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Comprehensive Assessment of the Safety of *Eucommia ulmoides* Leaf Extract for Consumption as a Traditional Chinese Health Food

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ABSTRACT

To ensure the export quality of *Eucommia ulmoides* leaf extract (ELE) and facilitate *E. ulmoides* leaf inclusion in the directory of traditional Chinese health foods, an overall safety assessment of ELE was performed, including genotoxicity and long-term toxicity, according to the national food safety standards of China. No variations in the reverse mutation number of the nominal bacterial strains were observed under ELE treatment in comparison with the solvent control. Additionally, the micronucleus rates of *in vivo* mammalian erythrocytes and *in vitro* mammalian cells under ELE treatment were equivalent to or significantly lower than those of the solvent control. The fold change in the trifluorothymidine resistance mutation frequency of the thymidine kinase gene under ELE treatment was less than three times in comparison with the solvent control, suggesting that ELE did not cause genotoxicity. Moreover, animal experiments showed that the growth performance of rats under ELE treatment was enhanced because the body weights of rats increased. No oxidative injury or inflammatory responses were induced and no histopathological lesions of tissues were detected under ELE treatment. In addition, plasma triglycerides and low-density lipoprotein cholesterol levels significantly decreased, and plasma high-density lipoprotein cholesterol levels significantly increased with ELE treatment, suggesting that ELE was health-promoting. Furthermore, moderate to excellent antimicrobial activities, a favorable anticancer capacity, and superior antioxidant abilities of ELE were found, implying ELE possesses good bioactivities. Therefore, we affirmed ELE is safe to consume as a traditional Chinese health food.

KEYWORDS

Eucommia ulmoides leaf extract; genotoxicity; long-term toxicity; growth performance; bioactivity

1 Introduction

Eucommia ulmoides (*E. ulmoides*) Oliver, a traditional Chinese medicinal plant generally known as "duzhong" in China [1,2], is a monotypic deciduous and dioecious tree and is the only species of the genus *Eucommia* [3,4]. *E. ulmoides* originated in China during the glacial movement in the third century and is currently cultivated at an altitude range of 300–1300 m in China, such as in the Hunan, Henan,

and Yunnan provinces [5]. *E. ulmoides* has a long application history, particularly the raw bark. As recorded in the Chinese medical classics *Shen Nong Ben Cao Jing* and *Ben Cao Gang Mu*, the raw bark of *E. ulmoides* has been used as traditional Chinese medicine and has been included in the Chinese Pharmacopoeia [6]. Based on its pharmacological activities, *E. ulmoides* bark is commonly used as a tonic for organs (liver and kidney) and has been found to prevent miscarriage, regulate blood pressure, and act as an anti-aging agent [7–10]. Although *E. ulmoides* bark has shown multiple pharmacological effects, the bark is not conducive to large-scale development and utilization due to resource constraints. Therefore, a new renewable resource is urgently needed to compensate for the shortage of bark.

To find an alternative to the bark, an analysis of the chemical components of the bark was necessary. The chemical components of the bark were separated, purified, identified, and found to be lignans, iridoids, flavonoids, phenolics, steroids, terpenoids, and gutta-percha [5]. Of these, lignans and iridoids were identified as the two most important constituents, and are usually denoted as key chemotaxonomic markers. These two constituents were shown to exhibit excellent pharmacological activities, such as the lignans liriodendrin, (+)-pinoresinol di-O-β-D-glucopyranoside, and (+)-syringaresinol, which were found to have a satisfactory antihypertensive effect, and aucubin, genipin, and geniposidic acid of iridoids, which displayed remarkable antihypertension and anti-aging effects. Recently, evidence has shown that the chemical components of the bark are similar to those of the leaves, including lignans, iridoids, phenols, steroids, triterpenes, polysaccharides, and flavonoids [11,12], suggesting that the leaves of *E. ulmoides* can solve the dilemma of the bark shortage. In addition, numerous studies have reported that *E. ulmoides* leaves were found to increase bone strength, reduce fattiness, and increase energy metabolism, thus resulting in longevity and improved fertility in humans [13,14]. Moreover, flavonoids of *E. ulmoides* leaves (e.g., chlorogenic acid (CGA), rutin, ferulic acid, and caffeic acid) displayed good antioxidant activity [15]. Taken together, *E. ulmoides* leaves are a promising replacement for bark [16]. Furthermore, due to the process of peeling will cause trunk damage (even death) and the bark regeneration speed being slower than leaves, the *E. ulmoides* leaves may be more suitable for commercialization based on their advantages of picking easily, large amount, and growth every year. Therefore, *E. ulmoides* leaves are a better renewable material in comparison with the bark, which can meet the ever-increasing demand.

Because the utility and value of *E. ulmoides* have been widely recognized, it has attracted much attention from abroad and has been successfully traded with other countries, such as the United States, Russia, Britain, and France [17]. In 2019, we formulated the group standards for *E. ulmoides* leaf extract at the invitation of the China Chamber of Commerce for Import and Export of Pharmaceutical and Health Products, which ensured the export quality of *E. ulmoides* leaf extract. Furthermore, although the chemical components of the bark are similar to the leaves, some composition differences were determined between them, such as the characteristic component, CGA, which is highly enriched in leaves, whereas its concentration in the bark is below the detection limit. Simultaneously, divergent in other characteristic components composition is observed between leaves and barks, e.g., rutin is only determined in leaves, whereas geniposide and pinoresinol diglucoside are only detected in barks. In addition, apart from the composition difference, the concentrations of the same components (aucubin, geniposidic acid) in leaves also distinct from that of in barks, and their concentrations are significantly higher in barks [18]. Previous studies have demonstrated that CGA induces an antihypertensive effect [19] and significantly reduced the systolic and diastolic blood pressure of hypertensive patients and rats [20,21], and can potentially serve as an antihypertensive drug [22]. Meanwhile, geniposidic acid and aucubin show the abilities of free radical scavenging, anti-inflammatory, and anti-cancer [23]. Therefore, the composition and concentration difference of these characteristic components between the bark and leaves might lead to bioactivity variations, and an overall assessment of the safety of *E. ulmoides* leaf extract is urgently needed to ensure the continued trade of *E. ulmoides* leaf extract around the world.

In the present study, genotoxicity tests, cytotoxicity assay, and *in vivo* long-term animal toxicity studies of *E. ulmoides* leaf extracts were performed according to the national food safety standards of China. Furthermore, the bioactivity of *E. ulmoides* leaf extracts was also explored, including analysis of antimicrobial activity, anticancer capacity, and antioxidant abilities. We hypothesized that *E. ulmoides* leaf extract was safe for serving as a traditional Chinese health food based on its bioactivities. Our study will examine the safety of *E. ulmoides* extract, which contributes to the application of *E. ulmoides* leaf extracts.

2 Materials and Methods

All of the experiments regarding animals (rats) were approved by the Animal Welfare and Ethics Committee of the Institute of Analysis, Guangdong Academy of Sciences (China National Analytical Center, Guangzhou, China). The permission number is No. 2021002.

2.1 Standards and Reagents

The chemical standards (chlorogenic acid, geniposidic acid, aucubin, geniposide, pinoresinol diglucoside, and rutin) were provided by Yuanye Technology Co., Ltd. (Shanghai, China) with a purity ≥99%. Methanol and phosphoric acid were provided by Merck (Darmstadt, Germany), while hexane, petroleum ether, and chloroform were provided by Guangzhou Chemical Reagent Factory (Guangzhou, China). All of the chemical standards and solvents were commercially available and used without dilution unless otherwise specified. A Milli-Q filter system (Millipore, Bedford, MA, USA) was used to prepare ultrapure water. The cells and bacteria trains used in the present study were provided by iPhase (Beijing, China).

2.2 Preparation of E. ulmoides Leaf Extract (ELE)

The dry leaves of *E. ulmoides* were provided by Hengxin Co., Ltd. (Zhangjiajie, China). After extraction twice with ultrapure water (liquid:solid = 1:10) under the condition of 80°C for 1 h, the extracted liquid was concentrated to 20 Brix under vacuum rotary evaporation. Subsequently, the obtained concentrated liquid was dried by spray drying. During spray drying, the inlet air temperature was 150°C–170°C and, while the outlet air temperature was 90°C–95°C. The extraction yield of ELE is about 25%.

2.3 Composition Analysis of ELE

The chemical composition analysis of ELE was performed on an LC-20A HPLC system (Shimazu, Japan) equipped with a diode array detector. The standard curves of the six chemical standards were plotted first. A Thermo BDS HYPERSIL C-18 analytical column (250 mm× 4.6 mm, 5 μm) was used for target component separation. For separation, the mobile phase solutions consisting of solvent A (0.2% phosphoric acid in water) and solvent B (MeOH) were prepared. A linear gradient elution program was designed as follows: 95% A from 0–17 min; 95%–86% A from 17–45 min; 86%–65% A from 45–55 min; and 65%–55% A from 55–70 min. For analysis, a 10 μL sample was injected into the column, and then the column temperature was maintained at 30°C. The flow rate was set at 1 mL/min. Four characteristic components (geniposidic acid, chlorogenic acid, geniposide, and pinoresinol diglucoside) were detected at 238 nm, while the other two components (aucubin and rutin) were monitored at 208 and 254 nm, respectively. The analysis of physicochemical parameters in ELE (moisture and ash), pesticide residues (hexachlorocyclohexane (HCH), dichlorodiphenyl trichloroethane (DDT), quintozene (PCNB)), microbial content (aerobic bacterial count, coliforms, molds and yeasts, Salmonella, *Staphylococcus aureus*), and mineral elements were carried out following GB 5009.3-2016, GB 5009.4-2016, GB/T 5009.19-2003, GB/T 5009.136-2003, GB 4789.2-2016, GB 4789.3-2016, GB 4789.15-2016, GB 4789.4-2016, GB 4789.10-2016, and GB 5009.268-2016, respectively.

