



Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: <http://www.ginsengres.org>

Research Article

Genotoxicity and subchronic toxicological study of a novel ginsenoside derivative 25-OCH₃-PPD in beagle dogsWei Li^{1,2,*}, Xiangrong Zhang^{1,2,*}, Meng Ding¹, Yanfei Xin³, Yaoxian Xuan³, Yuqing Zhao^{1,2,*}¹ Department of Functional Food and Wine, Shenyang pharmaceutical University, Shenyang, China² Key Laboratory of Structure-Based Drug Design & Discovery of Ministry of Education, Shenyang, China³ Center of Safety Evaluation, Zhejiang Academy of Medical Sciences, Hangzhou, China

ARTICLE INFO

Article history:

Received 27 October 2016

Received in Revised form

30 August 2017

Accepted 25 May 2018

Available online 31 May 2018

Keywords:

Beagle dog

Subchronic toxicity

Ginsenoside

ABSTRACT

Background: Ginsenosides have been widely used clinically for many years and were regarded as very safe. However, a few researches on the toxicities of these kinds of agents showed that some ginsenosides may have side-effect on the rats or dogs. So it is extremely necessary to further clarify the potential toxicity of ginsenosides. This study was carried out to investigate long-term toxicity and genotoxicity of 25-methoxydammarane-3, 12, 20-triol (25-OCH₃-PPD), a new derivative of ginsenoside, in beagle dogs. **Methods:** Twenty-four beagle dogs were divided randomly into four treatment groups and repeatedly orally administered with 25-OCH₃-PPD capsule at 60, 120, and 240 mg/kg/day for 91 consecutive days. Ames, micronucleus, and chromosomal aberration tests were established to analyze the possible genotoxicity of 25-OCH₃-PPD.

Results: There was no 25-OCH₃-PPD-induced systemic toxicity in beagle dogs at any doses. The level of 25-OCH₃-PPD at which no adverse effects were observed was found to be 240 mg/kg/day. The result of Ames test showed that there was no significant increase in the number of revertant colonies of 25-OCH₃-PPD administrated groups compared to the vehicle control group. There were also no significant differences between 25-OCH₃-PPD administrated groups at all dose levels and negative group in the micronucleus test and chromosomal aberration assay.

Conclusion: The highest dose level of 25-OCH₃-PPD at which no adverse effects were observed was found to be 240 mg/kg per day, and it is not a genotoxic agent either in somatic cells or germs cells. 25-OCH₃-PPD is an extremely safe candidate compound for antitumor treatment.

© 2018 The Korean Society of Ginseng, Published by Elsevier Korea LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Cancer is one of the world's most serious illnesses whose mortality rate is second only to cardiovascular disease. With the advancement of therapeutic interventions such as surgery, chemotherapy, and radiotherapy, the survival rate of cancer patients has increased. Despite the progress of such treatments, the side-effects

of patient intolerance and the destruction of healthy cells are something that cannot be overlooked. Consequently, there has been an increased focus on investigating agents that have the potential to be more effective and less toxic from the Traditional Chinese Medicine [1]. Ginsenoside and notoginsenoside are the main constituents in the roots, leaves, and seeds of the plant *Panax ginseng* and *Panax notoginseng*. These derivatives have been shown to have activities

Abbreviations: 25-OCH₃-PPD, 25-methoxydammarane-3, 12, 20-triol; micronucleated polychromatic erythrocytes, MNPCE; polychromatic erythrocytes, PCE; normochromatic erythrocytes, NCE; mean corpuscular hemoglobin, MCH; Erythrocyte count, RBC; white blood cells count, WBC; mean corpuscular volume, MCV; hemoglobin concentration, HGB; hemoglobin concentration distribution width, HDW; hematocrit, HCT; red cell distribution width, RDW%; mean corpuscular hemoglobin concentration, MCHC; mean platelet volume, MPV; platelets, PLT; basophils, BASO; lymphocytes, LYMPH; neutrophil cell, NEUT; eosinophils, EOS; monocytes, MONO; reticulocyte count, RETIC; prothrombin time, PT; alanine aminotransferase, ALT; aspartate aminotransferase, AST; alkaline phosphatase, ALP; gamma-glutamyl transferase, γ -GT; creatinine, Crea; albumin, ALB; urea nitrogen, BUN; total triglyceride, TG; creatine phosphokinase, CK; glucose, GLU; total protein, T.P; total cholesterol, T.CHO; total bilirubin, T.BIL; total calcium, TCa; sodium, Na; potassium, K; chloride, Cl; SPSS, statistical package for social sciences.

* Corresponding author. Department of Functional Food and Wine, Shenyang Pharmaceutical University, Shenyang, 110016, China.

E-mail address: zyq4885@126.com (Y. Zhao).

* These authors contributed equally to this work.

<https://doi.org/10.1016/j.jgr.2018.05.005>

p1226-8453 e2093-4947/\$ – see front matter © 2018 The Korean Society of Ginseng, Published by Elsevier Korea LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

against many illnesses such as diabetes [2–4], delayed onset muscle soreness [5], diet-induced hypercholesterolemia [6], myocardial ischemia [7], inflammation [8], and atherosclerosis [9]. Notably attracting the attention of scientists, these constituents also have effects on a spectrum of cancers such as breast [10,11], colon [12], colorectal [13], cervical [14], prostate [15], liver [16], and lung cancers [17]. Investigations on the structure–activity relationship with ginsenoside show that the number of the sugars substituted on the basic skeleton and the type of glycoside play an important part to the anticancer activities of ginsenosides [18]. Researches have shown that these antitumor effects were inversely correlated to the number of sugar moieties [19].

25-methoxydammarane-3, 12, 20-triol (25-OCH₃-PPD) is a new derivative of ginsenoside first obtained from *P. notoginseng* and has been shown to exhibit antitumor activities against various human cancer cells lines such as breast, prostate, lung, and colorectal cancer cell lines [1,20–23]. In the previous studies, 25-OCH₃-protopanaxadiol (PPD) has been demonstrated to have the highest cytotoxic activity among all the tested ginsenosides (PPD and Rg₃). The half maximal inhibitory concentration (IC₅₀) of this compound for most cell lines were 5- to 15-fold lower than that of 20(S)-protopanaxadiol and 10- to 100-fold lower than that of Rg₃ [20], a newly clinically used ancillary drug in cancer treatment in China [24]. Initial mechanistic examination with prostate and lung cancer cell lines revealed that 25-OCH₃-PPD could inhibit proliferation, reduce survival, and induce cell apoptosis as well as cycle arrest in G1 phase and reactive oxygen species (ROS) production on human LNCaP and PC3 prostate cancer cells and human A549, H358, and H838 lung cancer cells [22,25]. Additionally, 25-OCH₃-PPD upregulated the phosphorylation levels of extracellular signal-regulated kinase (ERK) and p38 and downregulated the expression of cyclin D1, cyclin E, and MDM2 dose-dependently, which associated with the proliferative inhibition [26]. Moreover, 25-OCH₃-PPD dose-dependently inhibited the growth of prostate cancer and lung cancer xenograft tumors *in vivo* without affecting normal cell viability [25,26]. The mechanistic examination of 25-OCH₃-PPD in colorectal cancer indicated that it wields its anticancer effect by targeting β -catenin signaling, a key mediator in the Wnt pathway. β -catenin/T cell factor (TCF) transcriptional activity was also significantly suppressed [23]. Pharmacokinetics investigation in rats showed that the major metabolite of this agent was 25-OH PPD, and the metabolic pathway is considered to be phase I [27]. The CYP3A4 is one of the important enzymes in the metabolic process of C-20 hydroxyl group of 25-OCH₃-PPD [28]. In our previous studies, the oral long-term toxicity of 25-OCH₃-PPD in Sprague Dawley rats was reported [29]. Although some statistical changes were seen in the hematology, biochemistry, and organ weights relative to the vehicle control group, the parameters were within the normal limits and the histopathological examination show no abnormal responses. The highest oral dose level in rats with no toxic response after treated with 25-OCH₃-PPD was considered to be 600 mg/kg per day. Taking into account the different species and as an important part of preclinical safety evaluation, the genotoxicity and subchronic toxicity studies in beagle dogs were conducted. The new results of this article will be necessary and helpful to determine a suitable dose of 25-OCH₃-PPD for clinical applications.

2. Materials and methods

2.1. Ethics statements

All animal work in this study was conducted under the Good Laboratory Practice of P.R. China. All animals involved received human care in compliance with accredited facilities of the Chinese Association for Accreditation of Laboratory Animal Care. Our institutional animal care and use committee, in conjunction with

the veterinary staff, created a comprehensive environmental enrichment program that included social housing and social interaction with caretakers.

2.2. Subchronic toxicological study in beagle dog

2.2.1. Compound and animals

25-OCH₃-PPD (Fig. 1), with 99.0% pure, was prepared by our laboratory. The compound 25-OCH₃-PPD, weighing accurately, was put into hard capsule according to the dose level.

Twenty-four beagle dogs (12 of each sex) aged approximately 4–6 months and weighing between 5.5 and 7.5 kg each were obtained from the Zhejiang Laboratory Animal Center (Hangzhou, China; No. 0014658). Each dog was kept in a stainless steel cage separately. Other feeding conditions were as following: room temperature: 20–22°C; relative humidity: 45–65%; air ventilation: 15 times/h; light condition: 12-h light/dark cycles. Body weight, temperature, consumption, and clinical signs of all the dogs were recorded for 2 weeks before experiment. Hematology and biochemistry were conducted twice and urine, electrocardiography (ECG), and ophthalmic examination for one time during this period. Dogs that showed no abnormality of all above indexes were selected for the study.

2.2.2. Treatment schedule

All the dogs were divided randomly into four treatment groups (6 dogs/group): one control group (empty capsules) and three treatment groups administered orally with 25-OCH₃-PPD at 60, 120, and 240 mg/kg. The dose was selected based on the effective dose and the previous toxicity study. In the pharmacodynamic test, the effective dose was 20 mg/kg in mice [28] which calculated for 3.5 mg/kg in dogs. The administration doses 240, 120, and 60 mg/kg were respectively about 68-, 34-, and 17-fold the effective dose. For all the groups, two dogs were sacrificed after treatment for 46 days, another two dogs were sacrificed after treatment for 90 days, and the remaining dogs (two dogs) were autopsied after 4 weeks of recovery.

