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Safety Assessment of *Cistanche tubulosa* Extract: Genotoxicity and Repeated Dose Toxicity Tests

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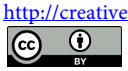

Abstract

Cistanche tubulosa (CTE) aqueous extract is already employed as a botanical prescription drug for treating dementia in China. CTE, derived from dried *C. tubulosa* stems, is freshly prepared with sterile distilled water. CTE requires acute and chronic toxicity studies to ensure its safety in clinical applications. This study evaluates the safety profile of CTE through both *in vitro* and *in vivo* tests. Ames tests conducted on five strains of *Salmonella typhimurium* showed no increase in reverse mutations following exposure to CTE. Micronucleus tests in mice indicated that CTE (0.5 - 2 g/kg) did not cause genetic damage in blood-forming cells. In acute studies, mice were administered CTE orally at doses of 2, 4, and 8 g/kg for 14 days, with controls receiving water as the vehicle. Chronic oral toxicity was evaluated over 6 months in rats and dogs at doses of 1.65 g/kg and 1.5 g/kg, respectively, to identify toxicity thresholds and calculate safety margins relative to the clinical dose. Results demonstrated that CTE administration, ranging from the no-observed-adverse-effect level (NOAEL) to the high-dose ceiling, induced no mortality, with all physiological parameters remaining within homeostatic ranges. These data provide a critical framework for defining the safety margin in human clinical translation.

Subject Areas

Food Science & Technology, Toxicology

Keywords

Cistanche tubulosa, Ames Tests, Micronucleus Tests, Chronic Oral Toxicity Studies

1. Introduction

Cistanche tubulosa (Schenk) R. Wight (CT), a cornerstone of traditional Chinese medicine for over 2000 years [1], is renowned for its diverse biological activities and anti-aging properties. The aqueous CTE is used as a prescription drug for dementia in China [2]. Over the past decade, CTE has demonstrated numerous pharmacological activities, including neuroprotection, learning and memory enhancement, cardiac function improvement, hyperlipidemia and hyperglycemia reduction, and prevention of obesity-induced diabetes and metabolic syndrome [3]. Historically, CTE has also been used to treat impotence, lumbar pain, and constipation induced by blood deficiency [4]. The major active components, phenylethanoid glycosides such as echinacoside and acteoside, exhibit various bioactivities, including anti-osteoporotic effects, vasorelaxation, protection against testis and sperm injury, and anti-hepatic fibrosis effects [5]. Acteoside has shown protective effects against amyloid-beta peptide-impaired memory, glutamate-induced neurotoxicity, and neurotoxicity in cultured neurons and rat cortical cells, functioning as a potent antioxidant. Echinacoside also demonstrates neuroprotective activity in Parkinson's disease models, suggesting potential therapeutic effects against Alzheimer's disease [6].

CTE, an extract from dried *Cistanche tubulosa* stems, is freshly prepared using sterile distilled water. While herbal medicines have fewer side effects than allopathic medicines, their use is not always risk-free. This study aims to evaluate the safety profile of CTE using both *in vitro* and *in vivo* tests, determining the no-observed-adverse-effect level (NOAEL) [7] to ensure safe clinical use.

2. Materials and Methods

2.1 Raw Material Extraction Process

The stem powder of *C. tubulosa* was extracted by refluxing with water. The resulting filtrate was collected. The filtrate was concentrated. Ethanol was then added to this concentrate, and the supernatant was collected. The supernatant underwent separation using macro-porous absorption resin and was subsequently spray-dried to yield the aqueous extract (CTE). The yield of the CTE is 10%. This extract was produced by Sinphar Tian-Li Pharmaceutical Co., Ltd., Hangzhou, Sinphar Group. The stem powder of *Cistanche tubulosa* used in this study was sourced from the standard reference material listed in the Chinese Pharmacopoeia. It was cultivated in Hetian, Xinjiang, in association with *Tamarix* ramosissima, following approved GAP guidelines.

2.2. Preparation of Test Substance

For the micronucleus assay, CTE was suspended in water for injection on each dosing day to achieve concentrations of 25, 50, and 100 mg/mL. For the oral acute toxicity study, CTE was similarly prepared in water for injection to reach concentrations of 100, 200, and 400 mg/mL. The dosing solution was freshly prepared on each dosing day and stirred continuously before administration. For the oral

chronic toxicity study, CTE was supplied by the Peking University School of Pharmaceutical Sciences from Prof. Tu Pengfei lab (Batch/Lot number: 980601), using the same technology as Sinphar Tian-Li Pharmaceutical Co., Ltd., China. The test substance was stored in a glass bottle sealed with airtight wrapping and kept at room temperature. Prior to testing, the powder was dissolved in distilled water by heating until fully dissolved, achieving a final concentration of 0.16 g/mL with a pH of 5 - 6.

2.3. Salmonella/Microsome Reversion Assay: Ames Test

In the Ames test, five histidine-requiring *Salmonella typhimurium* strains (TA97, TA98, TA100, TA102, and TA1535) were used, both with and without metabolic activation (S9) induced by Aroclor 1254. According to OECD Guideline 471 [8], the maximum dose level for non-cytotoxic substances is 5 mg/plate. Preliminary toxicity tests showed no cytotoxicity up to 5 mg/plate, which was selected as the top dose level for the Ames test, using DMSO as the solvent. Mutagenicity was assessed at five dose levels (0.3125, 0.625, 1.25, 2.5, and 5 mg/plate), incubated for 69 - 72 hours at 37˚C, and revertant colonies were counted. A dose level is considered toxic if it results in a >50% reduction in the mean number of revertants per plate relative to the mean negative control value. Cytotoxicity is indicated by bacterial lawn clearing and the appearance of pin colonies.

2.4. Human Peripheral Lymphocyte Chromosome Aberration

For the human peripheral lymphocyte chromosome aberration analysis, whole blood was collected in heparin-containing tubes and cultured in RPMI medium with 1.35% (v/v) PHA-M in T75 flasks. Cells were treated with distilled water or mitomycin C (1 μg/mL) and exposed to five concentrations (312.5, 625, 1250, 2500, or 5000 μg/mL) of the test substance. Three independent experiments were conducted, with and without S9 for 4 hours, and without S9 for 24 hours, to analyze the presence of mitotic cells. Colcemid (0.1 μg/mL) was added before trypsinization, followed by cell collection and treatment at 37˚C in Carnoy's solution (methanol: acetic acid, 3:1). Cells were then spread on glass slides and stained with 2% Giemsa solution in 0.075M phosphate buffer. Well-spread mitotic cells with chromosome numbers ranging from 44 to 48 were observed for the presence of aberrant chromosomes under a microscope at 1000× magnification. For each treatment concentration, two cultures were prepared, and 100 metaphases per culture were observed, with 200 metaphases examined in total across three independent experiments.

2.5. Animals

For the micronucleus assay, BALB/cAnNCrl mice were supplied by BioLASCO Taiwan Co., Ltd. The male mice were chosen at approximately 6 weeks old, weighing 21.1 - 23.4 g before testing. A total of 25 male mice were used and sacrificed at the study's completion. For the oral acute toxicity study, ICR mice were also

supplied by BioLASCO Taiwan Co., Ltd. Prior to treatment, all animals were weighed and observed. The required number of mice was randomly allocated into four groups (6 males and 6 females per group) using a computerized LIMS system. The mice were approximately 6 - 7 weeks old, with weight ranges of 22 - 32 g for males and 18 - 28 g for females. For the oral chronic toxicity study, Wistar rats and Beagle dogs were used. The Wistar rats, weighing 125 ± 8 g for males and 120 ± 8 g for females, were provided by the Resource Center for Experimental Animals. A total of 55 male and 55 female rats were fed an identical diet and housed in autoclaved plastic cages, with four rats per cage. After three months, each cage housed three or four rats. The room temperature was maintained at 22˚C ± 2˚C and humidity at 50% ± 10%. All animals were housed under the care of the Department of Laboratory Animal Science, Peking University (Housing Condition Certificate: 01-1057). The Beagle dogs used in the study had body weights ranging from 5.8 to 7.2 kg for males and 6.1 to 7.2 kg for females. Seven male and seven female dogs were provided by the Beijing Laboratory Animal Technology Services Collaborative. Each dog was fed an identical diet, with husbandry conditions certified under certificate 01-4003. Electronic scales (1/1000SE2020 and 1/100CT1200) were purchased from OHAUS company for accurate weight measurement.

2.6. Housing and Diet Conditions

For the micronucleus assay, all animals were confirmed to be in healthy condition before the study. The required number of mice was randomly allocated into five groups, with five animals per group, using Microsoft Excel before the first dosing day (Day 1). The weight variation of the animals did not exceed ± 20% of the mean body weight. Body weight of each mouse was recorded daily for five days, and animals were observed at least once daily for clinical signs during the study period. Peripheral blood was collected from each CTE-treated and non-treated animal between 36 and 48 hours after the last treatment. For the oral acute toxicity study, six mice per polycarbonate cage were housed in an AAALAC-accredited animal facility. The dose levels used in this study were based on results from a previous pilot study. The mice were fasted for approximately five hours before dosing and remained fasted until the end of dosing. The volume administered was adjusted according to individual body weight recorded before dosing. The oral route was chosen as it reflects the expected route of human consumption of the test article. The study's group numbers, dose levels, dose concentrations, dose volumes, and number of animals used are presented in Table 1.

For the oral chronic toxicity study in rats, the Wistar rats were quarantined, acclimatized, and randomized by weight into three groups, each comprising 18 males and 18 females. Treatments included CTE at 1.65 g/kg or 0.8 g/kg, or distilled water (control group). CTE was administered to the animals by oral gavage at a dosing volume of 1 ml/100 g. Each administration occurred at 2:00 pm on six consecutive weekdays for six months. The Beagle dogs were quarantined and acclimatized for two weeks, during which time they underwent hematology, clinical

chemistry, urinalysis, and electrocardiogram testing. The dogs were divided into three groups: high-dose (1.50 g/kg; 3 males and 3 females), low-dose (0.75 g/kg; 2 males and 2 females), and control (2 males and 2 females). The dosing was equivalent to 100 and 50 times of the clinically recommended dose based on body weight, respectively. The animals were fed twice daily (morning and afternoon), with the drug mixed into small pieces of feed, which the dogs consumed before their regular diet. The drugs were administered twice daily on six consecutive weekdays for 180 days, followed by a 14-day recovery period. Sterilized tap water was provided *ad libitum*.

Table 1. Diet experimental design of oral acute toxicity study.

Group No.	Dose level (mg/kg)	Concentration (mg/mL)	Dose volume (mL/kg)	Number of animals	
				Male	Female
1	0[a]	0	20	6	6
2	2000	100	20	6	6
3	4000	200	20	6	6
4	8000	400	20	6	6

[a]Vehicle.

2.7. Micronucleus Test

Male BALB/c mice (n = 5 per group) were administered test compounds orally by gavage at doses of 0.5, 1, and 2 g/kg body weight. Forty-eight hours after dosing, blood samples (10 μL) were collected from the tail vein, smeared on slides coated with acridine orange (40 μg/mL), and examined by fluorescence microscopy. The slides were blind coded by an individual not involved in the scoring process. A total of 2000 reticulocytes (1000 per slide) were examined under a fluorescence microscope for the presence of micronuclei (MN) in each animal. The proportion of reticulocytes to total erythrocytes was used as a bone marrow toxicity indicator induced by the test compound. The percentage of reticulocytes was measured using a flow cytometer (BD FACScan), analyzing 50,000 erythrocytes. The concurrent negative control MN/1000 reticulocytes (RETs) data were compared with historical control data. The acceptable range required a significantly increased mean frequency of micronucleated reticulocytes in the positive control group relative to the concurrent negative control ($p \leq 0.05$, U-test). The incidence of micronucleated reticulocytes per 1000 reticulocytes (MN/1000 RETs) was presented for each animal, with the Mean ± S.D. expressed for each group.