2.4 Genotoxicity Studies

The bacterial reverse mutation assay, mammalian erythrocyte micronucleus test, *in vitro* mammalian cell micronucleus test, and *in vitro* thymidine kinase (TK) gene mutation test on ELE were conducted following

GB 15193.4-2014, GB 15193.5-2014, GB 15193.28-2020, and GB 15193.20-2014, respectively. All of these standards were in line with the OECD guidelines with appropriate modification, including OECD 471 bacteria reverse mutation test, OECD 474 mammalian erythrocyte micronucleus test, OECD 487 *in vitro* micronucleus test, and OECD 490 *in vitro* mammalian cell gene mutation tests using the thymidine kinase gene.

For bacterial reverse mutation assay, the plate incorporation technique was used. Briefly, five nominal bacterial strains were adopted, including four *Salmonella typhimurium* strains (TA1535, TA100, TA98, TA97a) and one *Escherichia coli* strain (WP2uvrA). Four dose solutions of ELE (5, 2.5, 1.25, 0.625 mg/dish) were set with fold changes of two. Pure water was selected as solvent control. 2-aminoanthracene (2-AA), sodium azide (NaN_3), 2-aminofluerene (2-AF), methyl methanesulfonate (MMS), and sodium p(dimethylamino) benzenediazo sulfonate (Dexon) were used as the positive control. The culture medium (25 mL/dish) was prepared and stored at 37°C in dark for 24 h. Then, a mixture consisting of agar medium (2 mL), bacteria solution (0.1 mL), ELE solution (0.1 mL), and phosphate buffer (0.5 mL) was added. If the experiment was conducted under metabolic activation conditions, S9 solution (0.5 mL) was added too and denoted as $+S_9$; otherwise, it was defined as $-S_9$. After solidification, the plates were turned upside down and cultivated at 37°C for another 72 h. The reverse mutation number of bacterial strains was recorded.

For the mammalian erythrocyte micronucleus test, three dose solutions of ELE (10000, 5000, 2500 mg/kg) were prepared with a fold changes of two. The 10000 mg/kg was selected as the highest dose based on the rule of 50% lethal dose higher (LD50) than 10 g/kg. Pure water was selected as solvent control. Cyclophosphamide (CTX, 40 mg/kg) was used as the positive control. Ten rats (half male and half female) were used for each dose and ELE solution was given twice in 30 h by the method of gavage. Then, marrow fluids were harvested at 36 h and mixed with fatal bovine serum (FBS) on a slide. After smearing and drying, the slide was fixed with methanol for 5 min and stained with Giemsa for 15 min. Consequently, the slide was washed with phosphate buffer saline (PBS, 1/15 mol/L) and sealed. Two thousand cells were counted and the micronucleus rate (‰) was therefore calculated.

For *in vitro* mammalian cell micronucleus test, three dose solutions of ELE (5.0, 2.5, 1.25 mg/mL) were prepared with a fold changes of two. The highest dose was set based on the maximum concentration (5.0 mg/mL) without cytotoxicity. Pure water was selected as solvent control. CTX (20 ug/mL) and mytomycin-C (MMC, 0.25 ug/mL) were used as the positive control. If the experiment was conducted under metabolic activation conditions, S9 and CTX solutions were added and denoted as $+S_9$; otherwise, MMC was added and denoted as $-S_9$. Cells with a density of 1×10^5 cells/mL were cultured in a tissue culture flask under the condition of 5% CO_2 and 37°C. After removing the medium, ELE (1%) and S9 (10%) solutions were added, together with the complete medium (exclusion FBS). These cells were cultured for 4 h at the same condition and were washed with PBS three times. Consequently, a complete medium was added and cultured for another 24 h. After washing with PBS twice and digestion with 0.25% trypsin solution for 5 min, the cells were centrifuged under 1000 r/min for 5 min. The cells were harvested by discarding supernate and then fixed with stationary liquid (2 mL) three times. After natural drying, the cells were stained with Giemsa for 15 min. Two thousand cells were counted and the micronucleus rate (‰) was therefore calculated.

For *in vitro* TK gene mutation test, three dose solutions of ELE (5.0, 2.5, 1.25 mg/mL) were prepared with a fold changes of two. The highest dose was set based on the maximum concentration (5.0 mg/mL) without cytotoxicity. Pure water was selected as solvent control. CTX (50 ug/mL) and methyl methanesulfonate (MMS, 5.0 ug/mL) were used as the positive control. If the experiment was conducted under metabolic activation conditions, S9 and CTX solutions were added and denoted as $+S_9$; otherwise, MMs were added and denoted as $-S_9$. Cells with a density of 5×10^5 cells/mL were used and ELE solution (1%) was added. These cells were shaken under 37°C for 3 h and centrifuged to remove supernate. The obtained cells were

washed with PBS twice and resuspended by 10% RPMI 1640 medium. The cell density was adjusted to 2×10^5 cells/mL. The plating efficiency at 0 day (PE0) was measured. A cell density of 8 cells/mL (0.2 mL) was inoculated into a 96-well plate and cultured under the condition of 37°C and 5% CO_2 for 12 d. For expression, the cell suspension was cultured for 2 d and the cell density was kept below 10^6 cells/ml. The plating efficiency at the second day (PE2) was determined as the same procedure as PE0. Meanwhile, the trifluorothymidine (TFT) resistance mutation frequency (MF) was detected. The expression cells of the second day were adopted and the cell density was adjusted to 1×10^4 cells/mL. TFT (3 μg/mL) was added and inoculated into a 96-well plate (0.2 mL). The prepared plate was cultured under the condition of 37°C and 5% CO_2 for 12 d. The number of wells with mutant colony growth was counted.

2.5 Cytotoxicity Assay

The cell viability (%) of CHO, CHL, V79, and human hepatocellular liver carcinoma (HepG2) cells under ELE treatment was determined based on a methyl thiazolyl tetrazolium (MTT) cell proliferation assay. A cell density of 1×10^5/mL was prepared and then 100 μL of the cell suspension was added to the wells of 96-well plates. Medium alone (100 μL) was prepared as a negative control. The prepared plates were incubated at 37°C and 5% CO_2 for 12 h. Subsequently, MTT solution (5 mg/mL, 10 μL) was added to each well and incubated for another 6 h. After incubation, the medium of each well was removed and 100 μL of dimethyl sulfoxide (DMSO) was added. The obtained reaction system was mixed thoroughly and the OD value of each well was measured at 490 nm. Three concentrations of ELE (25, 12.5, and 6.25 mg/mL) were used for analyzing the cell viability of CHO, CHL, and V79 cells, while six concentrations of ELE (40.0, 20.0, 10.0, 5.0, 1.0, 0.1 μg/mL) were used for analyzing the cell viability of HepG2 cells. Cisplatin was chosen as the positive drug for evaluating an anticancer effect against HepG2 cells.

2.6 Long-Term Toxicity Testing

2.6.1 90-Day Repeated Dose Toxicity Study with the Following Subsections

Long-term toxicity testing of ELE was conducted following GB15193.13-2015. Sprague-Dawley rats, about 100 g each (n = 80, half male and half female), were randomly assigned to four groups: A group was the basal diet; B, C, and D groups were the basal diet supplemented with 1000, 500, or 200 mg/kg of ELE, respectively. The basal diet was provided by Jiangsu Xietong Shengwu Co., Ltd. (Nanjing, China). All of the rats were allowed free access to water and were fed under constant conditions of a 12 h light and 12 h dark cycle in the China National Analytical Center, Guangzhou (Zhongshan, China). The room temperature (23 ± 1°C) and relative humidity (50% ± 5%) were constant during the entire experimental period of 90 days.

2.6.2 Sample Collection

Blood samples of each group were obtained aseptically from the abdominal aortic vein of rats after 90 days of feeding. Then, the blood samples were immediately centrifuged at 3000 r/min for 15 min. All of the blood samples were stored at −80°C before use. Additionally, seven organs (brain, liver, spleen, heart, kidney, adrenal glands, and testis) were collected and weighed individually. Two intestinal tissues (duodenum and ileum) were also collected and stored in 4% paraformaldehyde until further use.

2.6.3 Biochemical and Hematological Analysis

Blood routine indexes (white blood cell (WBC), neutrophil (Neu), lymphocyte (Lym), monocyte (Mon), eosinophil (Eos), basophil (Bas), red blood cell (RBC), hemoglobin (HGB), hematocrit value (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpusular hemoglobin concerntration (MCHC), red blood cell distribution width-coefficient of variation (RDW-CV), red blood cell distribution width-standard deviation (RDW-SD), platelet (PLT), mean platelet volume (MPV), platelet distribution width (PDW), thrombocytocrit (PCT)) and blood biochemical indexes (albumin (ALB), alkaliphosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST),

creatinine (CREA-S), glucose (Glu-G), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total phosphorus (TP), UREA, γ-glutamyl transpeptidase (γ-GT)) were determined by using relevant detection kits (Mindray, Shenzhen, China). The blood antioxidant indexes (superoxide dismutase (SOD), glutathione (GSH), molondialdehyde (MDA), catalase (CAT)) and inflammatory factors (tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), Interleukin-6 (IL-6)) were also determined by using relevant detection kits from GuangZhou KYDbio Technology Co., Ltd. (Guangzhou, China). All of the analytical procedures were strictly in accordance with the instruction manual of each kit.

2.6.4 Histopathological Examination

Five organs (liver, spleen, kidney, stomach, and pancreas) and two intestinal tissues (duodenum and ileum) were dehydrated with graded alcohol and xylene, and the tissues were embedded in paraffin. After solidification, the obtained paraffin block was cut into 4-µm sections and stained with hematoxylin and eosin. An inverted microscope (ECHO, Chicago, Illinois, USA) was used to observe the tissue morphology. The villus height and the crypt depth of the duodenum and ileum were measured by using an automatic image analyzer (Olympus DP73 camera, Japan). A minimum of six villi and 10 crypts that were well-oriented were obtained from different parts of each intestinal tissue sample.