2.2.3. Clinical observations, body temperature, and weight

All beagle dogs were recorded for the mortality, gross toxicity, and clinical signs (including but not limited to the appearance, skin, and fur, behavior patterns, manure and urine, nausea, and vomiting) once a day during the whole experiment period. Body temperature and weight were taken once a week. Standard veterinary health-care practice was conducted to keep the dogs healthy during this experiment.

2.2.4. Electrocardiography and eye examination

Electrocardiograms were recorded using an Auto Electrocardiogram (SP2006, technical company, Beijing, P.R. China). All the

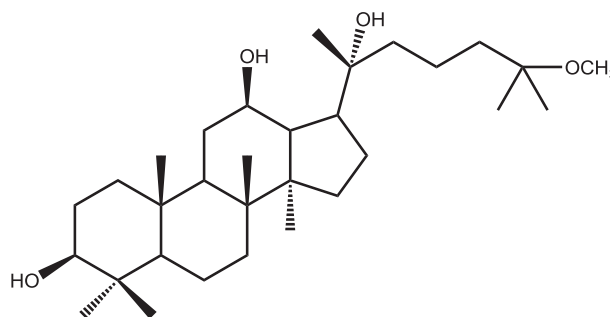


Fig. 1. Chemical structure of 25-OCH₃-PPD. 25-OCH₃-PPD, 25-methoxydammarane-3, 12, 20-triol.

dogs received this examination once every 2 weeks. The ECG parameters included Q-T interval, P-R interval, Q-T ratio, heart rate, QRS and S-T segment, II lead of P and QRS wave time, and wave voltage of P, R, and T.

The YZ6E1 ophthalmoscope (He, Suzhou, P.R. China) was used directly to detect the conjunctiva, cornea, pupil lenses, vitreous humor, retina, and optic papilla.

2.2.5. Urinalysis

Autoanalyzer H-800 (Dirui, Jilin, China) was used to test the urine samples. The parameters measured were bilirubin, pH, protein, vitamin C, occult blood, leukocytes, specific gravity, nitrite, and glucose. The urine color and transparency were recorded as well.

2.2.6. Hematology and biochemistry

Blood samples were obtained from dogs on Day 0, 46, and 91 of the study and again after recovery on Day 120 and collected into vacuum tube containing EDTA-K2. Autohematoanalyzer (ADVIA2120, Bayer, Germany) and automated coagulation analyzer (CA-560, Sysmex, Japan) were used to evaluate approximately 20 parameters about the hematology.

An electrolyte analyzer (Xilaiheng IMS-972, Shenzhen, China) and automatic serum analyzer (Hitachi 7020, Tokyo, Japan) were used to assess 17 blood chemistry parameters.

2.2.7. Pathological examination

For pathological examinations, two dogs (one of each sex) were euthanized respectively on Day 46, 91, and 120 (after 4 weeks of recovery). The appearance and the weights of all the organs including the kidneys, adrenals, brain, testes, heart, spleen, liver, thymus, lungs, epididymis, uterus, and ovaries were recorded in detail. About 33 tissues were obtained, paraffin embedded, and stained with hematoxylin and eosin. The pathology slices were then examined microscopically. All the methods used were carried out in accordance with the standard operation procedure (SOP) for laboratory animal use as outlined by the Zhejiang Institutional Experimental Animal Care and Use Committee (histopathological observation, SOP-ZJGLP-CZ03-47/1; pathological camera SOP-ZJGLP-CZ03-46/1).

2.3. Genotoxicity study

2.3.1. Bacterial reverse mutation test (Ames test)

Five strains of histidine auxotrophic *Salmonella typhimurium* including TA97, TA9, TA100, TA102, and TA1535 were used to test the mutagenic effect with or without S9 mix. Dose range was selected based on the results of preliminary work on TA100. The dose at 1,500 and 150 $\mu\text{g}/\text{plate}$ showed no obvious antibacterial and reverse mutation effect. Therefore, the 1,500 $\mu\text{g}/\text{plate}$ was selected as the highest dose level, and 25-OCH₃-PPD was assayed in five different concentrations of 1,500, 150, 15, 1.5, and 0.15 $\mu\text{g}/\text{plate}$. If a 2-fold increase in the number of revertants compared with the negative control was observed, the result was regarded as positive.

2.3.2. Micronucleus assay

Six male Institute of Cancer Research (ICR) mice with body weight from 17.0 to 19.0 g per dose were used for this experiment. The dose levels of 25-OCH₃-PPD were 2,400, 1,200, and 600 mg/kg according to the previous acute toxicology experiment on ICR mice with the highest dose level at 2,400 mg/kg [29]. Cyclophosphamide, 50 mg/kg, and 0.5% CMC-Na were selected as positive control and blank control, respectively. All the animals were sacrificed after 24 h of administration. The bone marrow was collected, fixed with menthol, and stained with 5% Giemsa [30]. 2,000 polychromatic erythrocytes (PCEs) per animal were scored using double-blind

method to determine the clastogenic/aneugenic property for the analysis of the micronucleated cells. PCE/(PCE + normochromatic erythrocyte) value in 200 erythrocytes/animal was calculated to assess the cytotoxicity of 25-OCH₃-PPD. If the ratio showed a significant dose response increase at one tested point or one dose group, the result was considered to be positive. The mean number of micronucleated PCEs in individual dog was used as the experimental unit, with variability (standard deviation) based on differences among animals within the same group.

2.3.3. Chromosomal aberration assay

The preliminary cytotoxicity assay of 25-OCH₃-PPD on Chinese Hamster lung fibroblast cells was calculated with IC₅₀ at 8.188 $\mu\text{g}/\text{ml}$. According to the Testing Guidelines for genotoxicity study of Drugs (Notification [ZH] GPT2-1) issued by the China Food and Drug Administration on Oct 2007, the highest dose level was selected as 10 $\mu\text{g}/\text{ml}$, which was slightly higher than the IC₅₀. Therefore, the dose ranges of 25-OCH₃-PPD were selected as 10, 5, 2.5, and 1.25 $\mu\text{g}/\text{ml}$. The Chinese Hamster lung cells were seeded on a plastic plate and incubated in a CO₂ incubator with 25-OCH₃-PPD of different concentrations in the presence and absence of S9 mix. Cyclophosphamide and mitomycin C were used as positive controls with 40 $\mu\text{g}/\text{ml}$ and 0.1 $\mu\text{g}/\text{ml}$, respectively. Colcemid was then added to the cell, incubated 2h, and harvested. The mixture with 5% Giemsa and phosphate buffer (1:9) was used to stain the cells for 20 minutes. All the chromosome deletion, exchange, and break were recorded.

2.4. Statistical analysis

All of the data are expressed as mean \pm standard deviation. The SPSS software, version 13.0, was used for the data analysis. One-way analysis of variance was performed, and the significant differences were confirmed by the Dunnett's test. *P* values of less than 0.05 and 0.01 were used to indicate the statistical significance in this study.

3. Results

3.1. The results of subchronic toxicity in beagle dogs

3.1.1. Clinical observations, body temperature, and weight

There was no death of animals during the whole experiment in all groups. In both the experimental and control groups, no obvious abnormalities were observed. All animals survived the length of the experiment and all were necropsied at the appropriate time point. The body weights were recorded with no statistical difference between compound 25-OCH₃-PPD groups and control group (Fig. 2A). All the tested indexes of ECG were all in the normal range. Furthermore, the body temperatures of animals were fluctuated in a normal range of 37.5–39.7°C (Fig. 2B). No statistical differences in food consumption and drinking patterns were observed with 25-OCH₃-PPD at various doses when compared to the control group (Fig. 2C).

3.1.2. Electrocardiography and eye examination

All the tested indexes of ECG were all in the normal range. The results of ophthalmoscopic examinations of conjunctiva, cornea, pupils' lenses, vitreous humor, retina, and optic papilla of the dogs appeared normal throughout the experiment regardless of doses.

3.1.3. Urine analysis

There were no significant differences observed in urine analysis between the control group and 25-OCH₃-PPD treatment groups for all the tested items (data not shown).

