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Full Length Research Paper

Toxicological evaluation of zerumbone on antitumor effects in mice

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Zerumbone (ZER), a bioactive compound isolated from *Zingiber zerumbet* Smith, was examined for single and repeated-dose toxicity at dosages with antitumor effects on imprinting control regions (ICR) mice and mice of either sex for repeated dose, respectively. For the single dose toxicity study, ZER was administrated to ICR mice at a dose of 500 mg/kg via intraperitoneal injection, while for the repeated dose toxicity study, mice of either sex were studied at dosages of 5, 25 and 50 mg/kg for a period of 28 days. The effects on body and organ weight, food and water consumption, hematology, serum biochemistry as well as histology, were evaluated. No mortality or significant changes in the clinical signs were produced at the single dose toxicity. There were no significant differences in the general condition, growth, organ weights, hematology, serum biochemistry, or histopathological analysis in the repeated dose toxicity as well. These results suggest that ZER is safe in a toxicity study for the cancer treatment in mice regardless of whether male or female mice.

Key words: Zerumbone, toxicity, antitumor effect, single and repeated dose, safety.

INTRODUCTION

Natural products obtained from plants have a long history of beneficial use by mankind for the treatment of diseases (Lucas et al., 2010). With developments of new bioassay methods, isolation of bioactive components from natural sources (plants and animals) and identification of their molecular mechanism of action in the living system has become important pharmacological research area (Aggarwal et al., 2009; Harvey, 2008; Shanmugam et al., 2011). Several promising natural products and natural product-inspired compounds are currently in clinical and pre-clinical developmental stages for cancer treatment (Prasannan et al., 2012). The rationale for the utilization of medicinal plants has rested largely on the long-term clinical experience with little or no scientific data on their efficacy and safety.

Zerumbone (ZER; 2,6,9,9-tetramethylcycloundeca-2,6, 10-trien-1-one) is a sesquiterpenoid found in large amounts in the rhizome of *Zingiber zerumbet* Smith, a plant traditionally used in Southeast Asian counties as an anti-inflammatory and anti-rheumatic agent and as a condiment. ZER has been reported to have chemo-preventive (Ohnishi et al., 2009), anti-inflammatory (Abdelwahab et al., 2010; Murakami et al., 2004) and free radical (hydroxyl free radicals, nitric oxide, singlet oxygen, etc) scavenging activities (Murakami et al., 2002) in several *in vitro* studies. Intrinsic antioxidant properties of ZER are believed to contribute to its antitumor action (Conti, 2006). ZER induces apoptosis in gliomablastoma multiforme, breast cancer, pancreatic cancer, ovarian and cervical cancer cell lines (Abdelwahab et al., 2012; Sehrawat et al., 2012; Weng et al., 2012). In addition, cytotoxic effect of ZER has been reported to be selective toward cancer cells as compared to normal cells (Abdul et al., 2009). ZER also has been reported to inhibit tumor growth in various animal models of inflammation and cancer (Huang et al., 2005; Kim et al., 2009; Prasannan

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et al., 2012; Sulaiman et al., 2010). ZER has been shown to suppress cervical intraepithelial neoplasia by diethylstilbestrol (DES) in female Balb/c mice, at an efficacy close to that of the anti-tumor drug cisplatin (Abdelwahab et al., 2010). Taha et al. (2010) reported that ZER protects rat livers from the carcinogenic effect of diethylnitrosamine (DEN) (single injected dose) and dietary 2- acetylaminoflurene (AAF). ZER has been suggested to modulate many inflammation-related molecular targets, and it has also been investigated as a chemopreventive agent. ZER has been used against colorectal and lung cancers, which are known to have chronic inflammation as a high risk factor for carcinogenesis (Azad et al., 2008; Xie and Itzkowitz, 2008). However, toxicity has not been reported in ZER for preclinical use.