2.7 Antimicrobial Activity

The antimicrobial activities of ELE against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Aspergillus niger* were determined following GB 15979-2002 with minor modifications. Two concentrations of each microbe (1×10^3 and 1×10^4 CFU/mL) and two concentrations of ELE (25.0 and 5.0 mg/L) were prepared. The prepared microbial suspension (100 µL) and ELE solution (0.5 mL) were added to a petri dish with 15 mL Mueller Hinton/potato dextrose agar medium. After solidification, the petri dish was turned over and incubated for 36 h. The incubation temperature of *E. coli*, *S. aureus*, and *P. aeruginosa* was 36°C, while the incubation temperature of *A. niger* was 15°C. The experiment was repeated three times. Pure DMSO was used as a negative control. The bacterial suspension was used as a blank control. The antimicrobial activity was then calculated.

2.8 Free Radical Scavenging Capacity

Four typical free radicals (hydroxyl radical (OH), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide radical (PTIO·), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid radical (ABTS)) were selected to assess the *in vitro* free radical scavenging rate of ELE. The detection process of the four free radicals was accorded to the method reported by Li et al. [24].

2.9 Statistical Analyses

All data were analyzed by using GraphPad 8.0 (Diego, California, USA) and SPSS 19.0. One-way analysis of variance (ANOVA) and the Tukey test were performed to conduct multiple comparisons. All data were expressed as mean ± standard deviation (SD), with $P < 0.05$ considered statistically significant.

3 Results and Discussions

3.1 Composition of ELE

The composition of ELE is listed in Table 1. The concentrations of crude polysaccharide, total polyphenol, total flavonoid, and protein in ELE were 2.06%, 17.35 g/100 g, 5.27 g/100 g, and 1.62 g/100 g, respectively. Four major characteristic components of ELE (chlorogenic acid, aucubin, geniposidic acid, and rutin) were determined and their concentrations were 81.73, 55.51, 86.55, and 7.55 mg/g, respectively. Based on these active ingredients, ELE likely confers multiple pharmacological activities, such as antiviral, anti-inflammatory, and antioxidant [25–28]. Physiochemical properties, pesticide residues, microbiological

components, and mineral element levels in ELE were also measured and all met the group standards for *E. ulmoides* leaf extract. ELE underwent a safety assessment and bioactivity analysis without findings unless otherwise specified. The chromatographic peaks of standards and ELE are shown in Fig. S1.

Table 1: The composition of *E. ulmoides* leaf extract (ELE)

Category	Index	Content	Category	Index	Content
Functional composition	Crude polysaccharide (%)	2.06 ± 0.18		K (mg/kg)	28994.45 ± 234.67
	Total polyphenol (g/100 g)	17.35 ± 0.61		Ca (mg/kg)	13187.64 ± 121.87
	Total flavonoid (g/100 g)	5.27 ± 0.06		Na (mg/kg)	2550.56 ± 25.89
	Protein (g/100 g)	1.62 ± 0.01		Mg (mg/kg)	4008.76 ± 33.48
	Chlorogenic acid (mg/g)	81.73 ± 3.73		P (mg/kg)	3169.25 ± 20.96
	Aucubin (mg/g)	55.51 ± 0.43		Fe (mg/kg)	39.74 ± 2.67
	Geniposidic acid (mg/g)	86.55 ± 0.56		Zn (mg/kg)	20.23 ± 1.81
	Rutin (mg/g)	7.55 ± 0.08		Cu (mg/kg)	0.85 ± 0.23
Physico-chemical tests	Moisture (%)	3.39 ± 0.05		Mn (mg/kg)	191.88 ± 4.43
	Ash (%)	11.6 ± 0.50		B (mg/kg)	25.16 ± 1.83
Pesticide residue	Hexachlorocyclohexane (mg/kg)	ND	Mineral elements	Sr (mg/kg)	30.45 ± 2.10
	Dichlorodiphenyl Trichloroethane (mg/kg)	ND		Al (mg/kg)	9.88 ± 0.67
	Quintozene (mg/kg)	ND		Cr (mg/kg)	2.37 ± 0.13
Microbiological Tests	Aerobic bacterial count (CFU/g)	<10		Ni (mg/kg)	1.70 ± 0.18
	Coliforms (MPN/g)	<0.3		Se (mg/kg)	0.33 ± 0.02
	Moulds and Yeasts (CFU/g)	<10		As (mg/kg)	0.22 ± 0.02
	Salmonella (25 g)	ND		Pb (mg/kg)	0.38 ± 0.03
	Staphylococcus aureus (25 g)	ND		Cd (mg/kg)	0.08 ± 0.01

3.2 Bacterial Reverse Mutation Assay of ELE

The bacterial reverse mutation assay is a fundamental genetic toxicology test, which is essential in assessing the genotoxic capabilities of food and drugs [29]. In this study, the reverse mutation number of the five nominal bacterial strains (TA1535, WP2uvrA, TA100, TA98, TA97a) under positive control $(+/-S_9)$ were either three or two times higher than that under solvent control (Table 2), suggesting that mutagenesis was triggered under positive control. Interestingly, the reverse mutation numbers of the five nominal bacterial strains under ELE treatment were equivalent to that under solvent control, and the fold changes of the reverse mutation numbers between ELE treatment and solvent control were less than two, no matter if the concentration of ELE was low or high, indicating that ELE did not cause mutagenicity.

Table 2: The bacterial reverse mutation assay of *E. ulmoides* leaf extract (ELE). 2-AA represents 2-aminoanthracene; NaN$_3$ represents sodium azide; 2-AF represents 2-aminofluerene; MMS represents methyl methanesulfonate; Dexon represents sodium p(dimethylamino) benzenediazo sulfonate. Different small letters indicate significant differences at $P < 0.05$ level of tukey test under different treatments

Strain	Positive control		Solvent control (Pure water)	Concentration of ELE			
				5000 µg/dish	2500 µg/dish	1250 µg/dish	625 µg/dish
TA1535	+S$_9$ (2-AA, 20 ug/mL)	176.45 ± 5.67 a	38.23 ± 4.03 b	36.43 ± 3.91 b	36.25 ± 4.12 b	39.23 ± 3.17 b	38.28 ± 1.72 b
	−S$_9$ (NaN$_3$, 15 ug/mL)	152.67 ± 4.32 a	29.14 ± 2.22 b	28.29 ± 2.92 b	31.45 ± 5.43 b	29.46 ± 2.59 b	26.45 ± 2.95 b
TA100	+S$_9$ (2-AF, 200 µg/mL)	429.34 ± 8.51 a	78.45 ± 3.74 b	76.44 ± 3.75 b	73.64 ± 4.57 b	78.36 ± 5.72 b	78.43 ± 8.67 b
	−S$_9$ (MMS, 10 uL/mL)	293.65 ± 6.62 a	97.98 ± 3.48 c	117.78 ± 6.72 b	81.28 ± 5.86 d	83.48 ± 4.23 d	86.29 ± 3.88 d
TA98	+S$_9$ (2-AF, 200 µg/mL)	198.85 ± 4.44 a	33.67 ± 3.36 b	37.20 ± 1.96 b	34.16 ± 2.22 b	37.25 ± 1.92 b	37.66 ± 2.15 b
	−S$_9$ (Dexon, 6 µg/dish)	121.36 ± 7.06 a	47.84 ± 3.19 b	46.35 ± 2.80 b	45.87 ± 2.85 b	49.65 ± 2.24 b	38.29 ± 2.63 c
TA97a	+S$_9$ (2-AF, 200 µg/mL)	369.78 ± 6.72 a	151.23 ± 4.23 c	144.66 ± 4.90 c	150.49 ± 3.86 c	165.78 ± 3.36 b	146.33 ± 5.52 c
	−S$_9$ (Dexon, 500 ug/mL)	352.24 ± 8.43 a	127.64 ± 7.04 c	160.79 ± 9.02 b	129.77 ± 7.37 c	162.66 ± 4.55 b	134.82 ± 5.97 c
WP2uvrA	+S$_9$ (2-AF, 200 µg/mL)	401.33 ± 9.72 a	148.66 ± 6.32 b	145.64 ± 8.64 b	146.77 ± 5.94 b	150.45 ± 6.38 b	143.44 ± 5.04 b
	−S$_9$ (MMS, 10 uL/mL)	361.23 ± 6.08 a	158.33 ± 5.31 b	151.58 ± 9.53 b	138.28 ± 3.95 c	154.78 ± 3.8 b	136.67 ± 1.25 c

3.3 Mammalian Erythrocyte Micronucleus Test of ELE

Currently, the *in vivo* mammalian erythrocyte micronucleus test is the most commonly performed regulatory test in the genotoxicity study [30]. Therefore, the mammalian erythrocyte micronucleus test (Table 3) was carried out on ELE. The micronucleus rates of mammalian erythrocyte under positive control was significantly higher than that under solvent control, indicating that elevated mammalian erythrocyte micronucleus rate was observed under positive control. In contrast to the positive control, the mammalian erythrocyte micronucleus rate under ELE treatment was close to the rate under solvent control, no matter if the concentration of ELE was low or high, implying that ELE did not cause an elevated mammalian erythrocyte micronucleus rate.

3.4 In Vitro Mammalian Cell Micronucleus Test of ELE

Similar to mammalian erythrocyte micronucleus test, the *in vitro* mammalian cell micronucleus test is one of the tests most often used and required by regulations targeting food safety [31] and the results of *in vitro* mammalian cells micronucleus test on ELE is listed in Table 4. The micronucleus rates of mammalian cells under positive control (+/−S$_9$) were all significantly higher than those under solvent control, indicating that elevated mammalian cells' micronucleus rates were determined under positive

control. In contrast to the positive control, the micronucleus rates of mammalian cells under ELE treatment were close to or significantly lower than the rates under solvent control, no matter if the concentration of ELE was low or high, suggesting that ELE did not cause an elevated mammalian cells micronucleus rate.