2.8. Fourteen-Day Oral Acute Toxicity Study

Mice were observed for mortality and clinical signs at 0, 1, 2, 3, and 4 hours after dosing. From Day 2 through Day 14, the animals were observed twice daily (at least six hours apart) for mortality and once daily for clinical signs. Any mortality or clinical signs were recorded and documented. Body weight was recorded for all

animals prior to the start of dosing (Day 1), then at a weekly interval (Day 8), and at the end of the study period (Day 15). At the end of the study, all mice were euthanized by carbon dioxide exposure followed by exsanguination and were necropsied in a randomized order. The external body surface and all organs/tissues in the thoracic and abdominal cavities were examined and recorded. Weekly body weights and body weight gains were assessed for homogeneity of variance (Equal Variance Test).

2.9. Oral Chronic Toxicity Study in Rats

Rats were quarantined, acclimatized, and randomized by weight into three groups, each consisting of 18 males and 18 females. Treatments included CTE at doses of 1.65 g/kg or 0.8 g/kg, or distilled water (control group). CTE was administered to the animals by oral gavage at a dosing volume of 1 mL/100g. Each administration was performed at 2:00 pm on six consecutive weekdays for six months. The animals were observed twice daily for clinical signs, activity levels, traits, responses to the environment, fur color, oral and nasal secretions, and feces. Any animals showing signs of illness were isolated and monitored closely. During the first three months, body weight and food consumption were recorded weekly, and subsequently, every two weeks. During the recovery period, body weight and food consumption were recorded weekly.

2.10. Oral Chronic Toxicity Study in Dogs

Dogs were quarantined and acclimatized for two weeks and underwent hematology, clinical chemistry, urinalysis, and electrocardiogram testing. They were divided into three groups: high-dose (1.50 g/kg; 3 males and 3 females), low-dose (0.75 g/kg; 2 males and 2 females), and control (2 males and 2 females). The animals were fed twice daily (morning and afternoon), with the test substance mixed into the diet in the form of small feed pieces, which were consumed first. The drugs were administered twice daily on six consecutive weekdays for 180 days, followed by a 14-day recovery period. Sterilized tap water was provided *ad libitum*. The animals were observed twice daily for clinical signs, activity levels, appetite, feeding behavior, nasal secretions, and feces, with afternoon observations conducted by the same person each time. Body weights were recorded weekly, and food consumption was recorded daily. Blood pressure, pulse, and electrocardiograph parameters were measured before treatment, at three and six months of treatment, and at the end of the recovery period in quiet, awake animals. Blood pressure was measured at the forelimb using a sphygmomanometer (EW273). Electrocardiogram recordings were made using an electrocardiograph (FK-11A).

2.11. Hematological, Clotting, and Serum Biochemical Analyses

For the oral chronic toxicity studies in rats and dogs, hematology and clinical chemistry parameters were measured at the end of months 3 and 6, and at the end of the recovery period. Wistar rats were fasted for approximately 8 hours, and 1 mL

of blood was collected from the vascular plexus in the eyes. Gross necropsy was performed at months 3 and 6, and at the end of the recovery period. Beagle dogs were similarly fasted, and blood was collected from the great saphenous vein in the forelimb. This study was approved by the Peking University School of Pharmaceutical Sciences, and all methods were performed in accordance with the relevant guidelines and regulations.

2.12. Urinalysis

Urinalysis was conducted before treatment, at three and six months of treatment, and at the end of the recovery period. On the day of urinalysis, urine was collected and tested using Multistix® 10 SG urinalysis strips and assessed with a urine chemistry analyzer (Clinitek 200).

2.13. Gross Necropsy and Histopathology

The Wistar rats and Beagle dogs were euthanized using carbon dioxide in the study. Gross necropsy of Wistar rats was performed at months 3 and 6, and at the end of the recovery period. The animals were fasted for approximately 8 hours before euthanasia. For Beagle dogs, gross necropsy was performed at month 6 and at the end of the recovery period. In accordance with the histopathology protocol, the heart, liver, spleen, lungs, kidneys, brain, thymus, adrenal glands, testes (or ovaries), prostate (or uterus), epididymis, and seminal vesicles were removed and weighed using electronic scales, then preserved in 10% formalin solution. Stomachs were removed and cut along the greater curvature, washed in saline solution, and the gastric mucosa was examined. Similarly, the small intestine, pancreas, thyroid, pituitary gland, and bladder were removed, preserved in 10% formalin solution, dehydrated with alcohol, embedded in paraffin, prepared for slide examination, stained with hematoxylin and eosin (HE), and examined microscopically. For Beagle dogs, the stomachs were removed and cut along the greater curvature, washed in saline solution, and the gastric mucosa was examined. Additionally, the small intestine, colon, rectum, bladder, pancreas, thyroid, pituitary gland, spinal nerve, submandibular gland, mesenteric lymph nodes, bone marrow (rib), and optic nerve were removed, preserved in 10% formalin solution, dehydrated with alcohol, embedded in paraffin, prepared for slide examination, stained with HE, and examined microscopically.

2.14. Statistical Analysis

All measured parameters are presented as means ± S.D. from at least three independent experiments ($n \geq 3$). Statistically significant differences between groups were determined using one-way analysis of variance (ANOVA). Differences with p-values of less than 0.05 were considered statistically significant. In the oral chronic toxicity study, semi-quantitative data were compared using frequency and level values for each group. All data were compared between the treated and control groups, performed using a t-test. If a significant difference was found in the t-test,

a chi-square test was then performed. The normal range for each physiological and biochemical parameter was compared with any treatment-induced changes.

3. Results

3.1. CTE Did Not Induce Mutagenicity or Clastogenicity *in Vitro* or *in Vivo*

In the Ames test, CTE did not increase the number of revertants at any dose level (0.3125, 0.625, 1.25, 2.5, and 5 mg/plate) with or without S9 metabolic activation across all five tester strains (TA97, TA98, TA100, TA102, and TA1535). There were no significant increases in the number of revertants compared to background spontaneous revertants in the Salmonella reverse gene mutation assay at doses ranging from 0.3125 to 5 mg/plate of CTE, with or without metabolic activation (Table 2). In the micronucleus assays, the treatment groups (0.5, 1, and 2 g/kg body weight) showed no significant differences in MN/1000 RETs compared to the negative control group treated with the vehicle (distilled water) (Table 3). These findings indicate that CTE did not induce mutagenicity or clastogenicity.

Table 2. Results of Ames test.

	TA97	TA98	TA100	TA102	TA1535
Without S9 metabolic activation					
Negative[a]	108 ± 15	21 ± 4	81 ± 15	246 ± 43	8 ± 3
Positive[b]	659 ± 59	164 ± 13	402 ± 28	1187 ± 151	145 ± 9
CTE (μg/plate)					
312.5	132 ± 16	22 ± 4	77 ± 13	253 ± 15	9 ± 3
625	109 ± 12	24 ± 8	83 ± 6	266 ± 35	7 ± 1
1250	124 ± 26	19 ± 6	84 ± 10	257 ± 9	8 ± 2
2500	135 ± 16	29 ± 19	93 ± 21	267 ± 34	10 ± 4
5000	154 ± 27	28 ± 4	108 ± 21	355 ± 21	10 ± 2
With S9 metabolic activation					
Negative[a]	117 ± 23	30 ± 2	68 ± 5	235 ± 35	9 ± 2
Positive[b]	829 ± 23	508 ± 26	366 ± 22	823 ± 28	175 ± 13
CTE (μg/plate)					
312.5	125 ± 7	32 ± 1	72 ± 10	244 ± 42	10 ± 1
625	119 ± 23	32 ± 6	76 ± 5	244 ± 24	7 ± 3
1250	119 ± 8	27 ± 6	74 ± 8	253 ± 9	9 ± 1
2500	124 ± 31	36 ± 3	85 ± 11	274 ± 21	8 ± 1
5000	121 ± 15	32 ± 6	97 ± 6	264 ± 3	13 ± 7

Data were presented as Mean ± S.D. (n ≥ 3). No significant changes of revertants were observed in five strains after treatment with CTE. [a]Negative control was dimethyl sulfoxide (DMSO); [b]Positive controls in –S9 plate: 2,4,7-trinitro-9-fluorenone (25 μg/plate for TA97, 6.25 μg/plate for TA98); Sodium Azide (1 μg/plate for TA 100, 0.4 μg/plate for TA 1535); Mitomycin C (125 μg/plate for TA 102). Positive control in +S9 plate: 2-aminofluorene (4 μg/plate for TA 97 and TA100, 1 μg/plate for TA 98); Danthron 25 μg/plate for TA 102; 2-aminoanthrene 1 μg/plate for TA 1535.

Table 3. CTE chromosome aberration assay in human peripheral lymphocytes.

	Aberrant cell (%)[δ]		Number of cells with structural aberrations (%)[γ]					
	With gap	Without gap	G	B	D	g	b	e
4 h without S9 metabolic activation								
Negative[α]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Positive[β]	20.0 ± 1.41***	19.5 ± 2.12***	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.71	2.5 ± 0.71**	17.0 ± 2.83***
CTE assay (μg/mL)								
312.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
625	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1250	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00 ±1.41	0.00 ± 0.00	0.00 ± 0.00
2500	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00
5000	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4 h with S9 metabolic activation								
Negative[α]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Positive[β]	17.5 ± 3.54***	19.0 ±1.41***	0.50 ± 0.71	1.00 ±1.41	0.50 ± 0.71	0.50 ± 0.71	2.5 ±2.12	13.5 ±2.12***
CTE assay (μg/mL)								
312.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
625	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1250	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00
2500	0.50 ± 0.71	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00
5000	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24 h without S9 metabolic activation								
Negative[α]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Positive[β]	22.5 ± 4.95***	21.5 ± 2.83***	0.00 ± 0.00	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00	2.00 ±0.00	19.5 ± 3.54***
CTE assay (μg/mL)								
312.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
625	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1250	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2500	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5000	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

No significant aberration was shown under the treatment groups. The data are expressed as the Mean ± S.D. (n = 2). Significant difference as ***$P < 0.001$ was compared with control and treatment group by one-way ANOVA. [α]1% DMSO in medium was used as negative control; [β]Mitomycin C 1 μg/mL was used as positive control in without S9 metabolic activation condition; Cyclophosphamide 40 μM was used as positive control in with S9 metabolic activation condition; [γ]G: chromosome gap; B: chromosome break; D: dicentric; g: chromatid gap; b: chromatid break; e: exchange; [δ]Chromatid and chromosome gaps were recorded but separated into two groups, with gaps and excluding gaps.