In a previous study, we investigated that ZER mitigates radiation resistance by heat shock protein 27 (HSP27). Overexpression of HSP27 in tumor cells increases tumorigenicity and protects against cell death triggered by a number of stimuli (Lelj-Garolla and Mauk, 2005). ZER induced cross-linking of HSP27 protein by its insertion between the disulfide bond, which resulted in a sensitizing effect to tumors. ZER-mediated altered cross-linking of HSP27 that modified normal HSP27 dimerization. ZER may be a novel strategy for inhibition of HSP27-mediated radio- and chemo-resistance (Choi et al., 2011). *In vivo* data using nude mice after grafting of human lung cancer cells indicated that ZER showed synergic tumor regression effects with radiation via radiation-sensitize effect.

Therefore, ZER would be beneficial for cancer therapy as a single and/or combination treatment, especially in tumor types with high HSP27 protein expression. As toxicity tests are important for clinical use, we have assessed the safety of ZER by employing single and repeated-dose toxicity studies in imprinting control regions ICR mice.

MATERIALS AND METHODS

Test and control reagents

ZER were isolated and purified from the dried rhizomes of *Z. zerumbet* Smith (Figure 1). The purities of all compounds (>97%) were identified and analyzed by high-performance liquid chromatography and nuclear magnetic resonance (Choi et al., 2011). The vehicle control was 1% dimethyl sulfoxide (DMSO) dissolved in phosphate buffer saline (PBS).

Experimental animals

All ICR mice (Japan SLC Inc.), aged 7 weeks and weighing 24 to 26 g in females and 27 to 29 g in males, were used after 1 week adaptation in this study. The animals were housed in autoclaved polycarbonate cages on hardwood bedding throughout the study.

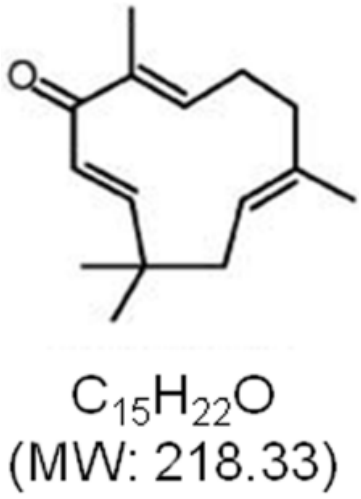

Figure 1. Structure of ZER (2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one).

The room was maintained at a controlled temperature (22 ± 1°C) and humidity (50 ± 5%) under a time-controlled system with 12 h light/12 h dark cycle. Food and water were supplied ad libitum. Studies were conducted according to the guidelines for the use and care of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences (KIRAMS).

Single-dose toxicity study

Six male and six female ICR mice were given a single dose intraperitoneal (*i.p.*) injection of zerumbone at 500 mg/kg. Since Ibrahim et al. (2010) reported that a 500 mg/kg single dose of ZER via oral administration produced severe liver and renal damage in female Sprague Dawley rats, we choose 500 mg/kg as a single injected dose. The control mice were given equal volumes of 1% DMSO in saline. When we compared 1% DMSO in PBS, there was no alteration related toxic effect to mice (Data not shown). All animals were examined for mortality and clinical signs twice a day. Body weights were measured on 0, 2, 4, 6, 8, 12 and 14 days. All of the animals were sacrificed on 14 days and examined by macroscopic lesions. Organs, including the brain, heart, lung, kidney, intestine, liver, reproductive organs, lymph nodes, salivary glands and thymus were collected from all animals in control (0 mg/kg) and maximum dose (intraperitoneal injection of 500 mg/kg) mice and were routinely processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic evaluation.

Repeated-dose toxicity study

After a one-week adaptation, the male or female mice were randomly assigned to each group: vehicle control, ZER at 5 mg/kg (low dose), 25 mg/kg (medium dose), or 50 mg/kg (high dose). The dosage levels of ZER were determined based on the findings in a tumor regression study with NCI460 and NCI1299 human lung cancer cells, in which a decrease in tumor growth was observed at doses of 5 mg/kg (Data not shown). Test drug and control articles

were administered as an intraperitoneal injection (0.1 ml/mouse) on every odd numbered day for 28 days. Animals were observed each day for signs of toxicity at 30 min, and 1, 3, and 6 h after injection. Daily food consumption was measured and estimated as gram of food eaten over 24-h period. Water was available to animals at all times. Body weights were recorded on all animals prior to the start of dosing (D0) and then at weekly intervals (D7, D14, and D21) until the end of the study period (D28). Measurements of body weight were carried out prior to dose injection.