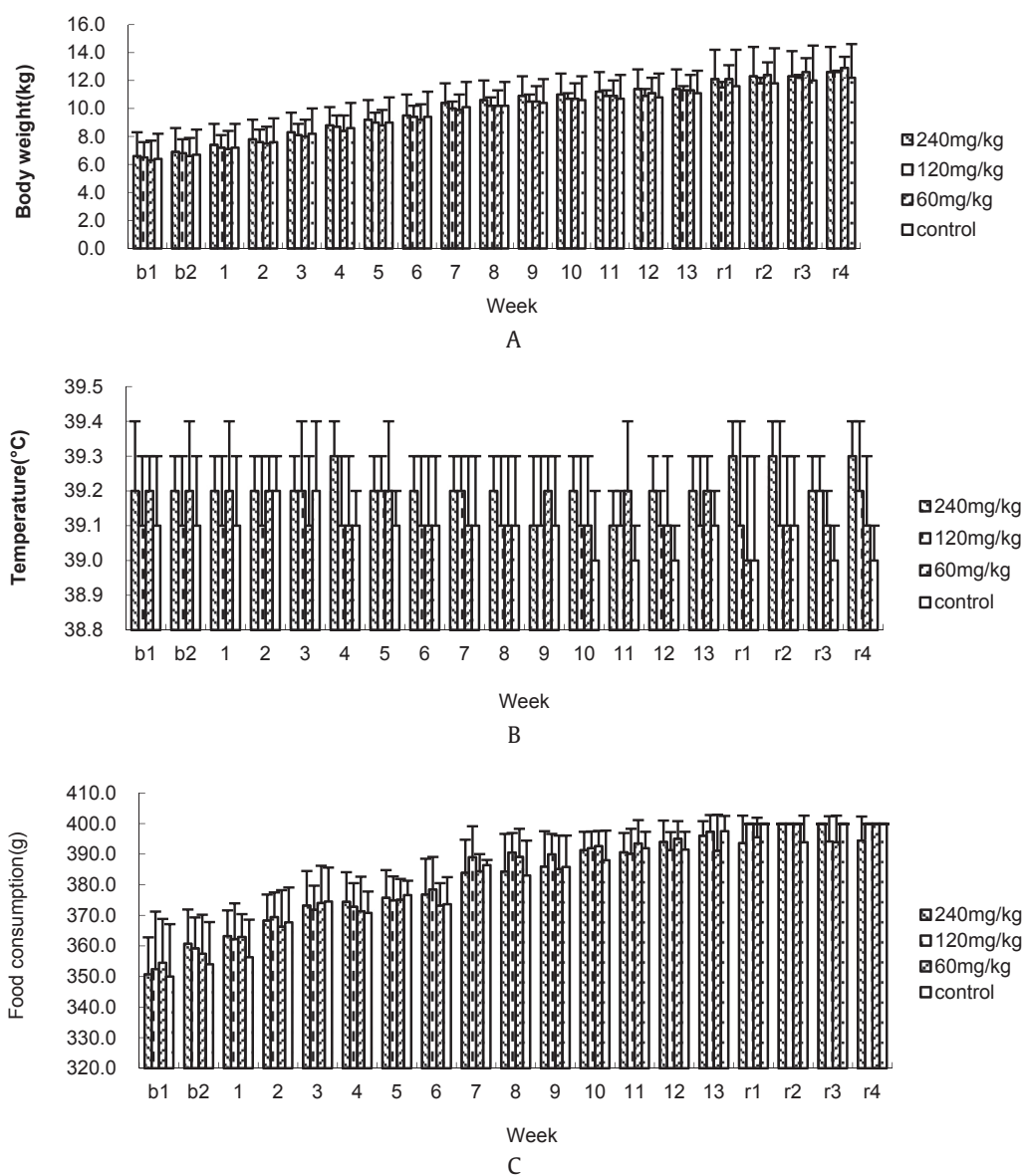


Fig. 2. The body weight change, temperature, and food consumption of beagle dogs during administration of different concentrations of 25-OCH₃-PPD. (A) Mean body weight of beagle dogs throughout the study. (B) Mean temperature of beagle dogs throughout the study. (C) Mean food consumption of beagle dogs for 13 weeks and 4 weeks recovery. 25-OCH₃-PPD, 25-methoxydammarane-3, 12, 20-triol.

3.1.4. Hematological and biochemical analysis

The hematological data were analyzed on Day 0, 46, 91, and 120 after administering 25-OCH₃-PPD and summarized in Table 1. On Day 46, the value of eosinophils and mean corpuscular hemoglobin (MCH) were decreased at 240 mg/kg/day and 60 mg/kg/day group, and mean corpuscular volume could also be observed, decreased both at 120 mg/kg/day and 60 mg/kg/day group. On Day 91, reticulocyte count (RETIC) at 240 mg/kg/day group and basophils, RETIC, mean haemoglobin concentration of reticulocytes (CHCMr) at 120 mg/kg/day group and CHCMr at 60 mg/kg/day group were decreased, while mean platelet volume was increased at 240 mg/kg/day group. On Day 120, RETIC was increased at 60 mg/kg/day group, while MCH and CH and red cell distribution width were decreased at 60 mg/kg/day and 120 mg/kg/day group, respectively.

In the serum biochemical analysis (Table 2), on Day 46, a decrease in the levels of TP, urea nitrogen, and creatinine (Crea) at high dose

group and glucose at high- and mid-dose group could be observed. On Day 91, total calcium and glucose were increased at high-dose group, while gamma-glutamyl transferase was decreased at mid-dose group. On Day 120, aminotransferase and Crea at high-dose group, TBIL at mid-dose group, and alkaline phosphatase at low-dose group were increased, while gamma-glutamyl transferase and Na⁺ at mid-dose group and alkaline phosphatase at low- and high-dose group were decreased. Other biochemical parameters presented no statistically significant changes.

3.1.5. Absolute organ weights, relative organ weights, and histopathological examination

There was an observed decrease in the absolute organ weights (Table 3) of the thymus of 25-OCH₃-PPD treatment group at 60 mg/kg/day ($p < 0.05$) and 120 mg/kg/day ($p < 0.01$) groups on Day 46 and 120 mg/kg/day group ($p < 0.01$) on. In addition, the relative organ weights (Table 4) of the thymus at doses of 120 mg/kg/day

Table 1
Hematological parameters in beagle dogs treated with 25-OCH₃-PPD for 13 weeks administration and 4 weeks recovery

Item	Control	60 mg/kg	120 mg/kg	240 mg/kg
<i>Day 0</i>				
WBC (10 ⁶ /μL)	15.34 ± 3.22	15.75 ± 4.32	16.14 ± 4.83	14.32 ± 3.90
RBC (10 ⁶ /μL)	5.86 ± 0.98	5.75 ± 4.32	5.66 ± 0.36	5.82 ± 0.66
HGB (g/dL)	114 ± 16	112 ± 12	113 ± 10	115 ± 11
HCT (%)	35.8 ± 4.8	35.1 ± 3.4	35.4 ± 3.1	36.2 ± 3.9
MCV (fl)	61.3 ± 2.8	61.1 ± 1.2	62.6 ± 1.7	62.3 ± 2.8
MCH (pg)	19.4 ± 0.7	19.6 ± 0.4	19.9 ± 0.6	19.8 ± 1.1
MCHC	317 ± 4	320 ± 4	318 ± 2	318 ± 5
CHCM	324 ± 5	325 ± 8	320 ± 3	322 ± 4
CH (pg)	19.8 ± 0.7	19.7 ± 0.3	19.9 ± 0.5	20.0 ± 0.9
RDW (%)	15.5 ± 1.6	15.8 ± 1.9	15.0 ± 1.4	15.6 ± 0.6
HDW (g/dL)	21.1 ± 1.2	21.4 ± 1.2	20.5 ± 0.8	20.8 ± 0.9
PLT (10 ³ /μL)	223 ± 82	222 ± 55	257 ± 84	261 ± 83
MPV (fl)	10.0 ± 1.0	10.4 ± 0.6	9.1 ± 0.9	9.5 ± 1.0
PT (s)	7.6 ± 0.5	7.6 ± 0.4	7.5 ± 0.4	7.7 ± 0.4
NEUT (10 ³ /μL)	5.7 ± 1.1	5.7 ± 1.2	7.0 ± 3.7	7.0 ± 3.3
LYMPH (10 ³ /μL)	7.2 ± 2.6	8.3 ± 3.6	7.0 ± 3.4	5.5 ± 1.4
MONO (10 ³ /μL)	0.94 ± 0.34	0.79 ± 0.11	0.93 ± 0.38	0.82 ± 0.33
EOS (10 ³ /μL)	1.17 ± 0.35	0.62 ± 0.18**	1.00 ± 0.51	0.68 ± 0.31*
BASO (10 ³ /μL)	0.14 ± 0.04	0.13 ± 0.04	0.13 ± 0.08	0.15 ± 0.04
RETIC (10 ⁹ /L)	129.3 ± 42.1	124.3 ± 28.0	119.1 ± 33.6	127.0 ± 33.6
<i>Day 46</i>				
WBC (10 ⁶ /μL)	14.61 ± 3.43	16.36 ± 3.47	14.28 ± 1.09	13.42 ± 1.91
RBC (10 ⁶ /μL)	6.08 ± 0.73	6.73 ± 0.89	6.40 ± 0.60	6.50 ± 0.56
HGB (g/dL)	127 ± 14	134 ± 14	130 ± 13	135 ± 12
HCT (%)	38.3 ± 4.2	40.2 ± 4.4	39.1 ± 4.3	40.6 ± 3.5
MCV (fl)	63.1 ± 1.9	60.0 ± 2.0*	60.9 ± 1.1*	62.6 ± 1.2
MCH (pg)	20.9 ± 0.7	20.0 ± 0.6*	20.4 ± 0.5	20.8 ± 0.6
MCHC	332 ± 9	333 ± 4	334 ± 9	332 ± 6
CHCM	326 ± 3	329 ± 3	327 ± 5	326 ± 3
CH (pg)	20.5 ± 0.6	19.7 ± 0.7	19.8 ± 0.5	20.3 ± 0.5
RDW (%)	13.0 ± 0.6	13.8 ± 0.9	12.9 ± 0.7	13.1 ± 0.6
HDW (g/dL)	20.0 ± 0.6	20.8 ± 1.1	19.9 ± 1.3	19.6 ± 1.0
PLT (10 ³ /μL)	307 ± 127	262 ± 46	315 ± 76	314 ± 108
MPV (fl)	8.6 ± 1.2	8.7 ± 1.8	8.8 ± 1.7	9.5 ± 1.2
PT (s)	6.4 ± 0.3	6.2 ± 0.4	6.7 ± 0.3	6.3 ± 0.3
NEUT (10 ³ /μL)	5.6 ± 1.7	6.9 ± 4.3	5.9 ± 1.5	6.9 ± 1.3
LYMPH (10 ³ /μL)	6.6 ± 1.9	7.3 ± 2.6	6.5 ± 1.6	5.0 ± 0.8
MONO (10 ³ /μL)	0.79 ± 0.22	0.80 ± 0.46	0.78 ± 0.16	0.72 ± 0.17
EOS (10 ³ /μL)	1.40 ± 0.47	1.19 ± 1.15	0.97 ± 0.52	0.59 ± 0.09**
BASO (10 ³ /μL)	0.09 ± 0.02	0.09 ± 0.03	0.08 ± 0.02	0.08 ± 0.03
RETIC (10 ⁹ /L)	87.1 ± 23.3	72.9 ± 25.8	97.7 ± 80.1	115.0 ± 99.4
<i>Day 91</i>				
WBC (10 ⁶ /μL)	12.77 ± 2.36	15.14 ± 2.94	14.61 ± 3.00	13.14 ± 4.08
RBC (10 ⁶ /μL)	7.52 ± 1.08	7.18 ± 0.92	6.33 ± 0.50	6.38 ± 0.44
HGB (g/dL)	158 ± 18	147 ± 17	131 ± 13	135 ± 12
HCT (%)	46.7 ± 5.5	43.7 ± 5.3	39.0 ± 4.0	40.1 ± 3.4
MCV (fl)	62.4 ± 2.7	61.0 ± 0.8	61.4 ± 1.4	62.8 ± 2.1
MCH (pg)	21.0 ± 0.8	20.5 ± 0.4	20.6 ± 0.5	21.1 ± 0.7
MCHC	337 ± 4	336 ± 4	335 ± 4	335 ± 2
CHCM	319 ± 5	322 ± 3	321 ± 6	324 ± 5
CH (pg)	19.9 ± 0.9	19.6 ± 0.3	19.6 ± 0.2	20.3 ± 0.6
RDW (%)	12.9 ± 1.1	13.3 ± 0.3	12.4 ± 0.3	12.6 ± 0.5
HDW (g/dL)	19.2 ± 1.4	19.6 ± 0.4	19.3 ± 0.6	19.5 ± 0.8
PLT (10 ³ /μL)	285 ± 101	198 ± 81	204 ± 41	232 ± 65
MPV (fl)	7.0 ± 0.3	8.0 ± 1.0	7.5 ± 0.6	7.6 ± 0.4*
PT (s)	6.6 ± 0.4	6.7 ± 0.5	6.7 ± 0.2	6.4 ± 0.3
NEUT (10 ³ /μL)	5.8 ± 1.8	6.3 ± 2.6	7.6 ± 4.0	6.6 ± 2.9
LYMPH (10 ³ /μL)	4.7 ± 0.6	7.3 ± 0.3**	4.9 ± 0.9	4.7 ± 0.9
MONO (10 ³ /μL)	0.57 ± 0.21	0.63 ± 0.20	0.58 ± 0.08	0.71 ± 0.29
EOS (10 ³ /μL)	1.44 ± 0.54	0.62 ± 0.16*	1.35 ± 0.68	0.92 ± 0.69
BASO (10 ³ /μL)	0.14 ± 0.03	0.14 ± 0.02	0.07 ± 0.01**	0.08 ± 0.02*
RETIC (10 ⁹ /L)	77.7 ± 15.3	67.6 ± 12.7	50.1 ± 8.2*	33.8 ± 11.7**
<i>Day 120</i>				
WBC (10 ⁶ /μL)	14.03 ± 0.93	16.32 ± 3.61	11.25 ± 1.55	14.21 ± 1.38
RBC (10 ⁶ /μL)	7.24 ± 0.12	7.30 ± 0.93	6.94 ± 0.37	7.42 ± 0.18
HGB (g/dL)	166 ± 1	157 ± 18	157 ± 18	169 ± 6
HCT (%)	57.0 ± 0.7	54.7 ± 6.6	54.7 ± 6.0	58.4 ± 3.0
MCV (fl)	78.8 ± 2.3	74.9 ± 0.6	78.6 ± 4.4	78.7 ± 2.1
MCH (pg)	22.9 ± 0.2	21.5 ± 0.4*	22.6 ± 1.4	22.7 ± 0.3
MCHC	291 ± 6	287 ± 2	287 ± 2	289 ± 4
CHCM	272 ± 4	270 ± 0	266 ± 1	270 ± 6
CH (pg)	21.4 ± 0.4	20.1 ± 0.1*	20.8 ± 1.1	21.2 ± 0.1
RDW (%)	13.4 ± 0.1	13.9 ± 0.9	13.0 ± 0.1*	13.1 ± 0.4