3.2. CTE Did Not Exert Adverse Toxic Effects in Acute and Chronic Oral Toxicity Studies

In the fourteen-day oral acute toxicity study, no animal deaths occurred. Additionally, there were no significant differences in body weights and total body weight gains between treated and control mice. The acute oral toxicity test results, including mortality and clinical observations, are summarized in Table 4. In the chronic oral toxicity studies in rats and dogs, no abnormal treatment-related effects were observed concerning clinical signs, fur appearance, activity levels, responses to the environment, and feces during both the treatment and recovery periods. After six months of oral administration at doses of 1.65 g/kg in rats and 1.5 g/kg in dogs, no significant toxic reactions were observed regarding body weight gains, clinical signs, or responses to the environment. While CTE administration decreased body weight gain in animals and temporarily affected some clinical chemistry parameters, these effects were low, reversible, and not significant in between-group comparisons (Table 5 and Table 6). In complete blood cell count (CBC) with differential and biochemical analyses (Table 7 and Table 8), ALT levels in male rats and AST levels in female rats were higher than controls after six months of CTE administration. In dogs, RBC and HGB levels in both sexes and ALT levels in males were significantly affected after three months of oral administration at a dose of 1.5 g/kg, but these effects were short-lived and reversible. No significant between-group differences were observed after six months of administration, except for a lower ALP value, and no significant differences were noted in any other biochemical parameters (Table 9 and Table 10). In urinalysis, aside from pH values in the male middle-dose group and urine volume and specific gravity in the female low-dose group, no significant changes were observed in other parameters, including semi-quantitative urinalysis and urinary sediment analysis, following six months of CTE administration.

Table 4. CTE micronucleus assay on male BALB/c mice.

Group No.	Negative	CTE (mg/kg b.w.)			Positive
	distilled water	500	1000	2000	MMC
Number of micronucleus (%)					
Day 1	2.00 ± 1.41	1.00 ± 1.41	2.00 ± 0.00	1.50 ± 0.71	19.5 ± 2.12***
Day 2	1.50 ± 0.71	1.50 ± 0.71	2.00 ± 0.00	0.50 ± 0.71	17.5 ± 2.12***
Day 3	1.50 ± 0.71	1.50 ± 0.71	0.50 ± 0.71	2.50 ± 0.71	15.0 ± 4.24***
Reticuloctes of 50,000 erythrocytes (%)					
Day 1	4.94	4.29	4.40	4.56	2.48
Day 2	5.23	3.27	4.18	4.31	3.24
Day 3	5.09	4.07	4.26	4.59	3.25

The data are expressed as the Mean ± S.D. (n = 2). Significant difference as ***$P < 0.001$ was compared with control and treatment group by one-way ANOVA.

Table 5. Body weight changes in male and female Wistar rats during the 6-month safety assessment.

Gender		Male			Female		
CTE (g/kg (body weight/week))		Control	0.8 g/kg	1.65 g/kg	Control	0.8 g/kg	1.65 g/kg
Time (weeks)	n	n = 16	16	16	16	16	16
		Week numbers relative to start date; Weight (g)					
First	16	124.59 ± 6.89	125.53 ± 7.33	124.81 ± 7.51	109.08 ± 6.52	109.70 ± 7.55	108.33 ± 7.78
1 week	16	183.57 ± 9.08	187.64 ± 7.64	186.76 ± 7.36	140.74 ± 8.45	143.03 ± 8.81	142.80 ± 6.96
2	16	226.11 ± 10.42	232.01 ± 9.88	232.71 ± 7.96	161.79 ± 8.13	164.83 ± 10.09	163.98 ± 7.64
3	16	262.36 ± 12.02	270.31 ± 10.86	272.13 ± 9.86	179.41 ± 9.32	184.74 ± 11.37	182.06 ± 7.98
4	16	307.64 ± 14.86	306.83 ± 11.67	315.81 ± 11.27	199.04 ± 11.08	203.93 ± 12.62	200.09 ± 9.56
5	16	335.66 ± 18.08	329.48 ± 13.48	334.91 ± 12.58	211.83 ± 10.18	216.70 ± 12.48	212.00 ± 12.80
6	16	364.45 ± 20.15	348.53 ± 16.38	357.53 ± 15.84	224.37 ± 11.78	229.41 ± 15.32	224.56 ± 15.15
7	16	384.29 ± 22.23	367.71 ± 17.32	378.34 ± 17.53	232.26 ± 12.73	238.02 ± 15.87	233.06 ± 15.8
8	16	403.40 ± 22.14	385.97 ± 18.03	395.61 ± 18.73	239.76 ± 12.74	244.40 ± 16.44	240.39 ± 17.31
9	16	417.27 ± 23.76	397.31 ± 17.75	406.81 ± 18.73	243.31 ± 11.92	248.25 ± 16.57	245.93 ± 17.38
10	16	424.61 ± 24.43	400.46 ± 19.28**	405.69 ± 17.03*	247.59 ± 12.63	254.26 ± 18.04	251.27 ± 18.58
11	16	433.46 ± 24.77	406.62 ± 21.48**	412.10 ± 18.96*	252.91 ± 14.00	258.04 ± 18.74	253.73 ± 18.78
12	16	442.09 ± 23.86	415.04 ± 21.96**	420.93 ± 19.24*	257.81 ± 13.45	261.43 ± 18.88	257.90 ± 19.08
13	16	452.64 ± 26.69	423.58 ± 22.75**	429.71 ± 19.76**	263.13 ± 12.89	265.58 ± 19.34	262.34 ± 19.93
14	11	461.91 ± 19.04	432.16 ± 11.14***	444.70 ± 13.86*	259.86 ± 8.73	258.59 ± 10.15	253.68 ± 12.85
16	11	476.93 ± 18.30	444.9 ± 13.25***	459.05 ± 17.23*	263.43 ± 8.25	260.30 ± 12.61	259.65 ± 10.15
18	11	494.32 ± 17.68	461.76 ± 12.48***	474.82 ± 20.57*	271.96 ± 11.32	264.05 ± 26.33	266.65 ± 10.93
20	11	490.53 ± 31.46	474.46 ± 14.05	486.68 ± 29.43	278.10 ± 11.42	276.53 ± 11.17	271.39 ± 12.34
22	11	488.63 ± 32.02	474.16 ± 13.86	494.44 ± 20.58	281.10 ± 10.35	280.35 ± 9.32	271.29 ± 13.06
24	11	513.76 ± 25.80	487.76 ± 15.53*	508.65 ± 18.96	287.03 ± 10.45	283.48 ± 12.92	279.43 ± 11.27
26	11	527.86 ± 28.30	494.45 ± 15.67**	513.86 ± 21.38	287.15 ± 7.11	282.77 ± 12.27	277.07 ± 13.12
Recovery first	4	510.85 ± 18.22	499.75 ± 17.78	509.50 ± 16.32	283.05 ± 7.94	277.85 ± 16.53	272.20 ± 14.76
1	4	496.75 ± 18.89	494.25 ± 14.89	505.00 ± 25.91	278.25 ± 7.89	272.50 ± 15.07	270.50 ± 12.18
2	4	506.50 ± 18.05	510.00 ± 16.87	520.75 ± 16.05	284.50 ± 12.07	284.50 ± 14.39	279.50 ± 12.18

Data were presented as Mean ± S.D. (n ≥ 11). *t*-test: compared with control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 6. Body weight changes in male and female Beagle dogs during the 6-month safety assessment.

Gender		Male			Female	
CTE (g/kg)	Control	0.75 g/kg	1.50 g/kg	Control	0.75 g/kg	1.50 g/kg
Time (weeks)	n = 2	n = 2	n = 3	n = 2	n = 2	n = 3
			Week numbers relative to start date; Weight (Kg)			
1	6.80 ± 0.42	6.80 ± 0.71	7.07 ± 0.31	6.55 ± 0.78	6.45 ± 0.64	6.73 ± 1.01
2	8.10 ± 0.00	7.60 ± 0.28	7.60 ± 0.56	7.10 ± 0.14	7.15 ± 0.49	7.60 ± 0.66
4	9.20 ± 0.42	8.85 ± 0.49	7.85 ± 0.35	7.85 ± 0.35	7.70 ± 0.282	8.57 ± 0.83
6	8.75 ± 0.50	8.55 ± 0.64	8.50 ± 0.40	7.45 ± 0.07	7.65 ± 0.35	8.00 ± 0.80
8	9.30 ± 0.71	9.15 ± 0.35	8.80 ± 0.35	7.65 ± 0.21	7.45 ± 0.64	8.20 ± 0.75
10	9.05 ± 0.78	9.35 ± 0.64	9.27 ± 0.29	7.15 ± 0.49	7.30 ± 0.57	7.97 ± 0.60
12	9.25 ± 0.78	9.70 ± 0.71	9.53 ± 0.67	7.05 ± 0.64	7.10 ± 0.43	8.20 ± 0.72
14	9.43 ± 1.45	9.45 ± 0.07	9.50 ± 0.17	7.08 ± 0.88	6.75 ± 0.35	7.97 ± 0.41
16	9.80 ± 1.71	9.65 ± 0.21	9.57 ± 0.21	7.05 ± 0.71	7.30 ± 0.71	8.43 ± 0.70
18	9.50 ± 1.41	9.60 ± 0.00	9.63 ± 0.25	7.80 ± 0.57	7.60 ± 0.85	8.50 ± 0.7
20	9.25 ± 1.06	9.75 ± 0.07	9.73 ± 0.23	8.20 ± 0.71	7.80 ± 0.85	8.80 ± 0.60
22	9.15 ± 1.06	9.55 ± 0.07	9.80 ± 0.17	8.05 ± 0.64	7.70 ± 0.85	8.73 ± 0.65
24	9.15 ± 0.92	9.50 ± 0.00	9.77 ± 0.21	8.10 ± 0.57	7.70 ± 0.99	8.80 ± 0.56
26	9.20 ± 0.89	9.50 ± 0.00	9.73 ± 0.15	7.95 ± 0.64	7.80 ± 0.99	8.77 ± 0.60
Recovery 1	9.15 ± 0.92	-	9.50 ± 0.00	8.10 ± 0.57	-	8.85 ± 0.92
Recovery 2	9.15 ± 0.92	-	9.60 ± 0.07	8.40 ± 0.21	-	8.88 ± 0.88

Data were presented as Mean ± S.D. (n ≥ 2).

Table 7. Subacute toxicity serum analysis after 6-month CTE treatment on Wistar rats.