Table 3: The mammalian erythrocyte micronucleus test of *E. ulmoides* leaf extract (ELE). CTX represents cyclophosphamide. Different small letters indicate significant differences at $P < 0.05$ level of tukey test under different treatments

Item	Positive control (CTX, 40 mg/kg)	Solvent control (Pure water)	Concentration of ELE		
			10000 mg/ kg	5000 mg/ kg	2500 mg/ kg
Cell number	2000	2000	2000	2000	2000
Micronucleus number	479.88 ± 23.24 a	17.66 ± 3.45 b	18.78 ± 2.61 b	17.28 ± 2.83 b	19.29 ± 1.69 b
Micronucleus rate (‰)	239.94	8.83	9.39	8.64	9.65

Table 4: The *in vitro* mammalian cells micronucleus test of *E. ulmoides* leaf extract (ELE). CTX represents cyclophosphamide; MMC represents mytomycin-C. Different small letters indicate significant differences at $P < 0.05$ level of tukey test under different treatments

Item	Positive control		Solvent control (Pure water)	Concentration of ELE		
				5.0 mg/mL	2.5 mg/mL	1.25 mg/mL
Cell number	2000		2000	2000	2000	2000
Micronucleus number	+S$_9$ (CTX, 20 ug/mL)	56.48 ± 6.62 a	8.33 ± 2.34 b	8.82 ± 1.16 b	4.66 ± 2.17 c	8.58 ± 2.24 b
Micronucleus rate (‰)		28.24	4.17	4.41	2.33	4.29
Micronucleus number	−S$_9$ (MMC, 0.25 ug/mL)	56.89 ± 5.84 a	6.67 ± 1.85 b	2.34 ± 1.26 c	3.66 ± 1.43 c	6.29 ± 1.62 b
Micronucleus rate (‰)		28.45	3.34	1.17	1.83	3.15

3.5 In Vitro TK Gene Mutation Testing of ELE

To better understand the mechanistic implications regarding *in vitro* genotoxicity test, the TK gene mutation assay was developed to provide higher specificity toward DNA-damaging reagents [32]. The TFT resistance mutation frequency (T-MF) of the TK gene under positive control (+/−S$_9$) was three times higher than under solvent control (Table 5), indicating that the TK gene mutagenicity was confirmed for the positive control. Although the T-MF of the TK gene under ELE treatment was higher than under solvent control, the fold changes of the T-MF under ELE treatment were less than three times the fold changes of the solvent control, no matter if the concentration of ELE was low or high, indicating that ELE did not cause TK gene mutations.

Table 5: The *in vitro* TK gene mutation test of *E. ulmoides* leaf extract (ELE). CTX represents cyclophosphamide; MMS represents methyl methanesulfonate; PE0 represents plating efficiency at 0 day; PE2 represents plating efficiency on the second day; RS represents relative survival; DSG represents relative suspension growth; RTG represents relative total growth; T-MF represents TFT resistance mutation frequency. Different small letters indicate significant differences at $P < 0.05$ level of the tukey test under different treatments

	Group		PE0 (%)	PE2 (%)	RS (%)	RSG (%)	RTG (%)	T-MF ($\times 10^{-5}$)
+S$_9$	Positive control (CTX, 50 ug/mL)		47.02 ± 1.21 d	96.28 ± 2.54 a	59.12 ± 1.23 d	79.70 ± 2.34 c	47.10 ± 1.69 e	150.45 ± 6.41 a
	Solvent control (Pure water)		79.54 ± 1.81 a	91.81 ± 2.16 b	100.00 ± 0 a	100.00 ± 0 a	100.00 ± 0 a	5.48 ± 1.23 d
	Concentration of ELE	5.0 mg/mL	47.08 ± 2.32 d	55.73 ± 2.15 d	59.15 ± 2.44 d	97.32 ± 2.37 b	57.00 ± 1.62 d	11.08 ± 1.62 b
		2.5 mg/mL	58.80 ± 0.94 b	68.62 ± 2.44 c	73.94 ± 2.33 b	97.44 ± 2.18 b	71.39 ± 1.95 b	9.54 ± 1.23 c
		1.25 mg/mL	52.76 ± 1.66 c	58.86 ± 1.83 d	66.33 ± 1.85 c	98.04 ± 1.87 b	65.15 ± 2.26 c	8.42 ± 1.54 c
−S$_9$	Positive control (MMS, 5 ug/mL)		31.60 ± 2.12 d	100.00 ± 0 a	37.86 ± 1.62 d	70.09 ± 1.57 d	26.50 ± 2.31 d	100.88 ± 4.54 a
	Solvent control (Pure water)		83.50 ± 2.43 a	96.22 ± 2.37 b	100.00 ± 0 a	100.00 ± 0 a	100.00 ± 0 a	4.23 ± 1.09 c
	Concentration of ELE	5.0 mg/mL	72.15 ± 2.23 c	79.57 ± 1.64 d	86.44 ± 1.64 c	95.36 ± 2.36 b	82.10 ± 2.33 b	6.25 ± 1.22 bc
		2.5 mg/mL	72.13 ± 2.74 c	87.54 ± 1.95 c	86.45 ± 1.45 c	90.37 ± 2.74 c	77.78 ± 2.82 c	7.36 ± 1.33 b
		1.25 mg/mL	79.54 ± 2.32 b	96.21 ± 2.06 b	95.34 ± 2.26 b	88.45 ± 2.33 c	84.18 ± 1.84 b	5.19 ± 1.67 bc
Blank			58.81 ± 2.25	91.82 ± 1.53	70.42 ± 1.81	96.70 ± 2.16	68.10 ± 1.47	5.65 ± 1.12

Evaluation of genotoxicity is a fundamental part of food safety assessment due to the potential health problems caused by genotoxicity. According to the national standards of food safety (GB 15193.4-2014, GB 15193.5-2014, GB 15193.28-2020, and GB 15193.20-2014), four genetic toxicity tests (including the bacterial recovery mutation test, mammalian erythrocyte micronucleus test, *in vitro* mammalian cells micronucleus test, and *in vitro* TK gene mutation test) were conducted in the present study. Interestingly, the bacterial reverse mutation assay results showed that the reverse mutation numbers of the tested bacterial strains under ELE treatment were equivalent to that under solvent control, suggesting that ELE does not cause genetic toxicity. Accordingly, the micronucleus rates of mammalian erythrocytes and *in vitro* mammalian cells under ELE treatment were close to or significantly lower than those of the solvent control, showing ELE did not cause chromosomal aberrations. In addition, the fold changes of the T-MF mutation frequency of the TK gene under ELE treatment were no greater than three times in comparison to solvent control, indicating that ELE did not cause TK gene mutation. Therefore, we concluded that ELE does not cause genotoxicity. Since these genotoxicity studies were in keeping with the OECD

guidelines, the results of no genotoxicity could not only meet the demand of China, but also be admitted by other counties, which benefited the normal trade of ELE.

3.6 Cytotoxicity Testing of ELE

To license a functional food, new material must undergo a cytotoxicity test. In this study, three typical cells (CHL, CHO, V79) were used to evaluate cytotoxicity [33]. The cell viabilities of CHL, CHO, and V79 cells under ELE treatment are listed in Table 6. The cell viability of V79 cells under ELE treatment was in a range of 65.22%–81.32%, indicating that ELE slightly induced V79 cell death. Although slight growth inhibition of ELE-treated V79 cells was determined, the inhibitory effect of ELE on CHL and CHO cells was acceptable as the cell viability of these ELE-treated cells surpassed 85%, no matter if the concentration of ELE was low or high, suggesting that ELE did not induce significant cytotoxicity. Since checking the cytotoxicity is a prerequisite to deciding the concentrations to be tested in a genotoxicity assay and also aids in the interpretation of positive results, our results can certify the concentrations used in genotoxicity tests are acceptable based on the standards and can support the obtained negative results from each test to some extent, which co-verify that ELE has no genotoxicity.

Table 6: The cytotoxicity test of *E. ulmoides* leaf extract (ELE). Different small letters indicate significant differences at $P < 0.05$ level of the tukey test under different treatments

Concentration of ELE	Cell viability (%)		
	CHL	CHO	V79
25 mg/mL	85.03 ± 2.6 c	85.31 ± 2.3 c	65.22 ± 5.2 c
12.5 mg/mL	94.46 ± 1.4 b	96.46 ± 1.2 b	74.20 ± 4.4 b
6.25 mg/mL	99.77 ± 0.2 a	99.87 ± 0.6 a	81.32 ± 3.8 a

3.7 Growth Performance of Rats under Long-Term ELE Treatment

Long-term toxicity testing is essential to evaluate the *in vivo* safety of plant extracts for serving as functional foods [34]. It can determine whether long-term administration induces tissue and organ damage, and also can determine the safe dose of administration. In the present study, no mortality in rats was observed during the entire long-term toxicity experiment. The results of ELE on the growth of rats are listed in Table 7. Rat body weight increases under ELE treatment were observed in comparison with the control, and the body weights of male rats significantly increased ($P < 0.05$), indicating that ELE was growth-promoting. Additionally, the heart and kidney weights of both male and female rats increased under ELE treatment, especially under high-level treatment (B group). Surprisingly, the brain weights of male rats significantly increased under ELE treatment when compared to control rats ($P < 0.05$), whereas this phenomenon was not observed in female rats. No significant variations were detected in the weights of other tissues (liver, spleen, adrenal gland, and testis) in both male and female rats among the four tested groups.

Many studies have reported that dietary medical plant extracts promoted animal growth [35,36], such as *Piper sarmentosum* extract for weaned piglets [37], *Moringa oleifera* extract for goats [38], *Crataegus pinnatifida* extract for juvenile golden pompano [39], and *Cassia abbreviata* extract for chickens [40]. Our previous work also showed that dietary supplementation with *E. ulmoides* leaf powder enhanced the growth performance of weanling piglets [41]. Similar to weanling piglets, the body weights of rats (both male and female) under ELE treatment increased in comparison to the control group, indicating that the growth performance of rats was strongly associated with ELE. The effects of ELE on elevated animal growth performance may be ascribed to numerous active ingredients of ELE belonging to phytoestrogens,

such as aucubin and wogonin [42], which can serve as estrogen receptor modulators in animals and strengthen the growth and development of animals, such as diet supplementation with ELE at a relatively high level shown to heighten the laying performance of hens [43].