Blood samples were collected from abdominal vein for hematological and biochemical analysis. Hematology parameters were measured using Hemavet950 Mulitspecies Hematology Analyzer (DREW Scientific Inc. Oxford, CT, USA) and included: white blood cell count (WBC), neutrophiles (NE), lymphocytes (LY), monocytes (MO), eosinophiles (EO), basophiles (BA), red blood cell count (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), and platelets (PLT). Serum biochemical analyses were performed using Dri-Chem4000i (Fujifilm, Japan) for glucose (GLU), total cholesterol (TCH), creatinine (CRE), blood urea nitrogen (BUN), total protein (TP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

During the autopsy, the brain, heart, lung, kidney, intestine, liver, reproductive organs, lymph nodes, salivary glands and thymus, were grossly evaluated, and were fixed in fixative solution for histopathological analysis. Fixed tissues were embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin and eosin. Any abnormalities in other organs were noted and the organs were collected for histopathological evaluation.

Data analysis

Data are presented as the mean ± standard deviation (SD). Statistical comparisons were conducted using the statistics software SPSS, version 14.0 (SPSS, Inc., Chicargo, IL, USA). In the case of body weight, one-factor (time) analysis of variance (ANOVA) with post hoc testing was used for determining group differences. The residuals were analyzed for homogeneity of variance and normality of distributions. Statistical comparisons were made using the Student's t-test (independent group) and Dunnett's test. Statistical significance was determined whenever a P value of 0.05 or less was observed.

RESULTS

General condition, symptoms and mortality

All tested animals survived the duration of the experiment. No adverse effects were observed in mice given ZER when compared to the control group. All animals did not show any clinical signs of toxicity. Body weight and food consumption were measured 1 to 3 times a week. Body weight and food consumption were similar in all groups.

Single-dose toxicity study

A single intraperitoneal injection of ZER at 500 mg/kg did not induce any clinical sign of toxicity. ZER in animals showed no differences in body weight, organ weight, and histological examinations when compared with the control during the 14 days study. Based on these data, we conducted a repeated-dose toxicity test in mice.

Repeated-dose toxicity study

ZER was injected at 0, 5, 25, and 50 mg/kg in 14 dosages over 28 days. During the treatment period, no mortality or clinical signs of general toxicity were observed at all the selected doses. Estimated food and water consumption did not differ in all ZER treated groups as compared to the control group (Data not shown). Body weights were measured weekly and no dose-related changes were found in males or females among ZER treated groups as compared to the control group (Figure 2). The organ weights (absolute and relative) did not show significant differences between the control and treated groups of either sex at the end of the treatment period (Table 1).

Hematological parameters

The hematology data indicated that ZER did not induced significant changes in hematological parameters such as erythrocyte, platelets and white blood cells including differential counts (Table 2). All parameters obtained from ZER treated and control groups were in the normal range. Serum biochemistry indicates toxicity of a test material. The results of the effect of the repeated administration of ZER on serum chemistry are summarized in Table 3. ZER did not exhibit any effect on the level of serum chemistry at all groups.

Histopathological evaluations

We analyzed hematoxylin and eosin stained tissue sections from brain, heart, lung, kidney, intestine, liver, reproductive organs, lymph nodes, salivary glands and thymus for general toxicity such as inflammation, hemorrhage, hyperplasia, necrotic cell death, etc. Some of the animals in the ZER treated and control groups showed mild inflammation in the lung, however, there was no statistical difference among the groups. No treatment related gross lesions or histopathological alteration under microscope were observed in all organs of ZER and control groups. The observations in this study imply that mice tolerated well in the single dose and repeated dose toxicity evaluations.