Table 1 (continued)

Item	Control	60 mg/kg	120 mg/kg	240 mg/kg
HDW (g/dL)	15.1 ± 0.6	16.1 ± 1.4	14.8 ± 0.8	14.6 ± 1.3
PLT (10 ³ /μL)	250 ± 24	173 ± 41	298 ± 70	240 ± 4
MPV (fl)	10.4 ± 1.1	13.3 ± 3.7	11.7 ± 1.3	11.6 ± 2.3
PT (s)	5.9 ± 0.5	6.0 ± 0.2	5.9 ± 0.4	6.4 ± 0.1
NEUT (10 ³ /μL)	7.8 ± 0.1	8.5 ± 2.9	5.6 ± 2.6	7.8 ± 0.1
LYMPH (10 ³ /μL)	4.8 ± 0.9	6.0 ± 0.0	4.0 ± 0.5	4.9 ± 0.9
MONO (10 ³ /μL)	0.68 ± 0.09	0.85 ± 0.16	0.57 ± 0.03	0.69 ± 0.04
EOS (10 ³ /ul)	0.57 ± 0.01	0.67 ± 20.52	0.81 ± 0.47	0.55 ± 0.33
BASO (10 ³ /μL)	0.14 ± 0.03	0.12 ± 0.02	0.08 ± 0.01	0.08 ± 0.04
RETIC (10 ⁹ /L)	41.7 ± 2.1	57.4 ± 1.1*	33.0 ± 7.5	48.5 ± 17.8

BASO, basophils; CH, corpuscular hemoglobin content; CHCM, corpuscular hemoglobin concentration mean; EOS, eosinophils; HCT, hematocrit; HDW, hemoglobin concentration distribution width; HGB, hemoglobin concentration; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume, MONO, monocytes; MPV, mean platelet volume; NEUT, neutrophil cell; PLT, platelets; PT, prothrombin time; RETIC, reticulocyte count; RBC, erythrocyte count; RDW, red cell distribution width; SD, standard deviation; WBC, white blood cell count.

The data represent mean ± SD.

**p* < 0.05.

***p* < 0.01.

The normal range: MCV (51.2~71.40); MCH (16.3~30.4); CH (16.7~24.6); RDW (7.2~22.9); MPV (7.0~49.3); LYMPH (0.18~10.02); BASO (0~0.1); EOS (0.06~2.52); RETIC (17.4~223.1).

(*p* < 0.05) on Day 46 could also be observed. However, the histopathological examination (Fig. 3) revealed no 25-OCH₃-PPD-related pathological changes.

3.2. The results of genotoxicity

3.2.1. Bacterial reverse mutation assay

No significant increase in revertant colonies of five strains could be observed at all concentration levels of 25-OCH₃-PPD (1,500, 150, 15, 1.5, and 0.15 μg/plate) with or without the S9 metabolic activation relative to the vehicle control (Table 5). The positive controls caused two-fold or greater increase in the number of revertant colonies in the absence and presence of S9 mix relative to the negative control which confirmed the validity of the test.

3.2.2. Mouse bone marrow micronucleus assay

Frequency of micronucleated polychromatic erythrocytes among 1000 PCEs were 2.75, 2.42, 1.75, and 2.83 and 57.83, for the vehicle control, at 600, 1,200, and 2,400 mg/kg of 25-OCH₃-PPD and positive control, respectively (Table 6). The 25-OCH₃-PPD administered groups of these three dose levels showed no significant difference relative to the negative control, while in contrast, the positive control group showed significant increase (*p* < 0.01) when compared to vehicle control.

3.2.3. Chromosomal aberration assay

The dose levels were obtained based on the preliminary cytotoxic experiment and 10 μg/ml of 25-OCH₃-PPD was determined as the highest dose for this assay. The results showed that the chromosomal aberration rates of administration group at all four dose levels were lower than 5% and had no significant difference compared with the vehicle control group with or without S9 mix. In contrast, the positive control indicated significant increases (*p* < 0.01) when compared to the negative control with or without S9 mix, and the chromosomal aberration rate was higher than 20% (Table 7).

4. Discussion

In recent years, there has been an increased interest in searching for antitumor treatments from Traditional Chinese Medicine. There

Table 2
Blood chemistry parameters in beagle dogs treated with 25-OCH₃-PPD for 13 weeks administration and 4 weeks recovery