Table 7: Effects of dietary supplementation *E. ulmoides* leaf extract (ELE) on the growth of rats. Different small letters indicate significant differences at $P < 0.05$ level of Tukey test under different treatments

		A	B	C	D
Male	Body weight (g)	334.20 ± 14.94 c	359.80 ± 13.07 b	375.80 ± 20.87 ab	390.60 ± 19.97 a
	Brain (g)	1.49 ± 0.02 b	1.83 ± 0.23 a	1.78 ± 0.20 a	1.95 ± 0.14 a
	Liver (g)	7.91 ± 0.52 b	7.90 ± 0.47 b	7.96 ± 0.48 b	8.83 ± 0.60 a
	Spleen (g)	0.54 ± 0.07 a	0.61 ± 0.07 a	0.59 ± 0.06 a	0.66 ± 0.14 a
	Heart (g)	0.89 ± 0.04 b	1.03 ± 0.05 a	0.97 ± 0.03 a	0.99 ± 0.04 a
	Kidney (g)	1.87 ± 0.13 b	2.11 ± 0.17 a	2.23 ± 0.22 a	2.31 ± 0.20 a
	Adrenal gland (g)	0.05 ± 0.01 a	0.05 ± 0.01 a	0.06 ± 0.01 a	0.06 ± 0.01 a
	Testis (g)	3.52 ± 0.04 a	3.59 ± 0.17 a	3.60 ± 0.10 a	3.74 ± 0.16 a
Female	Body weight (g)	243.80 ± 16.10 a	258.40 ± 18.97 a	260.80 ± 10.71 a	255.60 ± 12.10 a
	Brain (g)	1.77 ± 0.11 a	1.73 ± 0.01 a	1.76 ± 0.36 a	1.71 ± 0.16 a
	Liver (g)	6.59 ± 0.47 a	7.58 ± 0.92 a	6.93 ± 0.41 a	6.93 ± 0.36 a
	Spleen (g)	0.50 ± 0.05 a	0.57 ± 0.09 a	0.59 ± 0.11a	0.53 ± 0.05 a
	Heart (g)	0.78 ± 0.02 b	0.87 ± 0.04 a	0.87 ± 0.04 a	0.80 ± 0.02 b
	Kidney (g)	1.52 ± 0.10 b	1.71 ± 0.08 a	1.63 ± 0.09 ab	1.64 ± 0.04 ab
	Adrenal gland (g)	0.06 ± 0.01 a	0.05 ± 0.01 a	0.05 ± 0.01 a	0.05 ± 0.01 a

3.8 Blood Index Analysis of Rats under ELE Treatment

No significant alterations in the routine blood indexes of rats (both male and female) were observed under ELE treatment when compared to the control (Table S1). An evaluation of the blood biochemistry indexes (Table 8) showed that the AST concentration in rats significantly increased under dietary supplementation with ELE when compared to the control ($P < 0.05$), no matter if the rats were male or female, implying that ELE influenced liver function. The blood TG and LDL-C concentrations significantly decreased and HDL-C concentrations significantly increased under high-level ELE treatment (B group), indicating that dietary addition of ELE at a relatively high level contributed to lowering the blood lipids of rats. Interestingly, the blood TC concentrations in male rats significantly decreased under high-level ELE treatment in comparison with the control, whereas the TC concentrations in female rats significantly increased, indicating a divergent mechanism of rats to cope with ELE. Additionally, four typical antioxidant indexes (SOD, GSH, MDA, CAT) and three typical inflammatory factors (TNF-α, IL-1β, IL-6) were analyzed in both male and female rats. No significant difference in antioxidant indexes or inflammatory factors was determined among the four tested groups (Table S2), suggesting that ELE did not induce oxidative injury or an inflammatory response in rats.

Research on hyperlipidemia amelioration generally focuses on plant extracts or products that positively participate in lipid metabolism, such as *Ampelopsis grossedentata* extract and its characteristic component (dihydromyricetin) that was found to significantly lower plasma TG, TC, and LDL-C levels and increase plasma HDL-C levels in mice and hens [43–46]. In addition, evidence has shown that *E. ulmoides* can

also improve blood lipid levels [4]. In this research, plasma TG and LDL-C concentrations significantly decreased and the plasma HDL-C concentration significantly increased in both male and female rats under ELE treatments. Although a dose-response was not determined, the highest dose of ELE exerted a slightly stronger effect on HDL-C and LDL-C contents alterations if compared with the other two doses, which is in accordance with the findings in hens [43], indicating that ELE is beneficial for lowering blood lipid of rats. Previous research showed that the active ingredients of *E. ulmoides* (flavonoids and phenolics) ameliorated hypertriglyceridemia by upregulating the expression of genes related to hepatic α-, β-, and ω-oxidation [47] and thus participated in regulating blood lipid metabolism [48]. Since the complete mechanism of the lipid-decreasing effects of *E. ulmoides* is a complex network, the specific mechanisms should be further studied based on the abundant composition data of ELE.

Table 8: Effects of dietary supplementation *E. ulmoides* leaf extract (ELE) on the blood biochemistry indexes of rats. Different small letters indicate significant differences at $P < 0.05$ level of the Tukey test under different treatments

		A	B	C	D
Male	ALB (g/L)	33.37 ± 1.43 a	32.92 ± 1.77 a	34.96 ± 0.39 a	35.02 ± 2.89 a
	ALP (U/L)	113.36 ± 8.09 a	117.32 ± 20.68 a	108.68 ± 12.27 a	106.80 ± 16.55 a
	ALT (U/L)	35.31 ± 5.82 a	29.96 ± 4.17 a	30.48 ± 4.35 a	31.84 ± 7.82 a
	AST (U/L)	88.34 ± 3.43 b	98.76 ± 4.38 a	99.58 ± 3.17 a	97.88 ± 2.44 a
	CREA-S (μmol/L)	41.32 ± 11.34 a	33.52 ± 3.93 a	34.38 ± 5.44 a	43.20 ± 6.64 a
	Glu-G (mmol/L)	9.17 ± 1.03 a	6.94 ± 1.10 b	7.17 ± 0.87 b	7.60 ± 0.51 b
	TC (mmol/L)	2.45 ± 0.28 a	1.97 ± 0.24 b	2.02 ± 0.25 ab	2.43 ± 0.23 a
	TG (mmol/L)	0.68 ± 0.10 a	0.44 ± 0.10 b	0.43 ± 0.11 b	0.47 ± 0.11 b
	LDL-C (mmol/L)	2.57 ± 0.09 a	2.10 ± 0.10 c	2.15 ± 0.12 c	2.43 ± 0.05 b
	HDL-C (mmol/L)	1.40 ± 0.10 c	1.79 ± 0.07 a	1.65 ± 0.16 ab	1.54 ± 0.13 bc
	TP (g/L)	57.20 ± 2.16 a	56.81 ± 2.22 a	58.98 ± 1.48 a	60.75 ± 4.86 a
	UREA (mmol/L)	5.95 ± 2.10 a	6.06 ± 0.43 a	6.59 ± 0.38 a	6.53 ± 0.69 a
	γ-GT (U/L)	1.06 ± 0.93 a	-0.76 ± 0.55 b	-1.50 ± 0.65 b	-1.88 ± 1.10 b
Female	ALB (g/L)	37.82 ± 1.63 a	40.10 ± 4.43 a	38.20 ± 0.67 a	37.84 ± 1.89 a
	ALP (U/L)	76.31 ± 32.70 a	85.58 ± 27.82 a	57.90 ± 9.84	78.94 ± 34.64 a
	ALT (U/L)	25.60 ± 4.22 a	30.28 ± 8.69 a	28.94 ± 5.64 a	28.78 ± 6.67 a
	AST (U/L)	86.86 ± 13.22 b	102.46 ± 18.01 a	105.58 ± 10.99 a	106.58 ± 12.10 a
	CREA-S (μmol/L)	51.15 ± 12.26 ab	65.50 ± 13.13 a	42.54 ± 4.50 b	45.08 ± 3.50 b
	Glu-G (mmol/L)	7.23 ± 1.39 b	10.60 ± 1.46 a	6.51 ± 0.23 b	6.56 ± 1.72 b
	TC (mmol/L)	1.96 ± 0.24 b	2.28 ± 0.26 a	2.27 ± 0.37 a	1.95 ± 0.32 b
	TG (mmol/L)	0.82 ± 0.11 a	0.53 ± 0.15 b	0.47 ± 0.10 b	0.40 ± 0.10 b
	LDL-C (mmol/L)	2.62 ± 0.09 a	2.13 ± 0.10 b	2.25 ± 0.12 b	2.23 ± 0.05 b
	HDL-C (mmol/L)	1.51 ± 0.10 c	1.89 ± 0.07 a	1.74 ± 0.04 b	1.58 ± 0.11 c
	TP (g/L)	63.32 ± 2.56 a	68.60 ± 8.01 a	64.85 ± 0.94 a	65.27 ± 4.08 a
	UREA (mmol/L)	7.38 ± 1.43 a	8.59 ± 1.43 a	7.19 ± 1.53 a	7.25 ± 0.48 a
	γ-GT (U/L)	1.03 ± 1.06 a	-1.30 ± 0.83 b	-1.30 ± 0.88 b	-1.62 ± 1.65 b