		Male			Female		
		Control	0.8 g/kg	1.65 g/kg	Control	0.8 g/kg	1.65 g/kg
Parameter	Unit	Time: 3 months; n = 10					
RBC	10^6/dL	8.53 ± 0.17	9.61 ± 0.33***	8.46 ± 2.67*	8.36 ± 0.90	8.49 ± 0.41	8.47 ± 0.41
HGB	g/dL	159.00 ± 6.29	174.80 ± 9.00*	183.40 ± 12.00*	174.00 ± 17.49	172.20 ± 11.22	161.20 ± 5.94
HCT	%	0.46 ± 0.02	0.52 ± 0.03*	0.52 ± 0.04*	0.49 ± 0.06	0.48 ± 0.02	0.48 ± 0.03
MCV	fL	54.24 ± 1.50	53.53 ± 1.53	55.32 ± 0.83	57.80 ± 1.02	56.92 ± 0.72	56.76 ± 0.89
MCH	Pg	18.76 ± 0.46	18.00 ± 0.59	19.56 ± 0.37	20.50 ± 0.92	20.28 ± 0.99	19.08 ± 0.62
MCHC	g/dL	345.80 ± 8.63	337.00 ± 3.27	353.40 ± 6.79	355.20 ± 19.60	356.40 ± 13.61	335.60 ± 10.177

Continued

PLT	10^3/μL	807.00 ± 101.64	748.20 ± 92.31	809.00 ± 184.33	882.60 ± 130.78	802.20 ± 137.44	906.40 ± 171.21
Clotting time	sec	57.75 ± 27.85	79.25 ± 23.15	66.75 ± 22.85	69.25 ± 21.82	66.25 ± 21.36	72.50 ± 14.43
WBC	10^3/μL	14.59 ± 2.22	16.47 ± 2.30	15.63 ± 3.93	16.62 ± 4.01	15.00 ± 2.70	18.54 ± 3.88
Neutrophils	%	22.50 ± 3.63	26.80 ± 5.96	24.20 ± 6.41	30.00 ± 7.09	29.20 ± 7.84	22.60 ± 9.07
Lymphocytes	%	73.20 ± 4.61	68.50 ± 6.43	70.50 ± 4.55	66.00 ± 6.53	67.20 ± 7.80	73.70 ± 9.41
Monocytes	%	3.00 ± 0.94	3.00 ± 1.56	3.10 ± 1.52	1.80 ± 1.03	2.00 ± 0.82	2.90 ± 1.10
Eosinophil	%	1.30 ± 0.82	1.60 ± 0.84	1.70 ± 1.06	1.80 ± 0.79	1.20 ± 0.79	0.80 ± 0.79
Basophils	%	0.00 ± 0.00	0.10 ± 0.32	0.50 ± 0.71	0.20 ± 0.42	0.30 ± 0.48	0.10 ± 0.32
Parameter	Unit	Time: 6 months; n = 10					
RBC	10^6/dL	7.44 ± 0.42	7.34 ± 0.63	7.36 ± 0.61	6.28 ± 0.28	6.71 ± 0.34	6.70 ± 0.31*
HGB	g/dL	153.33 ± 3.56	155.57 ± 6.71	155.71 ± 5.49	141.43 ± 6.02	145.50 ± 5.21	149.71 ± 4.46*
HCT	%	0.440 ± 0.02	0.425 ± 0.04	0.436 ± 0.03	0.384 ± 0.02	0.410 ± 0.02	0.404 ± 0.01*
MCV	fL	59.17 ± 1.09	57.86 ± 1.21	59.30 ± 2.16	61.14 ± 1.43	60.35 ± 1.75	60.39 ± 1.51
MCH	Pg	20.67 ± 1.45	21.29 ± 1.35	21.21 ± 1.32	22.57 ± 1.58	21.68 ± 1.2	22.39 ± 0.79
MCHC	g/dL	349.50 ± 21.81	368.14 ± 25.68	357.86 ± 15.57	369.29 ± 22.01	360.00 ± 16.52	370.00 ± 8.58
PLT	10^3/μL	820.17 ± 143.17	699.57 ± 69.30	704.57 ± 57.27	730.43 ± 116.58	694.00 ± 67.69	715.00 ± 56.12
Clotting time	sec	57.80 ± 19.96	75.00 ± 14.15	63.86 ± 42.26	31.33 ± 13.26	30.50 ± 10.82	41.86 ± 13.42
WBC	10^3/μL	11.77 ± 1.36	12.36 ± 2.07	10.20 ± 1.66	9.56 ± 1.20	9.22 ± 1.71	9.63 ± 1.30
Neutrophils	%	22.60 ± 5.00	22.67 ± 5.02	27.00 ± 8.04	26.30 ± 5.80	27.75 ± 4.83	27.20 ± 5.31
Lymphocytes	%	71.02 ± 6.30	71.11 ± 6.48	69.50 ± 8.16	71.00 ± 6.21	69.38 ± 4.90	69.33 ± 4.18
Monocytes	%	2.14 ± 0.76	3.12 ± 0.89	2.50 ± 0.76	2.50 ± 1.18	1.80 ± 0.81	2.11 ± 1.05
Eosinophil	%	0.67 ± 0.50	0.67 ± 0.50	0.75 ± 0.71	0.90 ± 0.74	0.87 ± 0.60	0.44 ± 0.53
Basophils	%	0.22 ± 0.44	0.22 ± 0.44	0.13 ± 0.35	0.00 ± 0.00	0.10 ± 0.32	0.10 ± 0.32
Parameter	Unit	Time: After 2 months recovery; n = 4					
RBC	10^6/dL	8.10 ± 0.59	7.89 ± 0.75	7.84 ± 0.24	6.48 ± 0.37	6.99 ± 0.61	6.73 ± 0.39
HGB	g/dL	165.75 ± 9.22	160.00 ± 11.75	159.00 ± 8.12	149.75 ± 3.30	152.50 ± 11.45	143.50 ± 7.33
HCT	%	0.467 ± 0.02	0.461 ± 0.05	0.462 ± 0.01	0.420 ± 0.04	0.426 ± 0.03	0.407 ± 0.03
MCV	fL	57.68 ± 1.44	58.30 ± 1.74	59.00 ± 1.53	61.08 ± 2.08	61.00 ± 0.79	60.48 ± 1.23
MCH	Pg	20.50 ± 0.71	20.80 ± 1.12	20.35 ± 1.56	22.60 ± 1.07	21.80 ± 0.40	21.40 ± 1.91
MCHC	g/dL	354.75 ± 5.50	357.50 ± 23.87	344.25 ± 21.00	370.25 ± 9.36	358.00 ± 6.06	354.50 ± 36.41
PLT	10^3/μL	775.00 ± 27.70	734.75 ± 69.14	764.25 ± 99.87	748.25 ± 49.69	723.00 ± 137.03	754.00 ± 52.06

Continued

Clotting time	sec	45.50 ± 3.51	44.50 ± 4.65	48.75 ± 9.00	42.25 ± 5.62	43.25 ± 10.05	46.00 ± 2.82
WBC	10^3/μL	13.85 ± 2.67	10.75 ± 2.18	10.45 ± 1.42	8.53 ± 1.17	8.78 ± 1.87	8.48 ± 1.28
Neutrophils	%	21.00 ± 4.24	26.00 ± 4.55	22.00 ± 5.60	28.75 ± 8.18	27.00 ± 4.55	28.50 ± 7.33
Lymphocytes	%	75.75 ± 3.50	69.80 ± 3.96	74.50 ± 5.74	69.25 ± 8.46	69.25 ± 4.92	67.50 ± 6.55
Monocytes	%	2.00 ± 0.82	2.75 ± 0.50	3.00 ± 0.82	1.50 ± 0.58	2.50 ± 0.58	2.50 ± 0.58
Eosinophil	%	1.00 ± 0.82	1.25 ± 0.50	0.75 ± 0.96	0.50 ± 0.58	1.00 ± 0.82	1.25 ± 0.96
Basophils	%	0.00 ± 0.00	0.25 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data were presented as Mean ± S.D. (n ≥ 4). *t*-test: compared with control: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelets; WBC: white blood cells.

Table 8. Subacute toxicity serum analysis after 6-month CTE treatment on Beagle dogs.

Time: 3 months		Male			Female		
		Control	0.75 g/kg	1.50 g/kg	Control	0.75 g/kg	1.50 g/kg
Parameter	Unit	n = 2	n = 2	n = 3	n = 2	n = 2	n = 3
RBC	10^6/dL	8.12 ± 0.51	6.59 ± 0.04	6.53 ± 0.34*	7.34 ± 0.33	7.65 ± 0.10	6.34 ± 0.94
HGB	g/dL	152.5 ± 6.4	148.5 ± 9.2	143.3 ± 7.4	153.5 ± 6.4	161.0 ± 25.5	140.0 ± 14.1
HCT	%	0.52 ± 0.01	0.45 ± 0.01	0.44 ± 0.00*	0.51 ± 0.05	0.52 ± 0.03	0.43 ± 0.05
MCV	fL	64.1 ± 3.0	68.5 ± 0.9	67.2 ± 1.3	68.8 ± 3.4	67.2 ± 3.5	68.1 ± 3.2
MCH	Pg	18.85 ± 1.91	22.50 ± 1.27	22.0 ± 0.1	21.0 ± 0.1	21.1 ± 3.0	22.2 ± 1.4
MCHC	g/dL	293.0 ± 17.08	329.0 ± 22.6	327.0 ± 4.6*	304.5 ± 16.3	312.0 ± 29.7	326.0 ± 6.6
PLT	10^3/μL	216.0 ± 77.8	291.5 ± 54.5	213.3 ± 81.8	259.0 ± 17.0	302.5 ± 21.9	251.0 ± 38.9
Clotting time	sec	50.00 ± 7.07	49.50 ± 2.13	42.33 ± 11.24	70.50 ± 27.58	55.50 ± 7.78	48.67 ± 3.21
WBC	10^3/μL	12.70 ± 1.13	13.3 ± 0.4	13.3 ± 1.6	11.2 ± 0.9	10.6 ± 1.1	12.0 ± 2.6
Neutrophils	%	63.00 ± 16.97	59.50 ± 2.12	65.67 ± 5.86	67.00 ± 7.07	67.50 ± 9.20	61.00 ± 8.54
Lymphocytes	%	31.50 ± 14.85	36.50 ± 2.12	28.00 ± 7.00	26.00 ± 4.24	28.00 ± 9.20	32.67 ± 8.08
Monocytes	%	3.50 ± 2.12	2.50 ± 0.71	3.00 ± 1.00	4.00 ± 1.41	2.50 ± 0.71	3.33 ± 1.15
Eosinophil	%	2.00 ± 0.00	1.50 ± 0.71	3.33 ± 3.21	3.00 ± 4.24	2.00 ± 0.00	3.00 ± 1.00
Basophils	%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
				Time: 6 months			
RBC	10^6/dL	7.10 ± 0.69	6.54 ± 0.40	6.53 ± 0.35	6.29 ± 0.66	6.49 ± 0.06	6.34 ± 0.79
HGB	g/dL	147.00 ± 4.24	143.00 ± 14.10	147.67 ± 5.51	139.50 ± 20.51	143.50 ± 4.95	145.00 ± 20.0
HCT	%	0.473 ± 0.04	0.465 ± 0.04	0.461 ± 0.01	0.432 ± 0.06	0.448 ± 0.01	0.440 ± 0.04