Item	Control	60 mg/kg	120 mg/kg	240 mg/kg
<i>Day 0</i>				
ALT (nmol/s.L)	369 ± 99	383 ± 52	431 ± 96	411 ± 55
AST (nmol/s.L)	561 ± 129	606 ± 123	626 ± 55	588 ± 102
ALP (nmol/s.L)	3759 ± 897	3456 ± 1297	3884 ± 1084	3400 ± 871
GLU (mmol/L)	5.374 ± 0.191	5.476 ± 0.784	5.419 ± 0.458	4.743 ± 0.873
T.BIL(μmol/L)	0.890 ± 0.724	0.511 ± 0.231	0.442 ± 0.211	0.393 ± 0.110
T.CHO (mmol/L)	4.230 ± 0.604	4.776 ± 0.869	4.201 ± 0.441	4.876 ± 1.145
ALB (g/L)	21.30 ± 1.05	22.40 ± 1.35	24.28 ± 2.05*	23.43 ± 1.06**
T.P (g/L)	58.32 ± 12.12	56.13 ± 8.73	54.17 ± 6.61	53.13 ± 6.27
BUN (mmol/L)	3.069 ± 0.832	3.476 ± 1.109	3.134 ± 1.197	2.969 ± 1.127
Crea (μmol/L)	63.09 ± 8.56	66.14 ± 7.81	65.98 ± 7.78	68.46 ± 12.91
γ-GT (nmol/s.L)	65 ± 19	65 ± 20	50 ± 21	61 ± 35
CK (nmol/s.L)	5677 ± 2065	6023 ± 1368	6189 ± 1583	5622 ± 1642
TG (mmol/L)	0.507 ± 0.134	0.454 ± 0.112	0.481 ± 0.149	0.494 ± 0.097
Na ⁺ (mmol/L)	143.5 ± 1.2	142.9 ± 0.7	143.0 ± 0.9	143.5 ± 1.0
K ⁺ (mmol/L)	5.01 ± 0.35	5.19 ± 0.09	5.37 ± 0.27	5.27 ± 0.25
Cl ⁻ (mmol/L)	107.8 ± 0.8	107.8 ± 0.4	107.7 ± 0.9	107.9 ± 0.8
TCa (mmol/L)	2.50 ± 0.08	2.51 ± 0.02	2.51 ± 0.04	2.53 ± 0.03
<i>Day 46</i>				
ALT (nmol/s.L)	576 ± 435	484 ± 94	373 ± 140	566 ± 170
AST (nmol/s.L)	497 ± 48	544 ± 101	467 ± 30	550 ± 71
ALP (nmol/s.L)	2769 ± 742	2516 ± 459	2625 ± 746	3014 ± 1432
GLU (mmol/L)	6.997 ± 0.527	6.550 ± 0.633	5.874 ± 0.226**	5.359 ± 0.549**
T.BIL(μmol/L)	0.107 ± 0.127	0.099 ± 0.118	0.166 ± 0.121	0.075 ± 0.091
T.CHO (mmol/L)	5.036 ± 0.953	5.294 ± 0.659	5.505 ± 0.753	5.133 ± 0.928
ALB (g/L)	30.04 ± 3.06	29.52 ± 1.73	28.01 ± 2.39	28.29 ± 1.47
T.P (g/L)	59.85 ± 2.94	61.07 ± 7.20	55.65 ± 4.80	54.31 ± 2.89**
BUN (mmol/L)	4.064 ± 1.826	4.043 ± 1.478	2.995 ± 1.791	2.1111 ± 0.500*
Crea (μmol/L)	82.07 ± 5.72	84.67 ± 14.16	71.42 ± 16.03	73.72 ± 5.57*
γ-GT (nmol/s.L)	68 ± 18	54 ± 27	48 ± 29	49 ± 29
CK (nmol/s.L)	4447 ± 1234	4524 ± 1081	3325 ± 1162	5033 ± 1418
TG (mmol/L)	0.392 ± 0.064	0.320 ± 0.064	0.380 ± 0.117	0.412 ± 0.098
Na ⁺ (mmol/L)	144.9 ± 1.9	145.1 ± 1.0	145.5 ± 1.0	144.7 ± 1.6
K ⁺ (mmol/L)	4.66 ± 0.31	4.61 ± 0.38	4.78 ± 0.25	4.58 ± 0.10
Cl ⁻ (mmol/L)	109.0 ± 1.7	109.4 ± 0.8	110.3 ± 0.6	109.4 ± 1.6
TCa (mmol/L)	1.81 ± 0.09	1.85 ± 0.12	1.88 ± 0.10	1.87 ± 0.13
<i>Day 91</i>				
ALT (nmol/s.L)	523 ± 86	548 ± 115	499 ± 164	606 ± 71
AST (nmol/s.L)	626 ± 232	601 ± 158	528 ± 93	830 ± 301
ALP (nmol/s.L)	2284 ± 585	3183 ± 1144	2806 ± 613	2060 ± 231
GLU (mmol/L)	5.164 ± 0.209	5.348 ± 0.153	5.564 ± 0.398	5.770 ± 0.376**
T.BIL(μmol/L)	0.159 ± 0.218	0.351 ± 0.265	0.466 ± 0.315	0.511 ± 0.207
T.CHO (mmol/L)	3.983 ± 1.058	5.106 ± 1.573	4.702 ± 0.804	4.894 ± 0.673
ALB (g/L)	31.04 ± 4.33	28.10 ± 2.40	27.74 ± 1.36	29.15 ± 2.17
T.P (g/L)	56.58 ± 3.09	55.94 ± 5.45	53.65 ± 1.08	55.68 ± 3.34
BUN (mmol/L)	4.088 ± 2.768	3.532 ± 1.646	3.163 ± 1.713	2.337 ± 0.390
Crea (μmol/L)	92.01 ± 6.81	80.78 ± 25.33	93.59 ± 13.74	92.44 ± 5.41
γ-GT (nmol/s.L)	84 ± 7	73 ± 11	61 ± 17*	65 ± 21
CK (nmol/s.L)	3229 ± 823	4398 ± 2242	4201 ± 849	4225 ± 2129
TG (mmol/L)	0.386 ± 0.132	0.320 ± 0.060	0.372 ± 0.066	0.444 ± 0.122
Na ⁺ (mmol/L)	147.5 ± 0.7	147.5 ± 1.4	147.1 ± 1.6	146.8 ± 2.3
K ⁺ (mmol/L)	5.27 ± 0.26	5.33 ± 0.15	4.91 ± 0.17	4.90 ± 0.20
Cl ⁻ (mmol/L)	113.1 ± 0.7	113.1 ± 1.2	112.7 ± 1.5	112.0 ± 1.5
TCa (mmol/L)	2.60 ± 0.05	2.58 ± 0.05	2.62 ± 0.11	2.73 ± 0.05*
<i>Day 120</i>				
ALT (nmol/s.L)	386 ± 12	619 ± 85	619 ± 95	606 ± 67*
AST (nmol/s.L)	646 ± 114	597 ± 18	509 ± 21	597 ± 71
ALP (nmol/s.L)	3694 ± 233	1624 ± 530*	2559 ± 380	1490 ± 69**
GLU (mmol/L)	5.255 ± 0.488	4.668 ± 0.087	4.522 ± 0.831	4.110 ± 0.279
T.BIL(μmol/L)	0.153 ± 0.039	0.463 ± 0.356	0.751 ± 0.163*	0.599 ± 0.450
T.CHO (mmol/L)	7.032 ± 0.519	6.331 ± 0.139	6.591 ± 0.697	5.954 ± 1.358
ALB (g/L)	34.58 ± 1.93	33.57 ± 4.19	34.20 ± 4.12	37.63 ± 0.97
T.P (g/L)	70.85 ± 3.78	78.55 ± 6.61	76.52 ± 0.52	74.66 ± 1.23
BUN (mmol/L)	4.438 ± 1.713	8.144 ± 3.772	3.935 ± 0.721	4.927 ± 0.197
Crea (μmol/L)	79.59 ± 2.93	108.01 ± 15.49	93.50 ± 10.88	99.12 ± 1.26*
γ-GT (nmol/s.L)	58 ± 15	72 ± 12	0 ± 0*	15 ± 21
CK (nmol/s.L)	9150 ± 1809	3973 ± 55	5210 ± 626	4749 ± 68
TG (mmol/L)	0.661 ± 0.192	0.365 ± 0.030	0.547 ± 0.199	0.563 ± 0.150
Na ⁺ (mmol/L)	148.9 ± 0.1	148.0 ± 1.2	146.8 ± 0.6*	148.2 ± 1.3
K ⁺ (mmol/L)	4.71 ± 0.01	4.75 ± 0.05	4.96 ± 0.16	4.35 ± 0.28

Table 2 (continued)

Item	Control	60 mg/kg	120 mg/kg	240 mg/kg
Cl ⁻ (mmol/L)	110.4 ± 0.1	110.2 ± 0.1	110.1 ± 0.2	110.7 ± 0.9
TCa (mmol/L)	2.93 ± 0.01	2.91 ± 0.03	2.88 ± 0.04	2.92 ± 0.00

ALB, albumin; ALP, alkaline phosphatase; ALT, aminotransferase; AST, aspartate aminotransferase; BUN, urea nitrogen; CK, creatine phosphokinase; Crea, creatinine; γ-GT, gamma-glutamyl transferase; GLU, glucose; SD, standard deviation; TG, total triglyceride; T.P., total protein; TCHO, total cholesterol; T.BIL; total bilirubin; TCa, total calcium.

The data represent mean ± SD.

**p* < 0.05.

***p* < 0.01.

The normal range: ALT (206~1004); ALP (360~3978); BUN (2.054~10.013); Crea (36.19~108.40); GLU (3.027~7.670); T.BIL (0~5.371); ALB (20.64~44.94); T.P (49.96~74.86); γ-GT (0~142); Na⁺ (145.5~172.9); TCa (2.29~3.19).

are some investigations focusing on antitumor properties and other pharmacological activities of active compounds in ginseng. The active compounds are the ginsenosides in *P. ginseng* and other *Panax* species. Ginsenosides (F1, F2, Rg₃, Rh1, Rh₂, and compound K) are the hydrolyzed products of the sugar moieties of the major ginsenosides [21–35]. However, although ginsenosides have been widely used clinical for many years and were regarded to be very safe, some studies on the toxicities of these kinds of agents showed that some ginsenosides may have side-effects on the rats or dogs [24,36–40]. The statistical changes could be observed in the hematological data in beagle dogs after treated with ginsenoside Rg₃ for 13 weeks [24]. The subchronic study on ginsenoside CK in dogs showed compound related toxicity on liver [38].

25-OCH₃-PPD is a novel ginsenoside derivative obtained from *P. notoginseng* with high effectiveness and low toxicity. Previous studies demonstrated that 25-OCH₃-PPD possessed broad-spectrum functions in antitumor activity. Moreover, this compound has the potential to inhibit the growth of cancer xenograft tumors without killing normal cells [20]. Our previous subchronic study in Sprague Dawley rats showed that no observable adverse effect level of 25-OCH₃-PPD was up to 600 mg/kg/day. Taking into account the different species and as an important part of the nonclinical safety evaluation program, the genotoxicity and subchronic toxicity study in beagle dogs were conducted. Based on the preliminary toxicity experiment for 14 days, the subchronic toxicity, and the effective dose in rats, the highest dose level of the present study was determined as 240 mg/kg/day in dogs. The mid- and low-dose levels were considered to be 120 and 60 mg/kg/day, respectively. In addition, the effective dose is 3.5 mg/kg in dogs calculated on the level of mice at dose of 20 mg/kg [28]. The administration doses 240, 120, and 60 mg/kg were, respectively, about 68-, 34-, and 17-fold the effective dose.

Under the conditions of our study, there was no 25-OCH₃-PPD treatment-related death and toxicity at three dose levels. The clinical condition including food consumption and body weights showed no statistical changes between 25-OCH₃-PPD treatment groups and vehicle control group. As shown in the result, the temperature of the dogs with administered 25-OCH₃-PPD has no observable abnormalities, and the data were all similar to that of the control group. The respiratory rate and heart rate were all within the normal limits during the whole experiment. No 25-OCH₃-PPD compound-related effects on ECG data or urinalysis parameters were observed.

