 ± 0.0	0.032 ± 0.005*
50 mg/kg	0.9 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	3.0 ± 0.1*	0.78 ± 0.05*	0.03 ± 0.00	0.2 ± 0.0	0.026 ± 0.006

Control: 5% DMSO.
[1] Data are expressed as the mean ± SD (n = 10).
* Significant difference between the control and treated groups at $p < 0.05$.

Table 12
Relative brain organ weight changes of rats treated with ovatodiolide in the 28-day oral toxicity study.

Sex/Group	Heart (ratio)	Thymus (ratio)	Liver (ratio)	Kidney (ratio)	Adrenal (ratio)	Spleen (ratio)	Testis/Ovary (ratio)
Male							
Control	0.6 ± 0.1[1]	0.3 ± 0.1	4.9 ± 0.3	1.3 ± 0.3	0.03 ± 0.00	0.3 ± 0.0	1.5 ± 0.1
Ovatodiolide							
10 mg/kg	0.6 ± 0.0	0.3 ± 0.1	5.1 ± 0.3	1.4 ± 0.2	0.03 ± 0.00	0.3 ± 0.0	1.4 ± 0.1
25 mg/kg	0.6 ± 0.0	0.3 ± 0.0	5.2 ± 0.5	1.4 ± 0.1	0.03 ± 0.00	0.3 ± 0.1	1.5 ± 0.1
50 mg/kg	0.6 ± 0.0	0.2 ± 0.0*	5.2 ± 0.5	1.3 ± 0.1	0.02 ± 0.00	0.2 ± 0.0	1.4 ± 0.1
Female							
Control	0.4 ± 0.0	0.2 ± 0.1	3.2 ± 0.2	0.9 ± 0.1	0.03 ± 0.01	0.2 ± 0.0	0.03 ± 0.01
Ovatodiolide							
10 mg/kg	0.4 ± 0.0	0.2 ± 0.1	3.3 ± 0.2	0.9 ± 0.1	0.03 ± 0.01	0.2 ± 0.1	0.03 ± 0.01
25 mg/kg	0.4 ± 0.0	0.2 ± 0.1	3.3 ± 0.2	0.9 ± 0.0	0.03 ± 0.00	0.2 ± 0.0	0.04 ± 0.01*
50 mg/kg	0.4 ± 0.0	0.2 ± 0.0	3.4 ± 0.2	0.9 ± 0.1	0.03 ± 0.01	0.2 ± 0.0	0.03 ± 0.01

Control: 5% DMSO.
[1] Data are expressed as the mean ± SD (n = 10).
* Significant difference between the control and treated groups at $p < 0.05$.

of micronuclei in the peripheral blood induced by cyclophosphamide is 2.97 % compared to the peak of micronuclei (2.53 %) in bone marrow, which is delayed 12 h [20]. In our study, the peak of micronuclei occurred after 48 h of treatment with cyclophosphamide. At the same time, the proportion of reticulocytes and erythrocytes apparently declined. After 72 h, due to a decrease in drug concentration, the bone marrow kept producing erythrocytes, so the number of micronuclei began to decline.

Acute toxicity tests are considered a preliminary study which provides the basis for classification and labelling. It also provides initial information about the toxicity of a test article. Furthermore, with the results of acute toxicity tests, the dose of a new compound can be adjusted and help determine an appropriate dose in animal studies [21]. Increased neutrophils are associated with responses to stress or excitement and infectious diseases [22]. In our study, the proportion of neutrophils increased but it was within the normal reference range in SD

rats. Furthermore, all rats were housed in a specific-pathogen-free room, which would remove the possibility of infection. As a result, it might be related to individual differences in rats.

Exposure to low doses of partial substances might cause effects that appear similar to those of repeated toxicities. Consequently, the repeated dose oral toxicity test plays an important role. There were increases in liver weight and relative body weight (%) compared to the control groups in the high dose male and female rats. However, there was no difference in the relative brain ratio. Furthermore, there were no lesions in the liver histology in the high dose male and female rats. In addition, Cattley and Cullen have indicated that with a significant variation of body weight, changes in liver weights relative to brain weights can be used for comparison because brain weight is not generally affected in typical studies [23]. To sum up, ovatodiolide revealed no treatment-related toxicity in the examined items of body weight, feed consumption, organ weight, hematology, urinalysis, biochemistry, or pathology in rats.

As it knows chemotherapeutic drugs used in clinical medicine have strong toxicity at low dosages, such as 5 mg/kg cisplatin cause nephrotoxicity in rats [24]. OVA is an isolated compound from *Anisomeles indica* that has been extracted the attention due to its anti-cancer effects [25]. The aim of the 28-day feeding toxicity study is to confirm that whether at the 10 folds higher than therapeutic dosage would have toxicity effects in rats. The results of the study, 50 mg/kg OVA would not cause any adverse effect in rats. Because of OVA has low production in synthesis or purified from *Anisomeles indica*, we could not get enough amounts of OVA for the high-dosage toxicity study. However, the results of the previous study can be a dose-select reference of subchronic toxicity study. When synthesis process improvement to increase the productivity of OVA, the toxicity study of 100 folds higher than the OVA effect dosage (500 mg/kg) would be conducted in the future.

5. Conclusion

In conclusion, the results of the three genotoxicity assays were all negative. Accordingly, ovatodiolide did not display any mutagenicity or genotoxicity. The results of the acute oral toxicity study showed the acute oral toxicity of ovatodiolide was over 1000 mg/kg b.w. in rats. The no observed adverse effect level (NOAEL) of ovatodiolide was 50 mg/ kg b.w. in the 28-day oral toxicity test in rats.

Author statement

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Conflict of interest

The authors declare no conflict of interest.

Declaration of Competing Interest

The authors report no declarations of interest.

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