Continued

MCV	fL	66.65 ± 0.92	71.00 ± 1.41	70.63 ± 3.14	68.60 ± 1.56	69.00 ± 1.48	69.43 ± 2.65
MCH	Pg	20.80 ± 1.41	21.80 ± 0.85	22.63 ± 0.45	22.15 ± 0.92	22.10 ± 0.57	22.93 ± 0.45
MCHC	g/dL	311.5 ± 17.68	308.0 ± 5.66	320.0 ± 8.66	322.5 ± 6.36	321.0 ± 1.41	330.0 ± 6.24
PLT	10^3/μL	254.50 ± 9.19	212.50 ± 54.41	239.67 ± 56.52	356.5 ± 58.69	238.0 ± 86.27	285.00 ± 54.58
Clotting time	sec	57.80 ± 19.96	75.00 ± 14.15	63.86 ± 42.26	31.33 ± 13.26	30.50 ± 10.82	41.86 ± 13.42
PT	sec	7.05	7.95	7.05 ± 0.00	7.43 ± 0.53	7.43 ± 0.53	7.05 ± 0.00
APPT	sec	24.00 ± 3.11	16.95 ± 3.04	21.17 ± 0.57	19.15 ± 4.17	20.20 ± 6.08	21.03 ± 3.71
FIB	mg/dL	121.00 ± 4.24	155.00	119.0 ± 35.7	105.0 ± 0.71	122.0 ± 161.2	113.33 ± 34.27
WBC	10^3/μL	12.30 ± 2.83	13.70 ± 3.25	11.33 ± 0.59	9.45 ± 1.06	9.20 ± 0.14	11.03 ± 3.56
Neutrophils	%	67.50 ± 4.24	64.50 ± 20.51	69.67 ± 10.21	74.00 ± 8.48	71.00 ± 0.00	68.00 ± 5.00
Lymphocytes	%	27.50 ± 1.41	34.50 ± 20.51	22.67 ± 10.21	23.00 ± 7.07	25.50 ± 3.53	28.60 ± 4.36
Monocytes	%	2.00 ± 0.00	1.00 ± 1.00	2.00 ± 1.00	2.00 ± 0.00	0.50 ± 0.71	2.33 ± 1.53
Eosinophil	%	3.00 ± 1.41	0.00 ± 0.00	2.33 ± 2.52	1.00 ± 1.00	3.00 ± 4.24	1.00 ± 1.00
Basophils	%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
				Recovery period 2 weeks			
RBC	10^6/dL	7.01 ± 0.08	-	7.18 ± 0.54	6.22 ± 0.25	-	6.69 ± 0.96
HGB	g/dL	146.5 ± 2.12	-	148.5 ± 13.44	133.0 ± 2.82	-	149.0 ± 19.8
HCT	%	0.476 ± 0.01	-	0.492 ± 0.02	0.435 ± 0.02	-	0.479 ± 0.06
MCV	fL	67.90 ± 2.12	-	68.65 ± 1.91	69.90 ± 0.99	-	71.65 ± 1.48
MCH	Pg	20.95 ± 0.07	-	20.70 ± 0.28	21.40 ± 0.42	-	22.30 ± 0.28
MCHC	g/dL	308.5 ± 10.61	-	301.5 ± 12.02	306.0 ± 9.90	-	311.0 ± 2.83
PLT	10^3/μL	291.5 ± 57.28	-	267.5 ± 79.9	216.0 ± 4.24	-	283.0 ± 56.57
Clotting time	sec	109.0 ± 15.56	-	97.5 ± 17.68	103.0 ± 9.90	-	94.00 ± 15.56
WBC	10^3/μL	2.00 ± 0.82	-	3.00 ± 0.82	1.50 ± 0.58	-	2.50 ± 0.58
Neutrophils	%	58.50 ± 19.09	-	60.00 ± 1.41	47.5 ± 21.92	-	64.0 ± 4.24
Lymphocytes	%	38.00 ± 16.97	-	35.50 ± 0.71	48.5 ± 24.75	-	31.0 ± 1.41
Monocytes	%	3.00 ± 1.41	-	2.00 ± 1.41	2.50 ± 0.71	-	2.50 ± 0.71
Eosinophil	%	0.50 ± 0.71	-	2.50 ± 0.71	1.50 ± 2.12	-	2.50 ± 2.12
Basophils	%	0.00 ± 0.00	-	0.00 ± 0.00	0.00 ± 0.00	-	0.00 ± 0.00

Data were presented as Mean ± S.D. (n ≥ 2). *t*-test: compared with control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelets; WBC: white blood cells.

Table 9. Chronic toxicity biochemical analysis after 6-month CTE treatment on Wistar rats.

		Male			Female		
		Control	0.8 g/kg	1.65 g/kg	Control	0.8 g/kg	1.65 g/kg
Parameter	unit	Time: 3 months; n = 10					
ALT	U/L	32.80 ± 3.57	33.50 ± 3.17	33.10 ± 4.46	26.90 ± 2.77	29.40 ± 3.00	26.40 ± 3.50
AST	U/L	85.30 ± 12.21	78.70 ± 10.08	86.90 ± 17.20	77.60 ± 18.06	80.50 ± 11.32	82.00 ± 12.74
TBiL	mg/dL	1.89 ± 0.22	1.45 ± 0.34	2.11 ± 0.15	2.09 ± 0.30	1.88 ± 0.25	2.15 ± 0.56
ALP	U/L	112.10 ± 16.00	115.80 ± 15.56	134.30 ± 16.12*	61.90 ± 10.02	68.70 ± 12.66	87.10 ± 27.13
TP	g/dL	73.70 ± 1.35	69.60 ± 1.85	72.30 ± 1.79	73.00 ± 3.32	74.70 ± 1.85	78.10 ± 2.88*
ALB	g/dL	36.20 ± 0.40	35.70 ± 0.90	37.00 ± 0.63	37.90 ± 1.97	38.30 ± 1.00	38.50 ± 1.96
CHOL	mg/dL	2.49 ± 0.12	2.39 ± 0.16	2.43 ± 0.12	2.39 ± 0.23	2.45 ± 0.28	2.18 ± 0.07
GLU	U/L	5.50 ± 0.34	5.31 ± 0.45	5.40 ± 0.34	6.05 ± 0.50	5.99 ± 0.69	5.76 ± 0.81
BUN	mg/dL	5.90 ± 0.76	5.63 ± 0.61	6.28 ± 0.59	5.35 ± 0.71	5.72 ± 0.66	6.61 ± 2.27
Crea	U/L	60.60 ± 3.32	55.00 ± 2.44	59.10 ± 4.50	60.80 ± 1.25	61.70 ± 2.00	64.50 ± 5.3
Parameter	unit	Time: 6 months; n = 10					
ALT	U/L	44.83 ± 4.17	52.43 ± 4.76*	57.00 ± 9.63*	35.71 ± 8.16	38.60 ± 5.03	42.57 ± 8.92`
AST	U/L	122.50 ± 16.12	129.86 ± 12.33	135.71 ± 20.86	84.57 ± 13.82	98.17 ± 11.05*	107.29 ± 13.59***
TBiL	mg/dL	1.96 ± 0.17	2.24 ± 0.24	2.06 ± 0.22	2.26 ± 0.40	2.10 ± 0.33	2.29 ± 0.34
ALP	U/L	91.33 ± 12.61	101.00 ± 15.52	108.14 ± 16.72	65.43 ± 20.53	53.17 ± 4.17	70.00 ± 13.53
TP	g/dL	77.17 ± 3.37	77.00 ± 4.00	76.86 ± 3.08	74.86 ± 2.26	76.17 ± 1.60	77.86 ± 3.33*
ALB	g/dL	38.50 ± 0.84	38.43 ± 0.98	38.00 ± 1.00	40.33 ± 1.75	41.33 ± 0.82	42.14 ± 1.21
CHOL	mg/dL	2.58 ± 0.28	2.42 ± 0.12	2.56 ± 0.27	1.98 ± 0.17	2.14 ± 0.16	2.19 ± 0.25
GLU	U/L	5.43 ± 0.51	4.75 ± 0.41	4.90 ± 0.46	4.79 ± 0.42	5.32 ± 0.41	4.74 ± 0.42
BUN	mg/dL	6.67 ± 1.04	6.84 ± 0.53	6.51 ± 0.57	5.41 ± 0.38	5.39 ± 0.64	5.60 ± 0.75
Crea	U/L	64.67 ± 4.93	59.57 ± 4.69	57.71 ± 1.98**	56.86 ± 2.97	57.00 ± 6.16	62.86 ± 4.88*
Parameter	unit	Time: After 2 months recovery; n = 4					
ALT	U/L	39.75 ± 4.35	39.75 ± 3.10	32.75 ± 2.06	32.75 ± 5.74	28.75 ± 6.02	29.00 ± 2.94
AST	U/L	136.25 ± 11.27	106.05 ± 6.45**	111.50 ± 8.43*	101.25 ± 7.72	96.50 ± 7.77	92.00 ± 7.48
TBiL	mg/dL	2.30 ± 0.26	2.15 ± 0.42	2.00 ± 0.29	2.33 ± 0.36	2.58 ± 0.13	2.58 ± 0.19
ALP	U/L	95.00 ± 19.92	87.75 ± 20.29	76.00 ± 8.76*	60.75 ± 11.79	61.00 ± 9.42	61.25 ± 10.69
TP	g/dL	75.50 ± 0.58	73.25 ± 4.79	74.00 ± 2.71	74.00 ± 2.16	74.50 ± 2.52	73.75 ± 1.89
ALB	g/dL	37.75 ± 0.50	37.50 ± 2.08	38.00 ± 0.82	40.75 ± 0.96	41.00 ± 1.64	40.75 ± 0.50
CHOL	mg/dL	2.73 ± 0.23	2.69 ± 0.22	3.02 ± 0.44	2.38 ± 0.12	2.89 ± 0.18	2.36 ± 0.32
GLU	U/L	4.18 ± 0.47	3.80 ± 0.25	4.90 ± 0.80	4.15 ± 0.50	4.55 ± 0.40	4.60 ± 0.50
BUN	mg/dL	5.63 ± 0.48	5.40 ± 0.90	5.88 ± 0.93	5.33 ± 0.82	5.45 ± 0.80	5.60 ± 0.84
Crea	U/L	57.50 ± 5.45	62.00 ± 8.17	60.50 ± 3.87	59.25 ± 2.22	65.75 ± 4.92	63.75 ± 3.86

Data were presented as Mean ± S.D. (n ≥ 4). *t*-test: compared with control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBiL: total bilirubin; ALP: alkaline phosphatase; TP: total protein; ALB: albumin; CHOL: cholesterol; GLU: glucose; BUN: blood urea nitrogen; Crea: creatinine.

Table 10. Chronic toxicity biochemical analysis after 6-month CTE treatment on Beagle dogs.