Hematological examinations were performed on Day 0, 46, 91, and 120. Despite some parameters including mean corpuscular volume, basophils, RETIC, CHCMr, and red cell distribution width of 25-OCH₃-PPD group showed statistical differences compared with the control group, the mean values of the parameters were in the normal range. Ultimately, there was no biological or toxicological

Table 3

The relative organ weights (organ weights/body weights) (%) in beagle dogs treated with 25-OCH₃-PPD for 13 weeks administration and 4 weeks recovery

Item	Control	60 mg/kg	120 mg/kg	240 mg/kg
<i>Day 46</i>				
Heart	8.5 ± 1.5	7.8 ± 0.6	7.8 ± 1.4	7.5 ± 0.6
Liver	39.8 ± 10.9	32.7 ± 6.0	33.2 ± 1.8	30.7 ± 2.4
Spleen	4.9 ± 0.7	3.8 ± 0.2	4.0 ± 1.0	4.1 ± 0.9
Lung	10.6 ± 1.9	9.6 ± 0.7	9.7 ± 1.1	10.1 ± 1.4
Kidney	8.0 ± 0.2	6.2 ± 1.0	6.9 ± 1.3	6.9 ± 2.1
Brain	10.4 ± 1.2	9.0 ± 0.6	8.8 ± 0.2	9.7 ± 1.0
Thymus	3.8 ± 0.0	2.5 ± 0.3*	2.1 ± 0.0**	3.5 ± 0.3
Adrenal	0.181 ± 0.09	0.103 ± 0.01	0.122 ± 0.03	0.128 ± 0.03
Testis	0.80	0.54	0.68	0.22
Epididymis	0.23	0.15	0.19	0.15
Uterus	0.08	0.04	0.11	0.10
Ovary	0.14	0.05	0.08	0.06
<i>Day 91</i>				
Heart	6.8 ± 0.6	6.9 ± 0.3	7.0 ± 0.0	6.6 ± 0.3
Liver	23.0 ± 2.6	23.9 ± 4.1	21.0 ± 0.1	22.7 ± 3.3
Spleen	2.5 ± 0.3	2.3 ± 0.2	2.5 ± 0.0	1.8 ± 0.5
Lung	7.7 ± 0.6	7.3 ± 1.1	6.9 ± 1.1	7.4 ± 0.6
Kidney	4.2 ± 0.3	4.5 ± 1.3	4.0 ± 0.1	4.2 ± 0.3
Brain	7.4 ± 1.0	8.1 ± 0.7	8.1 ± 0.3	7.5 ± 0.0
Thymus	1.6 ± 0.0	1.9 ± 0.0**	1.5 ± 0.0	1.5 ± 0.8
Adrenal	0.080 ± 0.01	0.098 ± 0.00	0.087 ± 0.01	0.103 ± 0.02
Testis	1.10	0.86	1.19	0.65
Epididymis	0.19	0.19	0.25	0.18
Uterus	0.08	0.10	0.07	0.07
Ovary	0.05	0.06	0.06	0.05
<i>Day 120</i>				
Heart	6.4 ± 0.7	6.5 ± 0.3	6.7 ± 0.0	6.3 ± 0.3
Liver	21.1 ± 2.1	19.5 ± 2.1	22.1 ± 1.1	20.7 ± 0.1
Spleen	2.6 ± 0.1	2.5 ± 0.5	2.6 ± 0.6	2.8 ± 0.8
Lung	6.3 ± 1.3	6.1 ± 0.5	7.2 ± 1.0	6.8 ± 0.5
Kidney	4.4 ± 0.4	3.7 ± 0.2	4.5 ± 0.2	4.0 ± 0.3
Brain	6.5 ± 1.3	7.0 ± 0.9	6.8 ± 0.4	6.3 ± 0.7
Thymus	1.7 ± 0.4	1.8 ± 0.1	1.7 ± 0.0	1.7 ± 0.3
Adrenal	0.092 ± 0.02	0.102 ± 0.00	0.092 ± 0.02	0.102 ± 0.01
Testis	1.10	1.22	1.19	1.31
Epididymis	0.24	0.28	0.24	0.28
Uterus	0.22	1.77	0.17	0.10
Ovary	0.06	0.21	0.05	0.06

SD, standard deviation.

The data represent mean ± SD.

**p* < 0.05.

***p* < 0.01.

Table 4

The effect on organ weights in beagle dogs treated with 25-OCH₃-PPD for 13 weeks administration and 4 weeks recovery

Item	Control	60 mg/kg	120 mg/kg	240 mg/kg
<i>Day 46</i>				
Heart	67.9 ± 6.2	66.2 ± 4.9	66.7 ± 12.1	62.1 ± 4.6
Liver	318.2 ± 59.9	276.6 ± 52.8	285.4 ± 15.5	255.1 ± 20.3
Spleen	39.4 ± 2.5	31.8 ± 2.4	34.0 ± 8.5	33.7 ± 7.4
Lung	85.1 ± 8.3	81.3 ± 6.6	83.1 ± 9.8	83.6 ± 11.7
Kidney	64.5 ± 4.2	52.0 ± 8.7	59.5 ± 11.6	57.3 ± 17.4
Brain	84.2 ± 2.0	76.1 ± 5.4	75.6 ± 2.1	80.5 ± 8.2
Thymus	30.8 ± 2.6	20.7 ± 2.6	17.7 ± 0.4*	28.7 ± 2.6
Adrenal	1.4 ± 0.6	0.9 ± 0.0	1.0 ± 0.2	1.1 ± 0.3
Testis	6.9	4.6	5.8	1.9
Epididymis	2.0	1.3	1.7	1.3
Uterus	0.6	0.4	1.0	0.8
Ovary	1.0	0.4	0.7	0.5
<i>Day 91</i>				
Heart	72.8 ± 8.5	73.0 ± 2.5	79.1 ± 1.0	72.0 ± 0.9
Liver	247.4 ± 36.5	251.9 ± 41.3	236.7 ± 3.1	248.8 ± 48.9
Spleen	26.4 ± 2.4	24.5 ± 1.8	28.6 ± 0.7	19.9 ± 4.9
Lung	83.4 ± 9.4	76.5 ± 11.5	77.8 ± 11.1	80.3 ± 11.2
Kidney	44.6 ± 1.9	47.0 ± 13.3	44.4 ± 0.0	45.5 ± 0.6
Brain	79.4 ± 13.7	85.3 ± 6.4	90.7 ± 5.5	81.9 ± 4.0
Thymus	17.2 ± 0.9	19.8 ± 0.1	16.7 ± 0.7	16.7 ± 7.9
Adrenal	0.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
Testis	12.1	9.0	13.6	7.4
Epididymis	2.1	2.0	2.9	2.0
Uterus	0.9	1.1	0.7	0.7
Ovary	0.5	0.7	0.7	0.5
<i>Day 120</i>				
Heart	77.7 ± 6.8	78.3 ± 10.1	84.1 ± 0.6	78.9 ± 14.6
Liver	255.0 ± 25.7	233.1 ± 14.8	277.8 ± 16.0	259.7 ± 34.7
Spleen	31.8 ± 7.7	31.1 ± 11.2	32.9 ± 6.9	35.8 ± 14.8
Lung	75.1 ± 0.6	73.2 ± 6.7	90.3 ± 12.4	85.9 ± 17.7
Kidney	53.7 ± 5.8	44.5 ± 9.8	56.1 ± 2.7	50.7 ± 11.0
Brain	78.3 ± 0.1	83.5 ± 3.9	85.2 ± 5.8	78.3 ± 2.0
Thymus	20.1 ± 0.3	21.2 ± 2.0	21.3 ± 0.5	22.2 ± 7.3
Adrenal	1.1 ± 0.0	1.2 ± 0.2	1.2 ± 0.2	1.3 ± 0.3
Testis	15.2	16.5	14.8	18.1
Epididymis	3.3	3.8	3.1	3.9
Uterus	2.3	1.8	2.2	1.2
Ovary	0.7	2.3	0.7	0.7

SD, standard deviation.

The data represent mean ± SD.

**p* < 0.05.

—related clinical response in the hematology parameters analyzed from blood samples collected in either male or female dogs following administration of 25-OCH₃-PPD. Blood chemistry examinations were also performed for four time points. Although some items such as albumin, aminotransferase, Crea, TP, and urea nitrogen showed significant differences between administration groups and control group, the parameters were all in the normal physiological range, and the dogs exhibited no obvious clinical response, indicating that these fluctuations are considered to be of no toxicological significance.

When compared to control, the absolute organ weight of thymus in mid-dose group decreased by 42.5% (*p* < 0.05) and the relative organ weights in mid-dose and low-dose group reduced by 44.7% (*p* < 0.01) and 34.2% (*p* < 0.05) on Day 46 while all the parameters as described above returned to normal and showed no statistical changes with 25-OCH₃-PPD withdrawal on Day 91 and 120. Moreover, no abnormal changes were found in the 240 mg/kg/day group. Additionally, no treatment-related pathological changes were found in the thymus, according to the histopathological examination. Considering that thymus is an important immunotoxicity target organ among all the lymphoid tissues, it is of great significance to evaluate the changes. Because the variation of changes in lymphoid organ weights is very large and complex, the

analysis and explanation of the weight changes of the thymus should be closely linked to histopathology changes and be assessed on a case-by-case basis. If the weight variation of the lymphoid organ lacks the corresponding changes on the histopathology, the changes of the weight may need to be ignored [41]. The results of histopathological study showed no abnormal changes in all three dose levels groups treated with 25-OCH₃-PPD. Hence, given the result of this experiment, the changes of thymus was sporadic in nature.

The subchronic toxicity of beagle dogs in this study lead to the conclusion that administration of 25-OCH₃-PPD at oral dosage level of 240 mg/kg/day for a 13 week consecutive regimen revealed no adverse effects in beagle dogs.

Ames assay, micronucleus test, and chromosomal aberration assay were conducted to evaluate the genotoxicity of 25-OCH₃-PPD. The result of Ames test showed that there was no significant increase in the number of revertant colonies of 25-OCH₃-PPD-administrated groups compared to the vehicle control group, which indicated that 25-OCH₃-PPD has no mutagenic effect. There were also no significant differences between 25-OCH₃-PPD administrated group of all dose levels and control group in the micronucleus test and chromosomal aberration assay. From all the above data, we can conclude that 25-OCH₃-PPD is not a genotoxic agent