3.9 Histopathological Analysis of Rats under ELE Treatment

Five tissues (liver, spleen, kidney, stomach, and pancreas) of rats were selected for conducting histopathological examinations (Fig. 1). Among the four groups, no significant changes or cytoplasmic vacuolation were observed. The intercellular spaces of the liver, spleen, kidney, stomach, and pancreas revealed no significant differences. The cell morphology of the liver, spleen, kidney, stomach, and pancreas under ELE treatment was regular without abnormal cellular changes when compared to the control; for example, the glomerulus was intact without distension. No inflammatory infiltration was observed in the cells of tissues under ELE treatment. Therefore, it was concluded that ELE did not cause a histopathological lesion in rats.
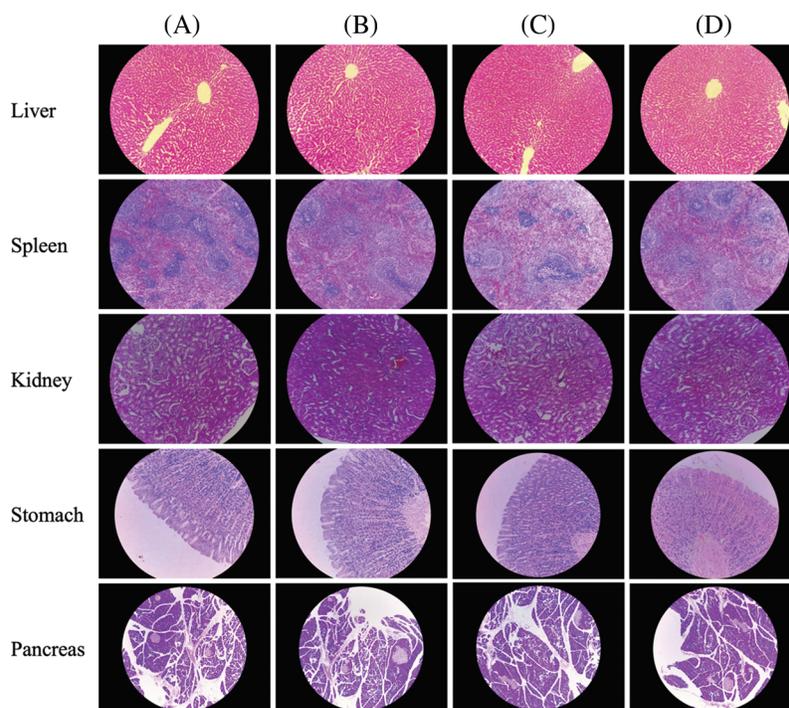
Figure 1: Effect of dietary supplementation *E. ulmoides* leaf extract (ELE) on the selected tissues of rats. (A−D) Representative photomicrographs of liver (20×), spleen (10×), kidney (20×), pancreas (10×), and stomach (20×) among the 4 tested groups

In our study, the long-term toxicity tests showed that the growth performance of rats (both male and female) were unaffected. The routine blood indexes, the antioxidant indexes, and the inflammatory factors were consistent between the ELE treatment and the control, indicating that long-term ingestion of ELE was harmless to the body because oxidative injury and inflammatory responses were not induced. No visible difference in the liver, spleen, kidney, stomach, or pancreas was identified between the ELE treatment and the control, which indicated that ELE did not cause histopathological lesions in rats. Taken together, we can therefore conclude that ELE does not cause long-term *in vivo* toxicity.

The duodenum villus heights of rats in the A group (both male and female) were significantly lower than those of rats under ELE treatment (Fig. 2), especially in female rats ($P < 0.05$), indicating that ELE promoted intestinal villus growth. No significant differences in the duodenum crypt depth of rats (both male and female) were observed between the ELE treatment groups and the A group ($P > 0.05$). A slight downregulation in the ileum villus height and crypt depth of female rats was observed under ELE treatment in comparison with the control condition (A group), without a significant difference ($P > 0.05$). In addition, the ileum villus height and crypt depth of male rats showed no difference among the four tested groups. In accordance with the alterations in villus height and crypt depth, the villus height to crypt depth ratio of the duodenum and ileum also changed.

The ratios of the A group were slightly lower than the ratios of the B, C, and D groups without significant differences ($P > 0.05$). These results implied that ELE was beneficial to intestinal morphology.
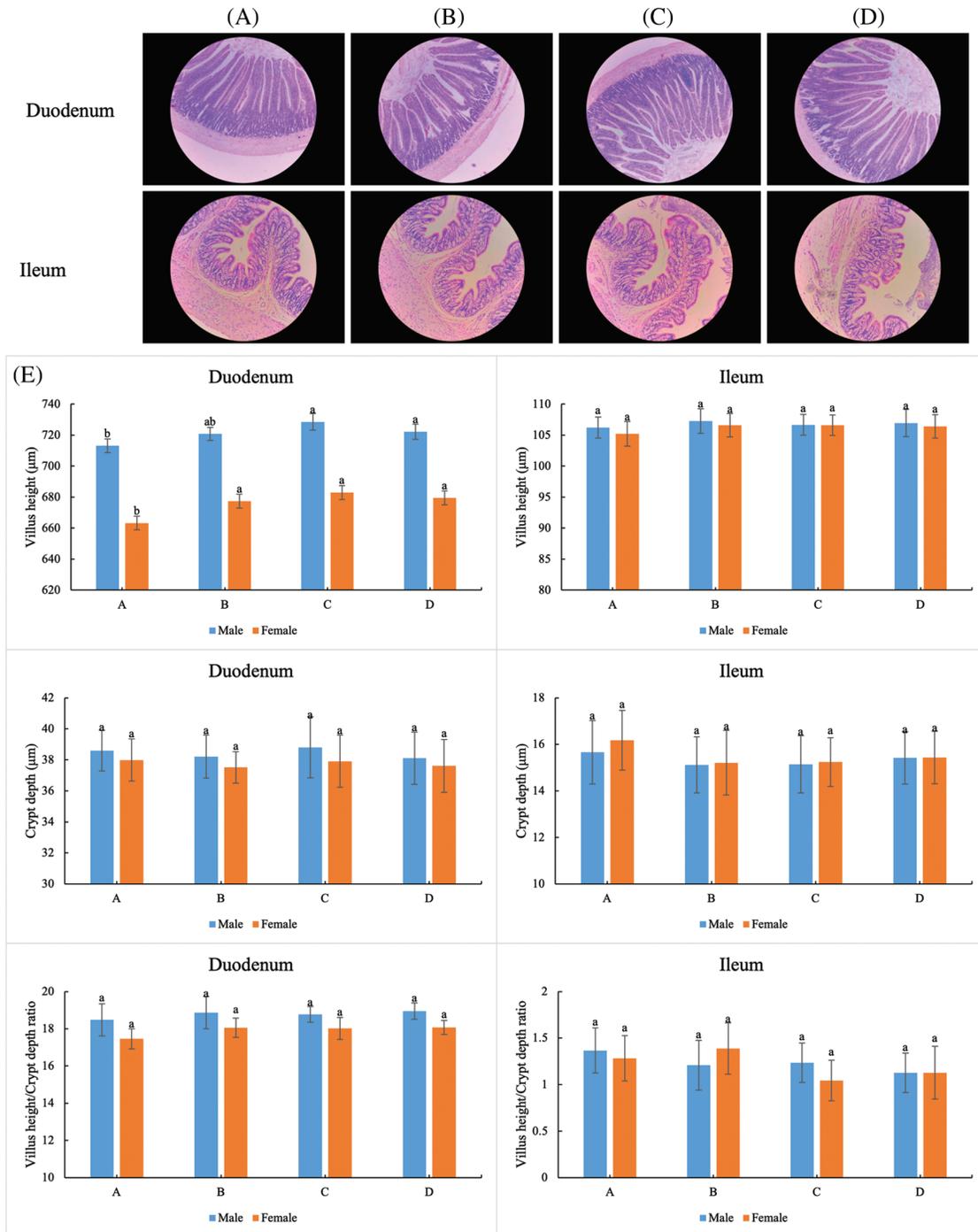

Figure 2: Effect of dietary supplementation *E. ulmoides* leaf extract (ELE) on the selected intestinal of rats. (A−D) Representative photomicrographs of villus in the duodenum (10×) and ileum (20×) of the 4 tested groups. (E) Villus height, crypt depth, and villus height to crypt depth ratio of duodenum and ileum in the 4 tested groups. Different small letters indicate significant differences at $P < 0.05$ level of tukey test under different treatments

Based on intestinal histomorphology, a higher villus height, as well as a higher villus height to crypt depth ratio, contribute to nutrient absorption [49,50]. Consistently, a higher villus height together with a higher villus height to crypt depth ratio of the duodenum and ileum were found under ELE treatment in comparison with the control condition, suggesting that dietary supplementation with ELE at a relatively high level was beneficial to nutrient absorption, as determined by intestinal histology changes. Therefore, another reason the growth performance was elevated may be attributed to ELE-mediated enhanced nutrient absorption via intestinal morphological changes. Furthermore, the alterations in villus height and crypt depth of female rats were more obvious than those of male rats, suggesting that ELE functioned as a phytoestrogen in stimulating the growth performance of rats.

3.10 Antimicrobial Testing of ELE

To further investigate the bioactivities of ELE, the antimicrobial activities, anticancer capacity, and antioxidant abilities of ELE were explored. The inhibition rates of ELE against *E. coli*, *S. aureus*, *P. aeruginosa*, and *A. niger* are listed in Table 9. Based on the results, ELE exhibited good to excellent antimicrobial activities against *S. aureus* and *P. aeruginosa* as the inhibition rate against *S. aureus* reached above 80% and the inhibition rate against *P. aeruginosa* ranged from 58.93%–98.19%. A moderate inhibition rate of ELE against *E. coli* and *A. niger* was noted as the inhibition rates were in a range of 34.08%–58.30%. These results imply that ELE has certain antimicrobial activity, which is in accordance with the former report [4].