Time: 3 months		Male			Female		
		Control	0.8 g/kg	1.65 g/kg	Control	0.8 g/kg	1.65 g/kg
Parameter	Unit	n = 2	n = 2	n = 3	n = 2	n = 2	n = 3
ALT	U/L	47.0 ± 5.7	40.5 ± 13.4	32.0 ± 6.1	30.0 ± 5.7	35.5 ± 7.8	26.7 ± 2.9
AST	U/L	32.5 ± 0.7	49.0 ± 5.7	40.3 ± 1.2**	48.5 ± 7.8	38.0 ± 0.0	45.7 ± 5.5
ALP	U/L	89.0 ± 31.1	112.0 ± 9.9	137.3 ± 49.1	164.0 ± 11.3	69.50 ± 48.8	94.7 ± 26.6*
TP	g/dL	69.5 ± 5.0	65.5 ± 2.1	64.3 ± 3.8	71.5 ± 0.7	70.0 ± 8.5	64.0 ± 5.0
ALB	g/dL	30.5 ± 0.7	30.0 ± 1.4	29.0 ± 0.0	30.0 ± 1.4	31.5 ± 2.1	26.3 ± 0.6
TBIL	mg/dL	1.20 ± 0.28	2.10 ± 0.00	1.43 ± 0.15	1.15 ± 0.21	1.20 ± 0.10	1.77 ± 0.06
TCHL	mg/dL	3.34 ± 0.08	3.54 ± 0.00	3.32 ± 0.88	3.24 ± 0.12	4.06 ± 0.04*	3.04 ± 0.28
BUN	U/L	3.95 ± 1.63	5.35 ± 2.05	4.73 ± 0.65	5.05 ± 0.07	5.00 ± 1.70	6.87 ± 029
CREA	U/L	72.5 ± 0.7	81.5 ± 6.36	75.67 ± 8.02	72.0 ± 2.83	74.0 ± 12.7	72.3 ± 5.8
GLU	U/L	3.85 ± 0.35	3.90 ± 0.14	4.13 ± 0.70	4.10 ± 0.85	3.60 ± 0.14	4.07 ± 0.23
Parameter	Unit	Time: 6 months					
ALT	U/L	47.00 ± 2.83	54.00 ± 16.97	41.33 ± 10.69	36.50 ± 0.14	32.5 ± 1.41	33.00 ± 6.55
AST	U/L	32.50 ± 0.71	49.00 ± 8.48	39.83 ± 7.36	36.00 ± 8.48	35.50 ± 9.19	44.67 ± 0.58
ALP	U/L	70.50 ± 16.26	63.00 ± 8.48	105.67 ± 50.21	154.50 ± 21.92	54.5 ± 21.92*	63.67 ± 22.50*
TP	g/dL	70.50 ± 0.71	69.50 ± 6.36	70.33 ± 3.51	69.50 ± 0.71	72.50 ± 3.53	73.67 ± 0.58
ALB	g/dL	30.50 ± 0.71	30.00 ± 0.00	30.33 ± 0.58	29.00 ± 1.41	30.00 ± 0.00	29.00 ± 1.00
TBIL	mg/dL	0.95 ± 0.07	1.45 ± 0.64	1.17 ± 0.42	1.05 ± 0.49	1.05 ± 0.35	1.23 ± 0.40
TCHL	mg/dL	3.99 ± 0.18	3.86 ± 0.03	4.27 ± 0.78	5.84 ± 0.04	4.35 ± 1.56	3.92 ± 0.70
BUN	U/L	4.65 ± 2.33	4.90 ± 1.41	5.07 ± 0.96	6.65 ± 0.78	4.40 ± 0.00	5.97 ± 1.32
CREA	U/L	83.00 ± 5.66	95.00 ± 14.1	82.67 ± 9.45	75.50 ± 13.44	74.00 ± 1.41	85.00 ± 10.54
GLU	U/L	5.50 ± 0.85	5.20 ± 0.14	4.70 ± 0.53	5.45 ± 0.49	5.20 ± 0.00	4.33 ± 1.04
Parameter	Unit	Recovery period 2 weeks					
ALT	U/L	49.00 ± 0.00	-	53.00 ± 1.41	32.50 ± 6.36	-	35.00 ± 2.03
AST	U/L	34.00 ± 8.48	-	38.00 ± 1.41	33.00 ± 2.83	-	37.50 ± 4.95
ALP	U/L	36.50 ± 2.12	-	58.5 ± 38.89	65.50 ± 13.44	-	37.00 ± 8.49
TP	g/dL	72.0 ± 4.24	-	68.0 ± 2.83	70.50 ± 3.54	-	74.50 ± 2.12
ALB	g/dL	30.0 ± 0.0	-	30.0 ± 1.41	29.00 ± 1.41	-	28.50 ± 0.71
TBIL	mg/dL	0.85 ± 0.07	-	1.20 ± 0.28	1.30 ± 0.28	-	1.45 ± 0.21
TCHL	mg/dL	3.48 ± 0.05	-	3.54 ± 0.56	3.92 ± 0.10	-	3.15 ± 0.30
BUN	U/L	3.45 ± 0.78	-	5.10 ± 1.56	5.40 ± 1.13	-	4.00 ± 0.85
CREA	U/L	78.5 ± 3.54	-	89.5 ± 13.4	73.00 ± 11.31	-	76.50 ± 4.95
GLU	U/L	3.90 ± 0.85	-	4.10 ± 0.28	4.40 ± 0.14	-	3.65 ± 0.21

Data were presented as Mean ± S.D. (n ≥ 4). *t*-test: compared with control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBiL: total bilirubin; ALP: alkaline phosphatase; TP: total protein; ALB: albumin; CHOL: cholesterol; GLU: glucose; BUN: blood urea nitrogen; Crea: creatinine.

3.3. No Macropathological or Histopathological Lesions Were Found in Chronic Oral Toxicity Tests for CTE

At six months and at the end of the recovery period, no significant gross lesions were found in either rats or dogs in the treatment or control groups. Gastric and intestinal mucosa examinations revealed normal mucosal folds with no evidence of ulcers, bleeding, or scarring. No abnormalities were observed in other organs either. The only significant finding in the Wistar rat gross necropsy was an increase in thymus weight and thymus-body weight ratio after three months of CTE treatment at 0.8 g/kg, compared to controls ($P < 0.05$). No other significant between-group differences were observed. After six months of CTE administration, no treatment-related lesions were found in major organs. Inflammatory cell infiltration was observed in some major organs across both treatment and control groups, with few animals showing lymphocyte infiltration in the renal interstitial, portal area of the liver, and alveolar septum, which may not be related to CTE administration (Table 11 and Table 12).

Table 11. Organ weight changes after 6-month CTE treatment on Wistar rats.

Time: 3 months							
Organ weight	(gram)	Control	0.8 g/kg	1.65 g/kg	Control	0.8 g/kg	1.65 g/kg
Organ-to-body weight ratio (%)			Males (n = 7)			Females (n = 7)	
Heart							
Absolute weight	gram	1.31 ± 0.13	1.17 ± 0.10	1.17 ± 0.15	0.75 ± 0.09	0.81 ± 0.09	0.87 ± 0.09
Ratio per body weight	%	0.30 ± 0.03	0.29 ± 0.01	0.30 ± 0.03	0.32 ± 0.03	0.32 ± 0.05	0.34 ± 0.04
Liver							
Absolute weight	gram	13.90 ± 1.20	13.60 ± 1.59	12.29 ± 0.95	7.65 ± 0.83	7.46 ± 0.75	8.21 ± 0.61
Ratio per body weight	%	3.16 ± 0.05	3.27 ± 0.29	3.13 ± 0.18	3.03 ± 0.17	2.96 ± 0.38	3.18 ± 0.13
Spleen							
Absolute weight	gram	0.80 ± 0.10	0.77 ± 0.10	0.67 ± 0.08	0.50 ± 0.06	0.50 ± 0.06	0.54 ± 0.13
Ratio per body weight	%	0.18 ± 0.01	0.19 ± 0.02	0.17 ± 0.01	0.20 ± 0.02	0.20 ± 0.03	0.21 ± 0.06
Lung							
Absolute weight	gram	1.67 ± 0.26	1.54 ± 0.33	1.83 ± 0.44	1.18 ± 0.10	1.23 ± 0.04	1.19 ± 0.13
Ratio per body weight	%	0.39 ± 0.11	0.38 ± 0.09	0.46 ± 0.10	0.46 ± 0.10	0.48 ± 0.17	0.49 ± 0.09
Kidney							
Absolute weight	gram	2.94 ± 0.26	2.79 ± 0.21	2.79 ± 0.29	1.70 ± 0.16	1.80 ± 0.01	1.87 ± 0.08
Ratio per body weight	%	0.67 ± 0.04	0.67 ± 0.06	0.71 ± 0.05	0.67 ± 0.03	0.71 ± 0.04	0.71 ± 0.04

Continued

Brain							
Absolute weight	gram	2.04 ± 0.08	2.01 ± 0.07	2.09 ± 0.11	1.75 ± 0.09	1.80 ± 0.02	1.90 ± 0.10
Ratio per body weight	%	0.48 ± 0.04	0.49 ± 0.05	0.53 ± 0.05	0.70 ± 0.05	0.71 ± 0.05	0.74 ± 0.05
Thymus							
Absolute weight	min gram	0.24 ± 0.06	0.22 ± 0.06	0.26 ± 0.05	0.21 ± 0.04	0.28 ± 0.03*	0.25 ± 0.04
Ratio per body weight	mg %	51.43 ± 15.73	54.29 ± 12.72	65.71 ± 11.33	81.25 ± 15.53	108.57 ± 15.74**	92.86 ± 7.56
Adrenal							
Absolute weight	min gram	99.14 ± 25.28	90.86 ± 20.92	121.29 ± 83.50	84.57 ± 12.43	87.57 ± 11.27	87.86 ± 7.78
Ratio per body weight	mg %	21.42 ± 6.90	22.86 ± 4.88	34.29 ± 25.07	30.38 ± 13.24	34.29 ± 5.35	34.29 ± 5.35
Testis or ovarian							
Absolute weight	gram	3.33 ± 0.28	3.16 ± 0.14	3.11 ± 0.30	0.14 ± 0.02	0.15 ± 0.03	0.16 ± 0.02
Ratio Per body weight	%	0.76 ± 0.08	0.76 ± 0.04	0.79 ± 0.05	52.50 ± 7.07	58.57 ± 10.69	57.14 ± 7.56
Epididymis or uterus							
Absolute weight	gram	1.43 ± 0.19	1.59 ± 0.23	1.34 ± 0.15	0.55 ± 0.21	0.57 ± 0.35	0.56 ± 0.20
Ratio per body weight	%	0.32 ± 0.04	0.38 ± 0.04	0.34 ± 0.03	0.22 ± 0.09	0.22 ± 0.10	0.21 ± 0.05
Postate							
Absolute weight	gram	1.07 ± 0.17	0.89 ± 0.20	0.89 ± 0.13			
Ratio per body weight	%	0.24 ± 0.03	0.21 ± 0.03	0.21 ± 0.04			
Seminal vesicle							
Absolute weight	gram	1.76 ± 0.50	1.80 ± 0.35	1.57 ± 0.42			
Ratio per body weight	%	0.40 ± 0.11	0.43 ± 0.07	0.40 ± 0.11			
Organ	Unit	Time: 6 months; n = 10					
Heart							
Absolute weight	gram	1.67 ± 0.36	1.31 ± 0.17	1.42 ± 0.12	0.89 ± 0.07	0.90 ± 0.09	0.87 ± 0.08
Ratio per body weight	%	0.32 ± 0.06	0.28 ± 0.04	0.29 ± 0.02	0.32 ± 0.03	0.33 ± 0.03	0.33 ± 0.03
Liver							
Absolute weight	gram	12.97 ± 0.78	12.10 ± 0.62	13.43 ± 0.96	7.29 ± 0.46	7.05 ± 0.42	6.86 ± 0.38
Ratio per body weight	%	2.52 ± 0.09	2.56 ± 0.10	2.53 ± 0.14	2.62 ± 0.18	2.60 ± 0.06	2.58 ± 0.14
Spleen							
Absolute weight	gram	0.88 ± 0.16	0.84 ± 0.08	0.87 ± 0.11	0.64 ± 0.08	0.67 ± 0.08	0.64 ± 0.05
Ratio per body weight	%	0.17 ± 0.03	0.18 ± 0.02	0.18 ± 0.02	0.23 ± 0.03	0.25 ± 0.04	0.24 ± 0.02