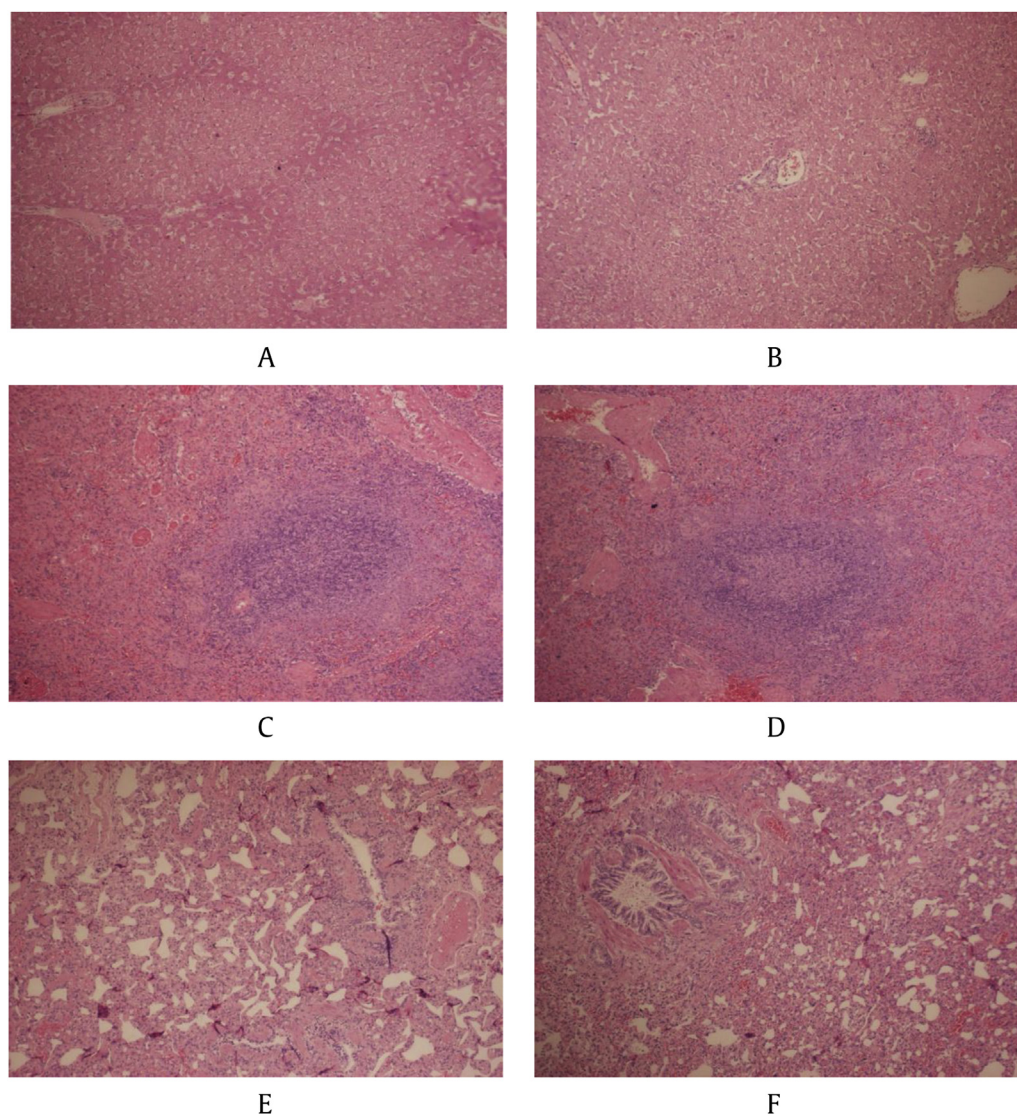


Fig. 3. Representative photographs of liver, spleen, and lung from the control and high-dose group at different stage during 120 days. (A–B) The comparison of liver section between a control and a high dose (240 mg/kg) with 25-OCH₃-PPD treatment on Day 46. (C–D) The comparison of spleen section between a control and a high dose (240 mg/kg) with 25-OCH₃-PPD treatment on Day 91. (E–F) The comparison of lung section between a control and a high dose (240 mg/kg) with 25-OCH₃-PPD treatment at the end of recovery on Day 120. 25-OCH₃-PPD, 25-methoxydammarane-3, 12, 20-triol.

Table 5
Effect of 25-OCH₃-PPD on bacterial reverse mutation without or with S9 metabolic activation

S9	Substance	dose(/plate)	TA97	TA98	TA100	TA102	TA1535
(-)	DMSO ¹⁾	0.1 ml	99.79 ± 7.9	22.3 ± 4.0	105.0 ± 11.1	247.7 ± 8.6	8.0 ± 1.7
	25-OCH ₃ -PPD	0.15 µg	94.3 ± 2.5	22.0 ± 2.6	107.3 ± 11.9	249.3 ± 16.3	8.7 ± 2.9
		1.5 µg	95.3 ± 10.5	23.7 ± 5.5	115.7 ± 8.0	254.0 ± 26.5	7.3 ± 1.2
		15 µg	111.3 ± 7.0	19.3 ± 6.5	97.3 ± 6.5	245.3 ± 22.0	9.0 ± 2.0
		150 µg	94.7 ± 9.3	19.0 ± 5.6	108.0 ± 6.0	250.7 ± 18.0	7.7 ± 0.6
		1,500 µg	103.3 ± 11.0	21.7 ± 3.8	102.3 ± 9.6	231.3 ± 14.0	8.0 ± 2.0
	Dexon ²⁾	50 µg	1770.7 ± 99.5**	1124.0 ± 124.4	—	1384.0 ± 50.3	—
	NaN ₃ ²⁾	2 µg	—	—	1673.3 ± 131.8	—	925.3 ± 50.3
	(+) DMSO ¹⁾	0.1 ml	140.3 ± 13.1	84.0 ± 9.2	141.7 ± 14.8	224.3 ± 11.1	65.0 ± 4.4
		25-OCH ₃ -PPD	0.15 µg	140.7 ± 11.7	75.3 ± 2.5	137.3 ± 6.1	212.0 ± 19.3
1.5 µg			132.3 ± 7.6	81.7 ± 9.3	148.0 ± 9.8	221.3 ± 13.5	59.0 ± 2.6
15 µg			132.7 ± 6.1	84.0 ± 6.6	134.3 ± 8.5	201.3 ± 19.9	61.0 ± 8.9
150 µg			133.0 ± 14.1	74.0 ± 2.6	138.3 ± 11.7	207.3 ± 15.6	65.3 ± 4.5
1500 µg			136.0 ± 3.6	79.7 ± 6.0	117.3 ± 7.6	186.7 ± 8.1**	59.0 ± 7.2
2-AF ²⁾		20 µg	1368.7 ± 187.2**	1581.3 ± 43.7**	1485.7 ± 131.0**	236.3 ± 15.9	—
CP ²⁾		200 µg	—	—	—	—	333.0 ± 35.5**

AF, 2-Aminofluorene; CP, cyclophosphamide; DMSO, Dimethyl Sulfoxide.

***p* < 0.01 compared to vehicle control.

¹⁾ Vehicle control.

²⁾ Positive control.

Table 6
Micronucleus assay results in mice following treatment with 25-OCH₃-PPD (n = 6)

Substance	Dosage(mg/kg)	MNPCE(‰)	PCE/(PCE + NCE)
0.5% CMC-Na ¹⁾	50 ml/kg	2.75 ± 0.69	0.42 ± 0.03
25-OCH ₃ -PPD	600	2.42 ± 1.07	0.41 ± 0.05
	1200	1.75 ± 1.41	0.44 ± 0.06
	2400	2.83 ± 2.16	0.31 ± 0.04
CP ²⁾	50	57.83 ± 10.66**	0.48 ± 0.03

CP, cyclophosphamide; MNPCE, micronucleated polychromatic erythrocytes; NCE, normochromatic erythrocyte; PCE, polychromatic erythrocyte.

**p < 0.01 compared to vehicle control.

¹⁾ Vehicle control.

²⁾ Positive control.

Table 7
Results of chromosomal aberration induced by 25-OCH₃-PPD

Substance	Dose (μg/ml)	Number of cells scored	Aberration rate(%)		
			-S9(6h)	-S9(24h)	+S9(6h)
DMSO ¹⁾	0.1 ml/hole	200	2	2.5	2.5
25-OCH ₃ -PPD	1.25	200	2.5	2.5	2.5
	2.5	200	2	1.5	2
	5	200	2.5	3.5	2.5
	10	200	1.5	2.5	2
MMC ²⁾	0.1	200	22**	24.5**	
CP ²⁾	40	200			22**

CP, cyclophosphamide; MMC, mitomycin C.

**p < 0.01 compared to vehicle control.

¹⁾ Vehicle control.

²⁾ Positive control.

either in somatic cells or germs cells. Combined with previous pharmacodynamics research, 25-OCH₃-PPD has proven to have low toxicity and high effectiveness as a potential candidate with anti-tumor activity, which further supports its candidacy for clinical use in cancer patients.

5. Conclusion

In conclusion, the evaluation of genotoxicity and subchronic oral toxicity of 25-OCH₃-PPD in beagle dogs was first reported. The level of 25-OCH₃-PPD at which no adverse effects were observed was found to be 240 mg/kg/day. Comparing with the effective dose levels of 3.5 mg/kg in beagle dog, the safety dose level is approximately 68-fold higher than the recommended dose from this subchronic toxicity study. The results of subchronic toxicity and genotoxicity study suggest that 25-OCH₃-PPD is an extremely safety candidate compound for antitumor treatment.

Conflicts of interest

The authors report no conflicts of interest.

Acknowledgment

This work was mainly supported by National Nature Science Foundation of China (Admission No. 81273389, 81302662, <http://www.nsf.gov.cn>), the growth plan for outstanding scholar of the University of Liaoning province (LJQ2015108), the Young Teacher Career Development Support Plan of Shenyang Pharmaceutical University (ZQN2015008) and the project for scientific and technological innovation of middle and young people in Shenyang (RC170479).

References

- Wang W, Zhang X, Qin JJ, Voruganti S, Nag SA, Wang MH, Wang Hui, Zhang RW. Natural product ginsenoside 25-OCH₃-PPD inhibits breast cancer growth and metastasis through down-regulating MDM2. *PLoS One* 2012;7:e41586.
- Yu XZ, Ye LF, Zhang H, Zhao J, Wang GQ, Guo C, Shang WB. Ginsenoside Rb1 ameliorates liver fat accumulation by upregulating perilipin expression in adipose tissue of db/db obese mice. *J Ginseng Res* 2015;39:199–205.
- Li DW, Cao JQ, Bi XL, Xia XC, Li W, Zhao YQ. New dammarane-type triterpenoids from the leaves of *Panax notoginseng* and their protein tyrosine phosphatase 1B inhibitory activity. *J Ginseng Res* 2014;38:28–33.
- Yang CY, Wang J, Zhao Y, Shen L, Jiang X, Xie ZG, Liang N, Zhang L, Chen ZH. Anti-diabetic effects of *Panax notoginseng* saponins and its major anti-hyperglycemic components. *J Ethnopharmacol* 2013;130:231–6.
- Kate LP, Kieran EF, Alan B, Shona P. The effects of *Panax notoginseng* on delayed onset muscle soreness and muscle damage in well-trained males: A double blind randomised controlled trial. *Complement Ther Med* 2013;21:131–40.
- Wu JH, Leung GP, Kwan Y, Sham TT, Tang J, Wang YH, Wan JB, Lee SM, Chan SW. Suppression of diet-induced hypercholesterolemia by saponins from *Panax notoginseng* in rats. *J Funct Foods* 2013;5:1159–69.
- Han SY, Li XH, Ma X, Zhang K, Ma ZZ, Jiang Y, Tu PF. Evaluation of the anti-myocardial ischemia effect of individual and combined extracts of *Panax notoginseng* and *Carthamus tinctorius* in rats. *J Ethnopharmacol* 2013;145:722–7.
- Fan JS, Liu DN, Huang G, Xu ZZ, Jia Y, Zhang HG, Li XH, He FT. *Panax notoginseng* saponins attenuate atherosclerosis via reciprocal regulation of lipid metabolism and inflammation by inducing liver X receptor alpha expression. *J Ethnopharmacol* 2012;142:732–8.
- Baek KS, Yi YS, Son YJ, Yoo S, Sung NY, Kim Y, Hong S, Aravinthan A, Kim JH, Cho JY. In vitro and in vivo anti-inflammatory activities of Korean Red Ginseng-derived components. *J Ginseng Res* 2016;40:437–44.
- Wang PP, Cui JG, Du XY, Yang QB, Jia CL, Xiong MQ, Yu XT, Li L, Wang WJ, Chen Y, Zhang T. *Panax notoginseng* saponins (PNS) inhibits breast cancer metastasis. *J Ethnopharmacol* 2014;154:63–67.
- Chan RYK, Chen WF, Dong A, Guo DA, Wong MS. Estrogen-like activity of ginsenoside Rg1 derived from *Panax notoginseng*. *J Clin Endocr Metab* 2002;87:3691–5.
- He NW, Zhao Y, Guo L, Shang J, Yang XB. Antioxidant, antiproliferative, and pro-apoptotic activities of a saponin extract derived from the roots of *Panax notoginseng* (Burk.) F.H. Chen. *J Med Food* 2012;15:350–9.
- Wang CZ, Xie JT, Zhang B, Ni M, Fishbein A, Aung HH, Mehendale SR, Du W, He TC, Yuan CS. Chemopreventive effects of *Panax notoginseng* and its major constituents on SW480 human colorectal cancer cells. *Int J Oncol* 2007;31:1149–56.
- Yang ZG, Sun HX, Ye YP. Ginsenoside Rd from *Panax notoginseng* is cytotoxic towards HeLa cancer cells and induces apoptosis. *Chem Biodivers* 2006;3:187–97.
- Chung VQ, Tattersall M, Cheung HT. Andrew Interactions of a herbal combination that inhibits growth of prostate cancer cells. *Cancer Chem Pharm* 2004;53:384–90.
- Toh DF, Patel DN, Chan ECY, Teo A, Neo SY, Koh HL. Anti-proliferative effects of raw and steamed extracts of *Panax notoginseng* and its ginsenoside constituents on human liver cancer cells. *Chin Med* 2011;6:4.
- Park SC, Yoo HS, Park C, Cho CK, Kim GY, Kim WJ, Lee YW, Choi YH. Induction of apoptosis in human lung carcinoma cells by the water extract of *Panax notoginseng* is associated with the activation of caspase-3 through down-regulation of Akt. *Int J Oncol* 2009;35:121–7.
- Wang K, Zhao YQ. Chemical constituents of the hydrolysate of saponins from the basal part of stem of *Panax notoginseng*. *Chin J Med Chem* 2008;18:288–90.
- Wang W, Zhao Y, Rayburn E, Hill DL, Wang H, Zhang R. In vitro anti-cancer activity and structure-activity relationships of natural products isolated from fruits of *Panax ginseng*. *Cancer Chem Pharm* 2007;59:589–601.
- Zhao Y, Wang W, Han L, Rayburn ER, Hill DL, Wang H, Zhang R. Isolation, Structural Determination, and Evaluation of the Biological Activity of 20(S)-25-methoxydammarane-3β, 12β, 20-triol [20(S)-25-OCH₃-PPD], a Novel Natural Product from *Panax notoginseng*. *Medicinal Chemistry* 2007;3:51–60.
- Wang W, Elizabeth RR, Hang J, Zhao YQ, Wang H, Zhang RW. Anti-lung cancer effects of novel ginsenoside 25-OCH₃-PPD. *Lung Cancer* 2009;65:306–11.
- Wang W, Wang H, Rayburn ER, Zhao Y, Hill DL, Zhang R. 20(S)-25-methoxydammarane-3beta, 12beta, 20-triol, a novel natural product for prostate cancer therapy activity in vitro and in vivo and mechanisms of action. *Br J Cancer* 2008;98:792–802.
- Bi XL, Zhao YQ, Fang WF, Yang WC. Anticancer activity of *Panax notoginseng* extract 20(S)-25-OCH₃-PPD: Targetting b-Catenin signalling. *Clin Exp Pharmacol P* 2009;36:1074–8.
- Liu JP, Lu D, Nicholson RC, Li PY, Wang F. Toxicity of a novel anti-tumor agent 20(S)-ginsenoside Rg3: A 26-week intramuscular repeated administration study in Beagle dogs. *Food Chem Toxicol* 2011;49:1718–27.

- [25] Wang W, Elizabeth RR, Zhao YQ, Wang H, Zhang RW. Novel ginsenosides 25-OH-PPD and 25-OCH₃-PPD as experimental therapy for pancreatic cancer anticancer activity and mechanisms of action. *Cancer Lett* 2009;278:241–8.
- [26] Zhang LH, Jia YL, Lin XX, Zhang HQ, Dong XW, Zhao JM, Shen J, Shen HJ, Li FF, Yan XF, et al. AD-1, a novel ginsenoside derivative, shows anti-lung cancer activity via activation of p38 MAPK pathway and generation of reactive oxygen species. *Biochim Biophys Acta* 1830;2013:4148–59.
- [27] Shi CH, Zhang X, Suo H, Yin T, Xu HY, Yuan B, Zhao YQ. Simultaneous determination by LC-MS/MS of 25-methoxydammarane-3b, 12b, 20-triol epimers and active metabolites in rat plasma after intravenous administration. *Xenobiotica* 2013;43:868–74.
- [28] Zhang XR, Zhang J, Li W, Liu L, Sun BS, Guo ZH, Shi CH, Zhao YQ. In vitro metabolism of 20(R)-25-methoxyl-dammarane-3,12,20-triol from *Panax notoginseng* in human, monkey, dog, rat, and mouse liver microsomes. *PLoS One* 2014;9:e94962.
- [29] Li W, Zhang XR, Xin YF, Xuan YX, Liu JP, Li PY, Zhao YQ. Oral subchronic toxicity evaluation of a novel antitumor agent 25-methoxydammarane-3,12,20-triol from *Panax notoginseng* in Sprague-Dawley rats. *Regul Toxicol Pharmacol* 2016;77:240–51.
- [30] Schmid W. The micronucleus test. *Mutat Res* 1975;31:9–15.
- [31] Shin JY, Lee JM, Shin HS, Park SY, Yang JE, Cho SK. Anti-Cancer Effect of Ginsenoside F₂ against Glioblastoma Multiforme in Xenograft Model in SD Rats. *J. Ginseng Res* 2012;36:86–92.
- [32] Shishtar E, Sievenpiper JL, Djedovic V, Cozma AI, Ha V, Jayalath VH, Jenkins DJ, Meija SB, de Souza RJ, Jovanovski E, et al. The effect of ginseng (the genus *panax*) on glycemic control: a systematic review and meta-analysis of randomized controlled clinical trials. *PLoS One* 2014;9(9). e107391.
- [33] Du J, Cui CH, Park SC, Kim JK, Yu HS, Jin FX, Sun C, Kim SC, Im WT. Identification and characterization of a ginsenoside-transforming β -glucosidase from *Pseudonocardia* sp. Gsoil 1536 and its application for enhanced production of minor ginsenoside Rg₂ (S). *PLoS One* 2014;9(6). <https://doi.org/10.1371/journal.pone.0096914>. e96914.
- [34] Cui CH, Kim JK, Kim SC, Im WT. Characterization of a ginsenoside-transforming β -glucosidase from *Paenibacillus mucilaginosus* and its application for enhanced production of minor ginsenoside F. *PLoS One* 2014;9(1). e85727.
- [35] Cui CH, Kim SC, Im WT. Characterization of the ginsenoside transforming recombinant β -glucosidase from *Actinosynnema mirum* and bioconversion of major ginsenosides into minor ginsenosides. *Appl Microbiol Biotechnol* 2013;97:649–59.
- [36] Hess FG, Parent RA, Stevens KR, Cox GE, Becci PJ. Effect of subchronic feeding of ginseng extract G-115 in beagle dogs. *Food Chem Toxicol* 1983;21:95–7.
- [37] Park ID, Yoo HS, Lee YW, Son CG, Kwon M, Sung HJ, Cho CK. Toxicological study on MUNOPHIL, water extract of *Panax ginseng* and *Hericium erinaceum* in rats. *J Acupunct Meridian Stud* 2008;2:121–7.
- [38] Gao YL, Liu ZF, Li CM, Shen JY, Yin HX, Li GS. Subchronic toxicity studies with ginsenoside compound K delivered to dogs via intravenous administration. *Food Chem Toxicol* 2011;49(8):1857–62.
- [39] Lu Dan, Liu Jinping, Zhao Wenjie, Li Pingya. Chronic toxicity of ginsenoside Re on Sprague-Dawley rats. *J Ethnopharmacol* 2012;144(3):656–63.
- [40] Park SJ, Lim KH, Noh JH, Jeong EJ, Kim YS, Han BC, Lee SH, Moon KS. Subacute oral toxicity study of korean red ginseng extract in sprague-dawley rats. *Toxicol Res* 2013;29(4):285–92.
- [41] Haley P, Perry R, Ennulat D, Frame S, Johnson C, Lapointe JM, Nyska A, Snyder PW, Walker D, Walter G. Best Practice Guideline for the Routine Pathology Evaluation of the Immune System. *Toxicol Pathol* 2005;33:404–7.