DISCUSSION

The present study was conducted to determine the single

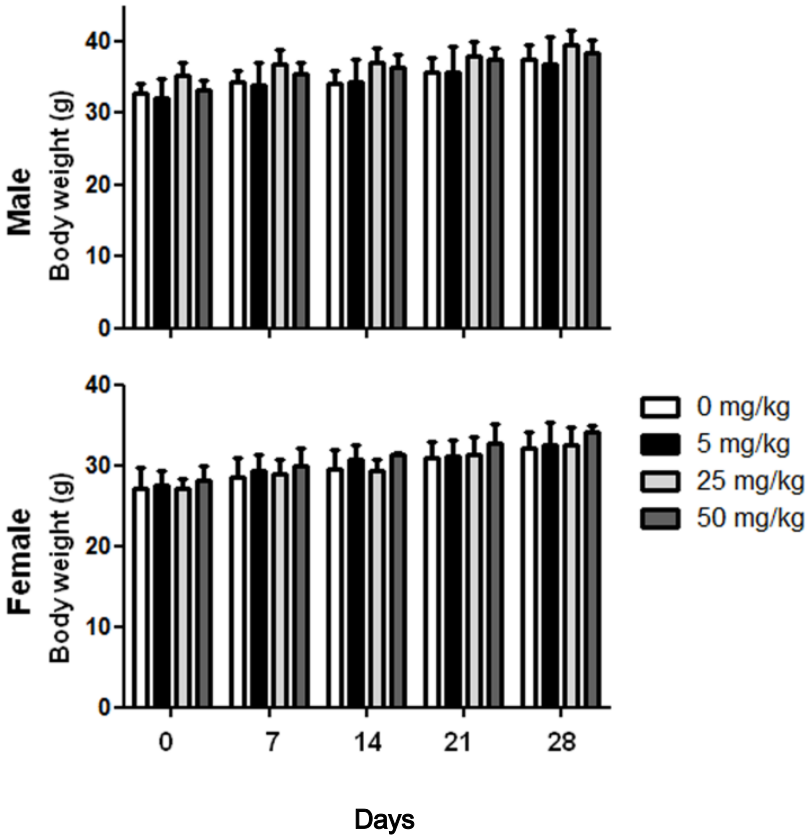

Figure 2. Effect of ZER on body weight changes in male and female mice in the 28-day repeated dose toxicity study. Data represented as mean and SD (n = 6). No statistical differences.

and repeated-dose general toxicity of ZER. In a previous study, we found that the combination of ZER and radiation showed synergistic antitumor effects as compared to ZER or radiation single treatment *in vivo* and *in vitro*. ZER produced cross-linking of HSP27, which is dependent on inhibition of the monomeric form of HSP27. ZER directly inserts between the disulfide bond in the HSP27 dimer and modifies normal HSP27 dimerization. Pretreatment with ZER before radiation inhibited the binding affinity between HSP27 and apoptotic molecules, such as cytochrome c and protein kinase C (PKC)δ, and induced sensitization *in vitro* and in an *in vivo* xenografted nude mouse system (Choi et al., 2011). It is well-known that overexpression of HSP27 in various tumors including breast, colorectal, ovarian, prostate cancers (Arrigo et al., 2007) increases malignancy and metastasis, and protects against cell death triggered by a number of stimuli (Lelj-Garolla and Mauk, 2005). However, in the case of HSP27, no strategies such as developing small molecules for HSP27 inhibitors in application to cancer therapy has been attempted, even though functional HSP27 inhibition may be a good strategy for combination therapy with chemotherapy agents or radiation. So, if it confirmed that ZER is safe as a HSP27 sensitizer with a therapeutic combination of chemotherapy and radiation, it would be a novel strategy for cancer therapy.

Our results showed no toxic effects on all organs including liver and kidney in both single and repeated dose of ZER. There are few reports which investigate the toxic effects of ZER *in vivo*, and are controversial. Ibrahim et al. (2010) showed that ZER causes severe renal and hepatic damage at the single dose of 500 mg/kg in Sprague Dawley rats, but not at the dose of 100 to 200 mg/kg. However, other group reported that several doses of ZER (15, 30, and 60 mg/kg for 11 weeks) protected rat livers in various indicators such as serum enzymes, proliferation and anti-apoptotic index of liver in the liver tumor model (Taha et al., 2010). And Fakurazi et al. (2008, 2009) reported that low concentration of ZER (0.05 to 0.5%) effectively mitigates liver damage induced by chemicals such as paracetamol and ethanol. Currently, it is also reported that ZER attenuates acute pancreatitis and pancreatitis-induced hepatic injury (Wenhong et al., 2012). We used ICR mice in our toxicity study and did not find toxicity in all organs including liver

Table 1. Absolute and relative major organ weights of mice in 28-day repeated dose toxicity of ZER.

Male (N=6)	Brain	Heart	Lung	Liver	Kidney	Spleen
Control	0.47±0.02 (12.4±0.6)	0.21±0.03 (5.4±0.7)	0.31±0.04 (8.2±1.1)	2.26±0.05 (59.4±1.32)	0.81±0.11 (21.3±1.3)	0.14±0.02 (3.6±0.5)
5 mg/kg	0.50±0.02 (13.0±0.6)	0.22±0.02 (5.7±0.5)	0.31±0.03 (8.1±0.7)	2.29±0.32 (59.8±8.3)	0.74±0.09 (19.3±2.2)	0.14±0.02 (3.7±0.5)
25 mg/kg	0.48±0.03 (12.4±0.7)	0.22±0.03 (5.7±0.8)	0.33±0.05 (8.3±1.2)	2.23±0.13 (57.0±3.3)	0.81±0.05 (20.7±1.3)	0.15±0.03 (3.8±0.8)
50 mg/kg	0.48±0.06 (12.5±1.4)	0.23±0.02 (6.1±0.5)	0.30±0.04 (7.8±0.9)	2.27±0.09 (58.9±2.2)	0.86±0.10 (22.4±2.7)	0.14±0.03 (3.7±0.8)
Female (N=6)						
Control	0.48±0.03 (14.3±0.9)	0.21±0.03 (6.3±0.9)	0.31±0.08 (9.2±2.3)	2.05±0.37 (61.4±11.0)	0.48±0.02 (14.3±0.6)	0.13±0.04 (4.2±1.2)
5 mg/kg	0.49 ± 0.06 (14.8±1.0)	0.21±0.04 (6.1±1.2)	0.30±0.04 (8.6±1.0)	2.20±0.14 (63.3±3.9)	0.50±0.04 (14.3±1.1)	0.16±0.03 (4.3±0.9)
25 mg/kg	0.48±0.05 (14.8±1.7)	0.21±0.05 (6.4±1.7)	0.29±0.04 (8.9±1.3)	2.01±0.31 (62.1±9.5)	0.46±0.06 (14.3±1.9)	0.14±0.03 (4.3±0.9)
50 mg/kg	0.50±0.05 (14.9±1.4)	0.20±0.03 (6.0±1.0)	0.30±0.05 (8.9±1.4)	2.04±0.23 (61.4±7.0)	0.50±0.02 (14.9±0.6)	0.15±0.03 (4.4±1.1)

No statistical difference. The unit of organ weight: g; ( ): relative organ weights were presented as the mg organ weight per 1 g body weight.

Table 2. Hematological values of mice in 28-day repeated dose toxicity of ZER.

Parameter	Male (N=6)			
	Control	5 mg/kg	25 mg/kg	50 mg/kg
RBC (10^6/µl)	7.05±1.33	7.21±2.14	7.63±0.63	7.19±1.00
Hb (g/dl)	13.1±2.0	14.2±3.2	13.5±1.3	13.3±1.7
HCT (%)	44.4 ±7.9	41.6±3.0	47.9±4.4	43.6±6.0
MCV (fL)	60.1±0.6	59.1±0.6	58.4±0.6	58.2±0.7
MCH (pg)	15.2 ±0.3	15.8±0.1	24.2±0.3	24.5±0.5
WBC (10^3/µl)	8.02 ±1.76	7.16±0.74	7.51±0.52	7.50±1.23
NE (%)	20.4 ±2.7	21.9±1.6	20.57±0.6	21.5±7.5
LY (%)	73.2±3.4	7.25±1.49	74.56±0.8	73.0±9.6
MO (%)	5.59±1.26	4.32±2.15	4.25±0.23	5.24±0.74
EO (%)	0.39±0.23	0.84±1.08	0.51±1.03	0.18±0.08
BA (%)	0.43 ±0.10	0.39±0.5	0.11±0.17	0.07±0.05
PLT (10^3/µl)	1226±341	982±308	1054±193	1300±272
	Female (N=6)			
	Control	**5 mg/kg**	**25 mg/kg**	**50 mg/kg**
RBC (10^6/µl)	9.59±0.54	9.84±1.44	9.17±1.26	9.72±1.60

Table 2. Contd.