Continued

Lung							
Absolute weight	gram	1.58 ± 0.21	1.44 ± 0.19	1.94 ± 0.32	1.20 ± 0.28	1.02 ± 0.12	1.11 ± 0.23
Ratio per body weight	%	0.31 ± 0.04	0.30 ± 0.04	0.36 ± 0.07	0.43 ± 0.10	0.38 ± 0.04	0.36 ± 0.09
Kidney							
Absolute weight	gram	3.22 ± 0.29	3.00 ± 0.12	3.19 ± 0.18	1.93 ± 0.10	1.88 ± 0.15	1.91 ± 0.17
Ratio per body weight	%	0.62 ± 0.04	0.63 ± 0.03	0.65 ± 0.04	0.69 ± 0.05	0.70 ± 0.05	0.72 ± 0.04
Brain							
Absolute weight	gram	2.13 ± 0.08	2.11 ± 0.07	2.11 ± 0.09	1.91 ± 0.09	1.88 ± 0.16	1.96 ± 0.05
Ratio per body weight	%	0.42 ± 0.01	0.45 ± 0.03	0.43 ± 0.03	0.69 ± 0.04	0.70 ± 0.08	0.73 ± 0.04
Thymus							
Absolute weight	min gram	0.17 ± 0.04	0.13 ± 0.02	0.16 ± 0.04	0.14 ± 0.05	0.15 ± 0.03	0.14 ± 0.03
Ratio per body weight	mg %	33.02 ± 6.88	27.70 ± 4.29	33.26 ± 7.22	54.20 ± 16.73	55.89 ± 11.50	52.13 ± 12.43
Adrenal							
Absolute weight	min gram	69.17 ± 4.92	60.00 ± 11.55	57.86 ± 8.09	72.14 ± 8.09	74.17 ± 4.92	75.71 ± 9.76
Ratio per body weight	mg %	13.45 ± 1.07	12.65 ± 2.29	11.78 ± 1.70	28.15 ± 4.16	27.39 ± 1.54	28.55 ± 3.52
Testis or ovarian							
Absolute weight	gram	3.40 ± 0.09	3.39 ± 0.13	3.22 ± 0.28	0.13 ± 0.02	0.15 ± 0.02	0.14 ± .0.02
Ratio per body weight	%	0.66 ± 0.04	0.72 ± 0.04	0.65 ± 0.06	48.13 ± 8.64	54.21 ± 4.48	52.24 ± 6.28
Epididymis or uterus							
Absolute weight	gram	1.42 ± 0.19	1.41 ± 0.02	1.40 ± 0.15	0.61 ± 0.29	0.48 ± 0.04	0.56 ± 0.10
Ratio per body weight	%	0.28 ± 0.04	0.30 ± 0.02	0.28 ± 0.04	0.22 ± 0.11	0.18 ± 0.01	0.21 ± 0.04
Postate							
Absolute weight	gram	1.20 ± 0.18	1.30 ± 0.21	1.31 ± 0.15			
Ratio per body weight	%	0.24 ± 0.03	0.27 ± 0.03	0.27 ± 0.04			
Seminal vesicle							
Absolute weight	gram	1.87 ± 0.45	1.87 ± 0.20	1.60 ± 0.37			
Ratio per body weight	%	0.36 ± 0.08	0.40 ± 0.04	0.33 ± 0.09			
Organ	Unit	Time: After 2 months recovery; n = 4					
Heart							
Absolute weight	gram	1.35 ± 0.13	1.35 ± 0.17	1.20 ± 0.08	0.83 ± 0.05	0.75 ± 0.13	0.80 ± 0.00
Ratio per body weight	%	0.28 ± 0.03	0.28 ± 0.03	0.24 ± 0.02	0.31 ± 0.03	0.30 ± 0.02	0.30 ± 0.01

Continued

Liver							
Absolute weight	gram	13.85 ± 0.73	14.43 ± 0.62	15.15 ± 0.79	7.93 ± 0.55	7.48 ± 0.70	7.13 ± 0.46
Ratio per body weight	%	2.80 ± 0.15	2.96 ± 0.09	3.06 ± 0.16	2.95 ± 0.31	2.80 ± 0.16	2.65 ± 0.08
Spleen							
Absolute weight	gram	0.85 ± 0.10	0.75 ± 0.06	0.80 ± 0.08	0.55 ± 0.06	0.55 ± 0.13	0.55 ± 0.06
Ratio per body weight	%	0.17 ± 0.02	0.16 ± 0.01	0.16 ± 0.02	0.20 ± 0.03	0.21 ± 0.04	0.20 ± 0.02
Lung							
Absolute weight	gram	1.63 ± 0.32	1.45 ± 0.13	1.35 ± 0.13	1.05 ± 0.17	1.00 ± 0.00	1.04 ± 0.11
Ratio per body weight	%	0.32 ± 0.06	0.30 ± 0.03	0.27 ± 0.02	0.39 ± 0.07	0.38 ± 0.02	0.39 ± 0.06
Kidney							
Absolute weight	gram	3.13 ± 0.15	3.18 ± 0.13	3.15 ± 0.17	1.93 ± 0.05	2.05 ± 0.13	1.93 ± 0.05
Ratio per body weight	%	0.63 ± 0.05	0.65 ± 0.03	0.64 ± 0.02	0.72 ± 0.03	0.77 ± 0.05	0.72 ± 0.03
Brain							
Absolute weight	gram	2.00 ± 0.14	2.03 ± 0.10	2.05 ± 0.10	1.88 ± 0.05	1.93 ± 0.10	1.88 ± 0.13
Ratio per body weight	%	0.40 ± 0.03	0.42 ± 0.03	0.42 ± 0.01	0.70 ± 0.04	0.72 ± 0.05	0.70 ± 0.04
Thymus							
Absolute weight	min gram	0.24 ± 0.02	0.22 ± 0.05	0.21 ± 0.03	0.15 ± 0.03	0.15 ± 0.01	0.13 ± 0.03
Ratio per body weight	mg %	47.42 ± 2.19	44.60 ± 9.99	41.40 ± 5.62	55.74 ± 9.77	56.48 ± 7.18	46.95 ± 13.58
Adrenal							
Absolute weight	min gram	71.25 ± 8.54	75.00 ± 5.77	75.00 ± 5.77	80.00 ± 14.14	70.00 ± 11.55	77.50 ± 9.57
Ratio per body weight	mg %	14.39 ± 1.52	15.40 ± 1.38	15.17 ± 1.34	29.69 ± 4.77	28.33 ± 5.93	28.92 ± 4.20
Testis or ovarian							
Absolute weight	gram	3.20 ± 0.14	3.23 ± 0.22	3.28 ± 0.22	0.13 ± 0.0	0.12 ± 0.03	0.12 ± 0.02
Ratio per body weight	%	0.65 ± 0.05	0.66 ± 0.05	0.66 ± 0.04	47.40 ± 4.46	45.27 ± 11.34	44.04 ± 10.08
Epididymis or uterus							
Absolute weight	gram	1.30 ± 0.00	1.30 ± 0.18	1.43 ± 0.17	0.43 ± 0.10	0.55 ± 0.06	0.63 ± 0.26
Ratio per body weight	%	0.26 ± 0.01	0.27 ± 0.03	0.29 ± 0.03	0.17 ± 0.02	0.21 ± 0.02	0.23 ± 0.09
Postate							
Absolute weight	gram	1.30 ± 0.21	1.10 ± 0.22	1.15 ± 0.06			
Ratio per body weight	%	0.26 ± 0.04	0.23 ± 0.04	0.23 ± 0.01			
Seminal vesicle							
Absolute weight	gram	1.68 ± 0.25	1.38 ± 0.22	1.58 ± 0.21			
Ratio per body weight	%	1.30 ± 0.21	1.10 ± 0.22	1.15 ± 0.06			

Data were presented as Mean ± S.D. (n ≥ 4). *t*-test: compared with control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 12. Organ weight changes after 6-month CTE treatment in Beagle dogs.

		Time: 6 months			
		Male		Female	
Organ weight (gram)		0.75 g/kg	1.5 g/kg	0.75 g/kg	1.5 g/kg
Organ-to-body weight ratio (%)		(n = 2)	(n = 1)	(n = 2)	(n = 1)
Heart					
Absolute weight	gram	69.45 ± 3.89	72.59	62.03 ± 13.25	57.53
Ratio per body weight	%	0.73 ± 0.04	0.66	0.79 ± 0.07	0.66
Liver					
Absolute weight	gram	306.55 ± 19.87	431.60	222.00 ± 42.28	229.40
Ratio per body weight	%	3.23 ± 0.21	3.92	2.84 ± 0.18	2.64
Spleen					
Absolute weight	gram	18.71 ± 1.49	22.83	20.20 ± 2.17	20.26
Ratio per body weight	%	0.20 ± 0.01	0.21	0.26 ± 0.00	0.23
Lung					
Absolute weight	gram	77.01 ± 4.39	85.90	59.92 ± 16.24	65.80
Ratio per body weight	%	0.77 ± 0.02	0.78	0.76 ± 0.11	0.76
Kidney					
Absolute weight	gram	45.73 ± 7.60	44.79	35.72 ± 10.06	36.92
Ratio per body weight	%	0.44 ± 0.01	0.41	0.45 ± 0.07	0.42
Pancreas					
Absolute weight	gram	24.58 ± 12.34	33.24	19.30 ± 1.03	23.11
Ratio per body weight	%	0.19 ± 0.03	0.30	0.25 ± 0.01	0.27
Brain					
Absolute weight	gram	76.63 ± 5.76	71.45	67.74 ± 2.31	58.58
Ratio per body weight	%	0.78 ± 0.11	0.72	0.88 ± 0.14	0.67
Thymus					
Absolute weight	gram	11.39 ± 4.53	9.41	10.45 ± 0.46	10.46
Ratio per body weight	%	119.85 ± 47.71	85.55	134.63 ± 11.19	120.23
Adrenal					
Absolute weight	gram	0.95 ± 0.04	1.08	1.07 ± 0.13	1.02
Ratio per body weight	%	10.00 ± 0.45	9.82	13.73 ± 0.11	11.72

Continued

Thyroid					
Absolute weight	gram	0.65 ± 0.04	0.69	0.58 ± 0.03	0.64
Ratio per body weight	%	6.85 ± 0.45	6.27	7.48 ± 0.59	7.36
Pituitary					
Absolute weight	gram	0.06 ± 0.00	0.06	0.07 ± 0.01	0.06
Ratio per body weight	%	0.63 ± 0.00	0.55	0.85 ± 0.20	0.69
Testes or ovaries					
Absolute weight	gram	14.98 ± 1.34	11.85	0.91 ± 0.28	1.36
Ratio per body weight	%	160.00 ± 10.00	110.00	11.92 ± 5.05	15.63
Epididymis or uterus					
Absolute weight	gram	5.10 ± 1.70	3.52	3.25 ± 0.92	7.87
Ratio per body weight	%	38.32 ± 4.76	51.82	23.18 ± 10.48	90.46
Prostate					
Absolute weight	gram	3.64 ± 0.45	5.70		
Ratio per body weight	%	53.69 ± 17.87	32.00		
Organ	Unit	Time: After 2 months recovery; n = 2			
Heart					
Absolute weight	gram	75.55 ± 9.13	74.69 ± 5.96	57.60 ± 1.98	66.65 ± 6.58
Ratio per body weight	%	0.72 ± 0.01	0.69 ± 0.03	0.69 ± 0.01	0.70 ± 0.08
Liver					
Absolute weight	gram	284.15 ± 27.79	236.85 ± 3.04	215.45 ± 7.99	231.85 ± 82.22
Ratio per body weight	%	2.71 ± 0.09	2.20 ± 0.11	2.57 ± 0.04	2.35 ± 0.37
Spleen					
Absolute weight	gram	26.44 ± 4.82	24.62 ± 6.24	17.69 ± 2.45	17.85 ± 1.34
Ratio per body weight	%	0.25 ± 0.01	0.23 ± 0.07	0.21 ± 0.03	0.19 ± 0.03
Lung					
Absolute weight	gram	56.36 ± 6.29	63.90 ± 1.41	48.00 ± 11.02	69.78 ± 6.89
Ratio per body weight	%	0.54 ± 0.01	0.60 ± 0.01	0.57 ± 0.11	0.73 ± 0.08
Kidney					
Absolute weight	gram	44.73 ± 7.46	38.76 ± 4.59	33.17 ± 2.32	40.15 ± 11.24
Ratio per body weight	%	0.42 ± 0.01	0.36 ± 0.06	0.40 ± 0.02	0.41 ± 0.03