Table 9: The antimicrobial test of *E. ulmoides* leaf extract (ELE). Different small letters indicate significant differences at $P < 0.05$ level of the tukey test under different treatments

Concentration of microbial Concentration of ELE	Inhibition rate (%)			
	1×10^3 CFU/mL		1×10^4 CFU/mL	
	25 mg/mL	5 mg/mL	25 mg/mL	5 mg/mL
Escherichia coli	58.30 ± 2.81 a	34.08 ± 2.23 b	44.10 ± 2.55 b	40.50 ± 2.27 a
Staphylococcus aureus	83.60 ± 1.92 a	80.68 ± 2.47 a	84.95 ± 2.84 a	80.20 ± 2.12 a
Pseudomonas aeruginosa	98.19 ± 1.32 a	97.91 ± 1.63 a	64.01 ± 2.92 a	58.93 ± 1.76 b
Aspergillus niger	50.40 ± 2.46 a	48.46 ± 2.66 a	55.67 ± 2.02 a	50.47 ± 2.35 b

3.11 Potential Anticancer Capacity of ELE

The cell viability of HepG2 cells under different ELE treatments was evaluated (Fig. 3). As the concentration of ELE increased, the viability of HepG2 cells decreased, which was in agreement with the effects of ELE on V79 cells. Besides, the IC50 of ELE against HepG2 was 3.98 mg/L, which was significantly lower than the IC50 of cisplatin (12.52 mg/L). Although the experimental design was the same as in cytotoxicity assays and the treatment time was only 12 h, a down-regulation in cells viability of HepG2 was observed along with ELE concentration increasing, suggesting ELE had the potential in inhibiting HepG2 cells proliferation based on the numerous active components.

3.12 Free Radical Scavenging Rate of ELE

Four typical free radicals (·OH, DPPH·, PTIO·, ABTS·) were chosen to assess the *in vitro* free radical scavenging rate of ELE (Fig. 4). Although scavenging rates (%) of ELE against the selected free radicals were lower than that of vitamin C, the free radical scavenging rate of ELE against ·OH, DPPH·, PTIO·, and ABTS· all increased as the ELE concentration increased, indicating that ELE at a relatively high

concentration was beneficial for free radical scavenging. Due to flavonoids of *E. ulmoides* leaves (e.g., CGA, rutin, ferulic acid, and caffeic acid) possessing good antioxidant activity [15], our results certified the ELE could also reveal preferable antioxidant activities, which had benefits in anti-aging [23].
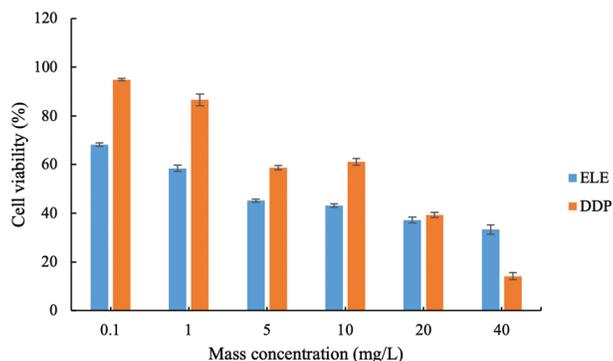

Figure 3: The cell viability of HepG-2 cells under different *E. ulmoides* leaf extract (ELE) treatment. DDP represents cisplatin
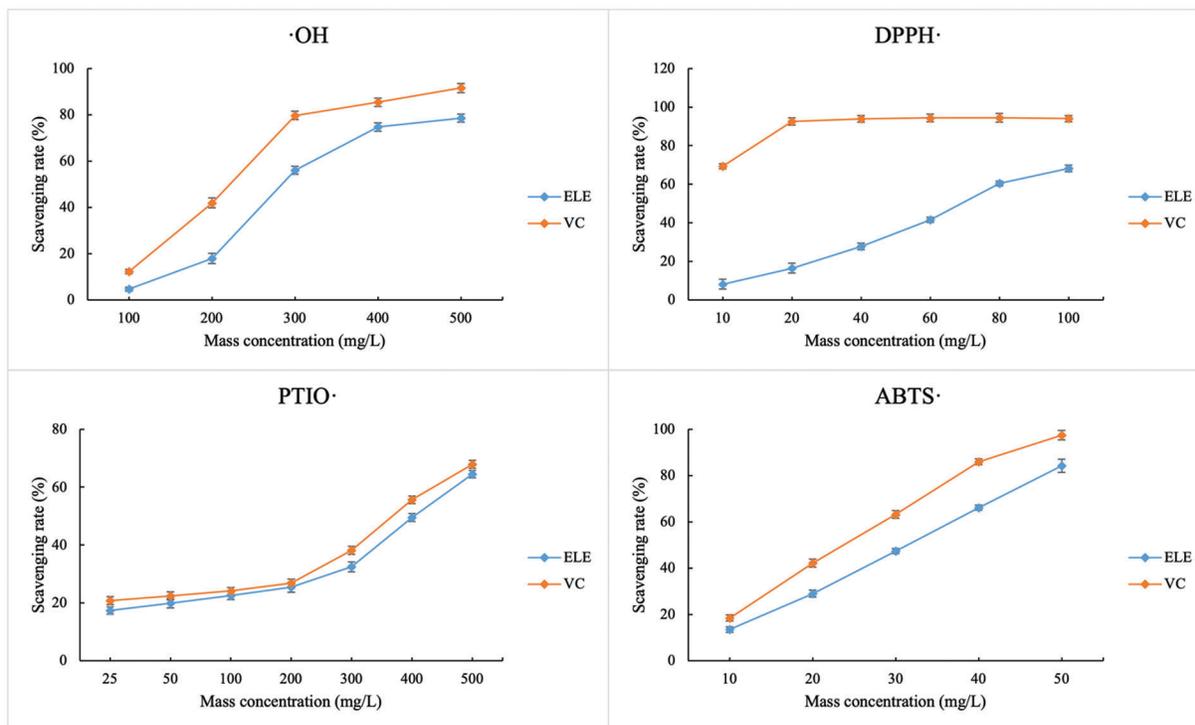

Figure 4: The free radical scavenging rates of *E. ulmoides* leaf extract (ELE). VC represents vitamin C

Because several types of active compounds were identified in *E. ulmoides* (either the bark or leaves) [5], *E. ulmoides* has been found to exhibit multiple pharmacological activities [4,51]. Numerous studies have shown that CGA, the most important characteristic component of *E. ulmoides* leaves, protected mice against Cd-induced hepatorenal injury [52], and the other characteristic components with high levels in *E. ulmoides* leaves, such as geniposidic acid (GPA) and aucubin (AU), caused free radical scavenging and

anticancer effects [53–55]. Therefore, these results are corroborative evidence showing that ELE has significant prospects for antimicrobial, anticancer, and antioxidant uses, based on the active ingredients. Meanwhile, our previous studies had proved *E. ulmoides* leaf extract revealed higher antihypertension effect than bark extract, and could enhance the body health and production of hens [18,43]. Together with our previous research showing that GPA and AU have tremendous potential for their anti-aging effects [23], ELE also shows outstanding potential in traditional Chinese health food fields, such as food, medicine, feeding, and daily chemical product. Many types of products derived from ELE have emerged, such as tea [56], beverages [57], feed additives [43], and healthy food [58]. Our work can facilitate *E. ulmoides* leaf inclusion in the directory of traditional Chinese health food as a new resource and can ensure the export quality of ELE.

4 Conclusion

In the present study, a comprehensive assessment of the safety of *E. ulmoides* leaf extract was conducted through a combination of genotoxicity tests, cytotoxicity assay, and *in vivo* long-term animal toxicity analysis. The conclusions from the preliminary data and discussion were as follows:

1. The reverse mutation number of nominal bacterial strains, the micronucleus rates of *in vivo* mammalian erythrocyte and *in vitro* mammalian cells, and the TFT resistance mutation frequency of the TK gene under ELE treatment were less than the rated fold change of revealing genotoxicity in comparison with the solvent control, indicating that ELE had no genotoxicity.
2. The growth performance of rats under ELE treatment was enhanced and no oxidative injury, inflammatory response, as well as histopathological lesions of tissues were detected between ELE-treated and control rats, indicating that ELE also had no *in vivo* long-term toxicity.
3. The blood lipid levels were ameliorated under ELE treatment, suggesting that ELE could potentially improve body health.
4. Good to excellent antimicrobial, anticancer, and antioxidant activities of ELE were determined, implying that ELE possessed preferable bioactivities.

These results could deepen our understanding of ELE as a traditional Chinese health food in addition to supporting the use of ELE in actual applications, such as the food, drug, and cosmetics industries.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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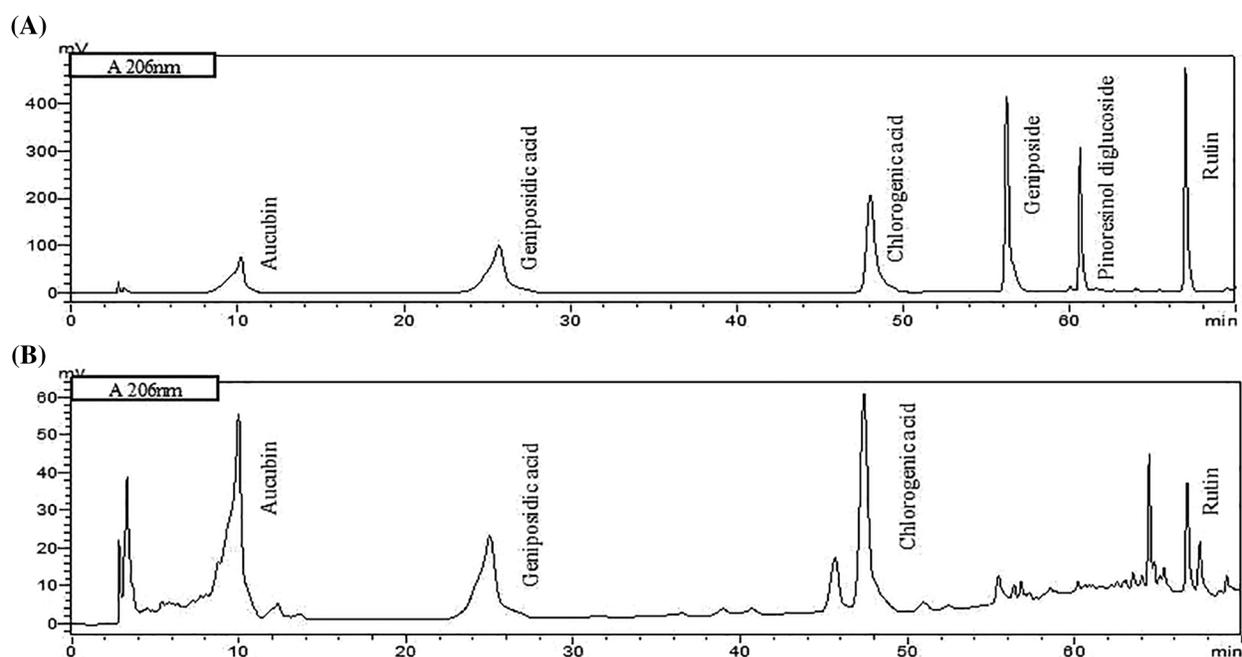
Appendix

Figure S1: Chromatograms of standards. (A) and characteristic components in *E. ulmoides* leaf extract (ELE) (B) using HPLC system.

Table S1: Effects of dietary supplementation *E. ulmoides* leaf extract (ELE) on the blood routine indexes of rats. Different small letters indicate significant differences at $P < 0.05$ level of Tukey test under different treatments

		A	B	C	D
Male	WBC (10^9/L)	4.88 ± 1.48a	4.60 ± 1.68a	4.82 ± 1.11a	5.46 ± 1.51a
	Neu (10^9/L)	0.65 ± 0.17b	1.10 ± 0.20a	0.67 ± 0.15b	0.61 ± 0.09b
	Lym (10^9/L)	3.38 ± 1.60a	3.22 ± 1.17a	3.88 ± 0.97a	4.55 ± 1.48a
	Mon (10^9/L)	0.24 ± 0.12a	0.25 ± 0.12a	0.25 ± 0.12a	0.27 ± 0.04a
	Eos (10^9/L)	0.03 ± 0.02a	0.03 ± 0.03a	0.02 ± 0.01a	0.02 ± 0.02a
	Bas (10^9/L)	0.01 ± 0.01a	0.01 ± 0.01a	0.01 ± 0.01a	0.01 ± 0.01a
	Neu (%)	17.13 ± 6.43ab	23.98 ± 3.48a	14.02 ± 4.77b	11.78 ± 2.83b
	Lym (%)	76.22 ± 6.92ab	70.00 ± 2.74b	80.32 ± 6.72a	82.26 ± 5.23a
	Mon (%)	5.60 ± 0.57a	5.12 ± 1.16a	5.18 ± 2.12a	5.24 ± 2.29a
	Eos (%)	0.88 ± 0.59a	0.72 ± 0.50a	0.46 ± 0.32a	0.42 ± 0.31a
	Bas (%)	0.17 ± 0.11a	0.18 ± 0.22a	0.02 ± 0.04b	0.30 ± 0.16a
	RBC (10^{12}/L)	9.15 ± 0.66a	9.52 ± 0.17a	9.45 ± 0.41a	9.36 ± 0.26a
	HGB (g/L)	163.09 ± 8.73a	173.40 ± 6.31a	170.80 ± 5.97a	167.80 ± 4.15a
	HCT (%)	46.70 ± 2.55a	47.72 ± 1.38a	48.96 ± 1.22a	47.74 ± 1.41a
	MCV (fL)	51.10 ± 1.40a	50.10 ± 0.99a	51.84 ± 1.05a	51.02 ± 0.94a
	MCH (pg)	17.85 ± 0.51a	18.20 ± 0.44a	18.08 ± 0.49a	17.92 ± 0.25a
	MCHC (g/L)	349.55 ± 5.16b	362.80 ± 7.66a	348.60 ± 5.27b	351.40 ± 3.78b
	RDW-CV (%)	12.70 ± 0.35a	12.78 ± 0.13a	12.58 ± 0.18a	12.72 ± 0.33a
	RDW-SD (fL)	26.35 ± 0.39a	26.82 ± 0.53a	26.66 ± 0.44a	26.50 ± 0.42a
	PLT (10^9/L)	894.18 ± 115.78a	1052.20 ± 58.90a	1018.00 ± 94.14a	946.40 ± 73.62a
	MPV (fL)	6.22 ± 0.33a	6.40 ± 0.29a	6.50 ± 0.29a	6.66 ± 0.26a
	PDW (%)	15.30 ± 0.14a	15.34 ± 0.09a	15.14 ± 0.11a	15.20 ± 0.14a
	PCT (%)	0.56 ± 0.09a	0.67 ± 0.03a	0.66 ± 0.04a	0.63 ± 0.06a
Female	WBC (10^9/L)	4.73 ± 1.19a	4.23 ± 0.62a	5.29 ± 1.40a	5.75 ± 2.59a
	Neu (10^9/L)	0.55 ± 0.16a	0.67 ± 0.08a	0.52 ± 0.20a	0.94 ± 0.56a
	Lym (10^9/L)	3.86 ± 1.02a	2.86 ± 1.16a	4.47 ± 1.08a	3.21 ± 2.06a
	Mon (10^9/L)	0.28 ± 0.07a	0.21 ± 0.10a	0.27 ± 0.12a	0.27 ± 0.19a
	Eos (10^9/L)	0.04 ± 0.02a	0.02 ± 0.02a	0.02 ± 0.02a	0.03 ± 0.04a
	Bas (10^9/L)	0.01 ± 0.01a	0.01 ± 0.01a	0.01 ± 0.01a	0.01 ± 0.02a
	Neu (%)	11.50 ± 1.94ab	16.82 ± 4.11a	9.56 ± 1.38b	13.36 ± 3.85ab
	Lym (%)	81.28 ± 2.63ab	75.98 ± 6.46b	84.94 ± 2.26a	79.02 ± 3.58ab
	Mon (%)	6.07 ± 1.64a	6.32 ± 1.54a	4.98 ± 0.84b	6.68 ± 1.84a
	Eos (%)	0.81 ± 0.41a	0.54 ± 0.26a	0.42 ± 0.37a	0.62 ± 0.41a
	Bas (%)	0.34 ± 0.13a	0.34 ± 0.41a	0.30 ± 0.07a	0.32 ± 0.16a

(Continued)

Table S1 (continued)

	A	B	C	D
RBC (10^{12}/L)	8.46 ± 0.33a	8.24 ± 0.60a	8.48 ± 0.38a	8.61 ± 0.54a
HGB (g/L)	157.90 ± 5.76a	156.80 ± 8.14a	155.80 ± 3.77a	158.80 ± 8.35a
HCT (%)	44.46 ± 1.68a	44.16 ± 2.52a	44.74 ± 0.95a	45.72 ± 2.86a
MCV (fL)	52.55 ± 0.91a	53.64 ± 1.70a	52.78 ± 1.65a	53.10 ± 0.60a
MCH (pg)	18.67 ± 0.33a	19.06 ± 0.71a	18.38 ± 0.64a	18.44 ± 0.21a
MCHC (g/L)	355.40 ± 3.78a	355.20 ± 2.86a	348.20 ± 1.92b	347.20 ± 5.63ab
RDW-CV (%)	12.13 ± 0.39a	12.20 ± 0.07a	12.08 ± 0.45a	12.28 ± 0.43a
RDW-SD (fL)	26.05 ± 0.81a	26.72 ± 0.68a	26.18 ± 1.28a	26.64 ± 0.89a
PLT (10^9/L)	1080.90 ± 143.44a	968.00 ± 42.08a	1082.40 ± 135.73a	1114.20 ± 98.58a
MPV (fL)	6.37 ± 0.17a	6.66 ± 0.17a	6.30 ± 0.17a	6.46 ± 0.29a
PDW (%)	15.27 ± 0.18a	15.32 ± 0.13a	15.10 ± 0.19a	15.18 ± 0.08a
PCT (%)	0.69 ± 0.09a	0.64 ± 0.01a	0.68 ± 0.09a	0.72 ± 0.07a

Table S2: Effects of dietary supplementation *E. ulmoides* leaf extract (ELE) on the antioxidant indexes and inflammatory factors of rats. Different small letters indicate significant differences at $P < 0.05$ level of Tukey test under different treatments

			A	B	C	D
Male	Antioxidant index	SOD (U/ml)	107.31 ± 17.70a	113.77 ± 17.16a	121.81 ± 31.57a	111.81 ± 32.02a
		GSH (umol/L)	1.95 ± 0.32a	2.07 ± 0.31a	2.21 ± 0.57a	2.03 ± 0.58a
		MDA (nmol/L)	8.43 ± 1.56a	7.89 ± 1.26a	7.60 ± 1.87a	8.34 ± 2.10a
		CAT (U/ml)	15.61 ± 2.57a	16.55 ± 2.50a	17.72 ± 4.59a	16.26 ± 4.66a
	Inflammatory factor	TNF-α (ng/L)	378.84 ± 36.92a	379.84 ± 32.47a	388.47 ± 39.01a	397.25 ± 17.78a
		IL-1β (ng/L)	42.04 ± 3.85a	41.59 ± 2.37a	43.54 ± 2.37a	43.83 ± 5.00a
		IL-6 (pg/ml)	122.95 ± 10.75a	128.95 ± 7.32a	120.21 ± 8.62a	114.03 ± 6.17a
Female	Antioxidant index	SOD (U/ml)	150.83 ± 40.62a	108.42 ± 25.05a	128.12 ± 21.33a	124.01 ± 27.19a
		GSH (umol/L)	2.74 ± 0.74a	1.97 ± 0.46a	2.33 ± 0.39a	2.25 ± 0.49a
		MDA (nmol/L)	6.19 ± 1.56a	8.51 ± 2.20a	7.03 ± 1.21a	7.36 ± 1.59a
		CAT (U/ml)	21.94 ± 5.91a	15.77 ± 3.64a	18.64 ± 3.10a	18.04 ± 3.96a
	Inflammatory factor	TNF-α (ng/L)	385.96 ± 26.09a	406.50 ± 27.83a	386.29 ± 28.64a	377.87 ± 21.99a
		IL-1β (ng/L)	41.95 ± 3.24a	41.80 ± 3.65a	43.59 ± 2.96a	40.71 ± 1.06a
		IL-6 (pg/ml)	119.73 ± 10.61a	115.07 ± 7.68a	119.66 ± 7.50a	132.09 ± 5.95a