Hb (g/dl)	14.6±0.7	15.6±2.4	16.9±2.1	16.1±2.3
HCT (%)	38.1±3.0	40.1±9.1	37.6±7.9	38.6±9.6
MCV (fL)	60.5±0.6	61.0±0.6	60.5±0.5	59.9±0.8
MCH (pg)	15.2±0.3	15.8±0.3	15.1±0.3	15.0±0.3
WBC (10^3/μl)	6.11±2.10	6.48±1.78	6.49±0.70	6.74±1.69
NE (%)	26.4±2.9	26.1±5.4	21.2±1.9	24.3±8.4
LY (%)	69.0±3.3	68.4±7.4	73.6±3.0	70.4±10.7
MO (%)	2.13±0.88	3.54±1.74	3.43±1.57	3.46±1.99
EO (%)	1.15±0.60	1.53±1.10	0.98±0.61	1.47 ±0.91
BA (%)	0.26±0.20	0.39±0.34	0.72±0.37	0.35±0.26
PLT (10^3/μl)	1301±284	836±405	1121±367	1153±543

No statistical difference.

Table 3. Biochemical parameters in mice after 28-day repeated administration of different dose of ZER.

Group	General parameter				Renal function parameter			
	GLU	P value	TCH	P value	CRE	P value	BUN	P value
Male								
Control	309±62	-	158±26	-	0.24±0.09	-	26.7±7.5	-
5 mg/kg	311±82	0.970	126±20	0.115	0.17±0.06	0.258	22.5±2.0	0.393
25 mg/kg	332±53	0.609	144±31	0.505	0.17±0.12	0.349	21.1±3.8	0.285
50 mg/kg	297±45	0.777	138±19	0.303	0.13±0.06	0.119	20.1±4.3	0.220
Female								
Control	206±14	-	122±20		0.13±0.05	-	25.7±1.4	-
5 mg/kg	219±51	0.561	107±24	0.307	0.12±0.04	0.662	24.6±2.8	0.415
25 mg/kg	202±17	0.652	107±14	0.206	0.10±0.02	0.186	24.2±3.0	0.829
50 mg/kg	208±35	0.927	108±40	0.473	0.10±0.01	0.186	25.8±1.4	0.931

	Hepatic function parameter							
	TP	p value	AST	p value	ALT	p value	ALP	p value
Male								
Control	5.52±0.48	-	49.6±10.0	-	30.2±7.0	-	235±36	-

Table 3. Contd.

5 mg/kg	5.27±0.32	0.452	45.7±8.3	0.591	31.0±2.7	0.859	234±27	0.967
25 mg/kg	5.47±0.40	0.877	55.3±19.7	0.596	36.0±1.7	0.219	194±49	0.212
50 mg/kg	5.73±0.29	0.516	44.7±7.2	0.490	31.3±9.1	0.848	218±59	0.638
Female								
Control	5.67±0.23	-	62.3±9.4	-	24.0±2.1	-	258±19	-
5 mg/kg	5.52±0.13	0.245	63.2±7.6	0.872	27.6±8.4	0.334	283±71	0.420
25 mg/kg	5.80±0.28	0.413	61.0±3.1	0.770	27.0±8.7	0.430	243±33	0.387
50 mg/kg	5.46±0.29	0.221	56.0±2.9	0.161	24.2±6.0	0.940	251±28	0.161

The values represent the mean and the standard deviation (SD). P values were calculated in relation to control.

and kidney. Most antitumor studies using ZER in mice induced tumors with the exception of the liver (Abdelwahab et al., 2010; Kim et al., 2009; Murakami et al., 2004). Mouse might have more tolerance to drug toxicity than rats (Finch et al., 1998; McKenna et al., 1997). We also investigated toxicity in ZER treated mice bearing human lung cancer cells at the autopsy and noticed no drug-related toxicity in preliminary study (Data not shown). Therefore, our results suggested that ZER at the dose of 500 mg/kg as a single treatment and 700 mg/kg as total repeated doses did not produce any toxicity to mice. Further studies are needed to clarify ZER toxicity to rat at the effective dosage which can inhibit tumor growth.

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