Continued

Pancreas					
Absolute weight	gram	26.66 ± 3.58	25.04 ± 3.49	22.08 ± 1.10	23.60 ± 5.09
Ratio per body weight	%	0.26 ± 0.01	0.24 ± 0.02	0.27 ± 0.02	0.24 ± 0.00
Brain					
Absolute weight	gram	72.44 ± 1.75	71.62 ± 0.35	65.96 ± 2.88	69.10 ± 13.44
Ratio per body weight	%	0.70 ± 0.08	0.67 ± 0.02	0.79 ± 0.05	0.72 ± 0.01
Thymus					
Absolute weight	gram	17.86 ± 4.95	8.36 ± 1.98	8.27 ± 3.49	11.54 ± 4.02
Ratio per body weight	%	174.27 ± 69.86	77.31 ± 15.58	97.96 ± 39.11	117.21 ± 16.63
Adrenal					
Absolute weight	gram	1.13 ± 0.24	1.17 ± 0.20	0.93 ± 0.04	0.90 ± 0.08
Ratio per body weight	%	10.68 ± 0.88	10.84 ± 1.45	11.07 ± 0.23	9.40 ± 1.11
Thyroid					
Absolute weight	gram	0.68 ± 0.25	0.72 ± 0.00	0.56 ± 0.06	0.73 ± 0.18
Ratio per body weight	%	6.36 ± 1.59	6.69 ± 0.24	6.62 ± 0.92	6.90 ± 0.53
Pituitary					
Absolute weight	gram	0.06 ± 0.00	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.00
Ratio per body weight	%	0.58 ± 0.08	0.42 ± 0.08	0.78 ± 0.06	0.53 ± 0.11
Testes or ovaries					
Absolute weight	gram	13.55 ± 5.17	11.73 ± 4.45	1.16 ± 0.14	1.05 ± 0.65
Ratio per body weight	%	104.88 ± 1.79	108.19 ± 37.44	13.80 ± 1.34	10.35 ± 4.52
Epididymis or uterus					
Absolute weight	gram	6.70 ± 1.58	7.09 ± 0.81	9.17 ± 6.13	11.57 ± 13.77
Ratio per body weight	%	65.15 ± 23.52	65.71 ± 5.11	108.72 ± 70.24	106.61 ± 119.39
Prostate					
Absolute weight	gram	3.40 ± 0.15	3.41 ± 0.04		
Ratio per body weight	%	32.45 ± 2.84	31.66 ± 0.75		

Data were presented as Mean ± S.D. ($n \geq 1$).

4. Discussion

Preventing Alzheimer's disease has become a major concern for scientists worldwide [9]. Over the past decade, CTE has been used as a prescription drug for dementia in China [4] [10], providing neuroprotection and enhancing learning and

memory. Despite its clinical use, comprehensive toxicity and long-term safety data for CTE remain insufficient [11] [12]. Our previous study [7] determined the NOAEL of CTE to be 7.8 g/kg bw (body weight) and 8.0 g/kg bw in a 90-day feeding study in rats. According to the Chinese Pharmacopeia, the safe dosage for officinal use of dried C. deserticola stem is 6 - 9 grams per day for humans [1]. In this fourteen-day oral acute toxicity study, mice were treated with doses of 2 g/kg bw and up to 8 g/kg bw (equivalent to 133 and 533 times the recommended adult daily intake of 0.015 g/kg bw, respectively) [13] [14]. In a six-month feeding test, Wistar rats were dosed at 1.65 g/kg and 0.8 g/kg bw (equivalent to 110 and 53.3 times the recommended adult daily intake, respectively). Beagle dogs were dosed at 1.50 g/kg and 0.75 g/kg bw (equivalent to 100 and 50 times the recommended adult daily intake, respectively). There were no significant differences in body weight, blood routine, blood biochemistry, organ ratios, and histopathological examination indicators between the test and control groups ($P > 0.05$).

Genotoxicity tests, including the Ames test and mammalian micronucleus test, are commonly used for safety evaluations [15]-[17]. Under Ames test conditions, with or without S9, treatment with CTE did not increase the number of revertants over spontaneous revertants in any of the five *S. typhimurium* test strains at all test doses. CTE was not mutagenic in the *S. typhimurium* reverse mutation analysis. Chromosomal aberrations induced by CTE were analyzed in three independent experiments, in which no aberration was found. In chronic oral toxicity studies in rats, alanine aminotransferase (ALT) levels were elevated in males and aspartate aminotransferase (AST) levels were elevated in females across both CTE dose groups relative to controls. These findings were not considered toxicologically significant, as they were not supported by adverse changes in clinical signs, integument, hematology, urinalysis, food consumption, coagulation parameters, organ weights, or histopathology. This study evaluated the CTE safety profile and found that acute and chronic toxicity studies indicated non-toxicity. Negative results were shown in both the Ames and micronucleus genetic toxicity tests.

5. Conclusion

The results of this study indicate that CTE is not genotoxic, as evidenced by the Ames test and mammalian micronucleus test, and that a NOAEL was established at 1.65 g/kg in rats and at 1.5 g/kg in dogs after six months of oral administration. No substantial histopathological lesions were observed in any organs. According to the ICH guideline M3 (R2) on non-clinical safety studies, the recommended clinical oral dose is approximately 1/50th of the NOAEL [18]. This study suggests that CTE, at the recommended adult clinical oral dose of 30 mg/kg, is relatively safe for long-term use.

Authors' Contributions

Conceptualization: M.-H.S.; methodology: H.-C.L.; formal analysis: C.-T.C.; investigation: L.L. and C.-J.W.; writing—original draft preparation: C.-T.C.; writ-

ing—review and editing: M.-H.S. All authors have read and agreed to the published version of the manuscript.

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Statement of Human

There is not relevant to human participation in this study.

Statement of Accordance with ARRIVE Guidelines

This study has been reported in accordance with the ARRIVE guidelines.

Ethical Approval

This study was approved by the Institutional Animal Care and Use Committee (IACUC), Animal Study Protocol Approval Number: 2008-TP-004.

Institutional Review Board Statement

All of the experimental procedures involving animals were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (AC00305) and approved by the Institutional Animal Care and Use Committee (IACUC) of Center of Toxicology and Preclinical Sciences Development Center for Biotechnology.

Data Availability

Data is provided within the manuscript or supplementary information files.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] Chinese Pharmacopoeia Commission (2010) Pharmacopoeia of the People's Republic of China: 2010. China Medical Science Press.

[2] Wang, T., Zhang, X. and Xie, W. (2012) Cistanche deserticola Y. C. Ma, "Desert Ginseng": A Review. *The American Journal of Chinese Medicine*, **40**, 1123-1141. https://doi.org/10.1142/s0192415x12500838

[3] Wu, C., Chien, M., Lin, N., Lin, Y., Chen, W., Chen, C., *et al.* (2019) Echinacoside Isolated from *Cistanche tubulosa* Putatively Stimulates Growth Hormone Secretion

via Activation of the Ghrelin Receptor. *Molecules*, **24**, Article 720. https://doi.org/10.3390/molecules24040720

[4] Chao, C., Huang, H., Huang, H., Chao, H., Yu, S., Su, M., *et al.* (2019) Inhibition of Amyloid Beta Aggregation and Deposition of *Cistanche tubulosa* Aqueous Extract. *Molecules*, **24**, Article 687. https://doi.org/10.3390/molecules24040687

[5] Wu, C.R., Lin, H.C. and Su, M.H. (2014) Reversal by Aqueous Extracts of *Cistanche tubulosa* from Behavioral Deficits in Alzheimer's Disease-Like Rat Model: Relevance for Amyloid Deposition and Central Neurotransmitter Function. *BMC Complementary and Alternative Medicine*, **14**, Article No. 202. https://doi.org/10.1186/1472-6882-14-202

[6] Liao, P.L., Li, C., Tse, L., Kang, J. and Cheng, Y. (2018) Safety Assessment of the *Cistanche tubulosa* Health Food Product Memoregain: Genotoxicity and 28-Day Repeated Dose Toxicity Test. *Food and Chemical Toxicology*, **118**, 581-588. https://doi.org/10.1016/j.fct.2018.06.012

[7] Gao, Y., Qin, G., Wen, P., Wang, Y., Fu, W., He, L., *et al.* (2017) Safety Assessment of Powdered *Cistanche deserticola* Y. C. Ma by a 90-Day Feeding Test in Sprague-Dawley Rats. *Drug and Chemical Toxicology*, **40**, 383-389. https://doi.org/10.1080/01480545.2016.1242013

[8] OECD (2020) Test No. 471: Bacterial Reverse Mutation Test. OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing.

[9] Abduljawad, A.A., Elawad, M.A., Elkhalifa, M.E.M., Ahmed, A., Hamdoon, A.A.E., Salim, L.H.M., *et al.* (2022) Alzheimer's Disease as a Major Public Health Concern: Role of Dietary Saponins in Mitigating Neurodegenerative Disorders and Their Underlying Mechanisms. *Molecules*, **27**, Article 6804. https://doi.org/10.3390/molecules27206804

[10] Li, Y., Peng, Y., Ma, P., Yang, H., Xiong, H., Wang, M., *et al.* (2018) Antidepressant-like Effects of *Cistanche tubulosa* Extract on Chronic Unpredictable Stress Rats through Restoration of Gut Microbiota Homeostasis. *Frontiers in Pharmacology*, **9**, Article 967. https://doi.org/10.3389/fphar.2018.00967

[11] Turck, D., Castenmiller, J., De Henauw, S., Hirsch-Ernst, K.I., Kearney, J., Maciuk, A., *et al.* (2021) Safety of Water Extract of *Cistanche tubulosa* Stems as a Novel Food Pursuant to Regulation (EU) 2015/2283. *EFSA Journal*, **19**, e06346. https://doi.org/10.2903/j.efsa.2021.6346

[12] Qin, G., Wang, Y., Wen, P., He, L., Fu, W. and Wang, F. (2015) Toxicological Security Evaluation of Herba *Cistanches granule*. *Chinese Journal of Health Laboratory Technology*, **16**, 2669-2671.

[13] Lu, L., Fan, Y., Yao, W., Xie, W., Guo, J., Yan, Y., *et al.* (2014) Safety Assessment of the Fermented Phylloporia Ribis (*Lonicera japonica* Thunb.) Mycelia by Oral Acute Toxicity Study in Mice and 90-Day Feeding Study in Rats. *Food and Chemical Toxicology*, **69**, 18-24. https://doi.org/10.1016/j.fct.2014.03.044

[14] Gu, C., Yang, X. and Huang, L. (2016) Cistanches Herba: A Neuropharmacology Review. *Frontiers in Pharmacology*, **7**, Article 289. https://doi.org/10.3389/fphar.2016.00289

[15] Hsu, L.C., Hsu, Y.W., Hong, C.C. and Pan, T.M. (2014) Safety and Mutagenicity Evaluation of Red Mold Dioscorea Fermented from *Monascus purpureus* NTU 568. *Food and Chemical Toxicology*, **67**, 161-168. https://doi.org/10.1016/j.fct.2014.02.033

[16] Lin, C.W., Chen, H., Yang, Y., Chen, Y., Hsu, Y. and Pan, T. (2022) Toxicological Evaluation of the Red Mold Rice Extract, ANKASCIN 568-R: 13-Week Chronic Toxicity, and Genotoxicity Studies. *Toxicology Reports*, **9**, 356-365.

https://doi.org/10.1016/j.toxrep.2022.02.008

[17] Peng, K.Z., Zhang, S.Y. and Zhou, H.L. (2016) Toxicological Evaluation of the Flavonoid-Rich Extract from Maydis Stigma: Subchronic Toxicity and Genotoxicity Studies in Mice. *Journal of Ethnopharmacology*, **192**, 161-169. https://doi.org/10.1016/j.jep.2016.07.012

[18] FDA Guidance for Industry (2010) M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals.