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Safety evaluation of mulberry leaf extract: Acute, subacute toxicity and genotoxicity studies

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Abbreviations:

1,8-DHAQ: 1,8-dihydroxyanthraquinone; 2-AF: 2-aminofluorene; 4-NO: 4-nitro-O-phenylenediamine ; ALB: albumin; ALP: alkalinephosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BG: blood glucose; BUN: urea nitrogen; bw: body weight; CHOL: cholesterol; CRE: creatinine; DNJ: 1-Deoxynojirimycin; FDA: United States Food and Drug Administration; GLP: Good Laboratory Practice; GSH: glutathione; GST: glutathione transferase; HG: hemoglobin; LD₅₀: median lethal dose; MLE: mulberry leaf extract; MMC: mitomycin C; NaN₃: sodium azide; NOAEL: no-observable-adverse-effect level; PCE: polychromatic erythrocytes; PLT: platelet count; RBC: red blood cells; TG: triglycerides; TPROT: total proteins; WBC: white blood cells

Abstract

Mulberry (*Morus alba* L.) leaves are of broad popular use for food or remedy purposes due to their bioactive properties, especially antidiabetic activity and antioxidative activity. The present study aimed to assess the toxicological profile of mulberry leaf extract (MLE), through acute, subacute toxicity and genotoxicity tests. Male and female rats received by gavage 15.0 g/kg bw of MLE in the acute toxicity test, and 0, 1.88, 3.75 and 7.50 g/kg bw/d of MLE for subacute toxicity test. In the acute toxicity study, no mortality or behavioral changes were observed, indicating the LD₅₀ is higher than 15.0 g/kg bw. In the subacute toxicity test, no significant changes were observed in hematological, biochemical or histopathological parameters in the animals exposed. The no-observed-adverse-effect level in the subacute toxicity study was considered to be 7.50 g/kg bw/d, the highest dose tested. In the genotoxicity study, MLE showed no mutagenic activity in the Ames assay and no evidence of potential to induce chromosome aberrations or sperm abnormalities in mice exposed to 10 g/kg bw. Collectively, aqueous extract of mulberry leaves could be considered safe, and the results support the application of MLE as novel food ingredient or product.

Keywords: Mulberry leaf extract; rat; subacute toxicity; genotoxicity; Ames assay; micronucleus; sperm abnormality; safety assessment

1. Introduction

Mulberry (*Morus alba* L.) is broadly distributed in Asia, native to China, Korea and Japan, and at present is cultivated throughout Europe (Gryn-Rynko et al., 2016; Hu et al., 2013; Jiang et al., 2013). The botanical and pharmaceutical names of mulberry tree are *Morus alba* L. and *Folium mori*, respectively (Chen et al., 1995). Mulberry leaves have been used as a remedy in China since ancient times (Song et al., 2009). Currently, the leaf and leaf-derived extracts are reported to be used as food or medicine in many countries (Aramwit et al., 2011; Ji et al., 2016; Tao et al., 2016; Wanyo et al., 2011). The leaves are used in reducing the risk and treatment of type 2 diabetes, and diseases of the cardiovascular system, urinary system, nervous system (e.g., Alzheimer's disease), as well as in weight loss (Hsu et al., 2012; Liu et al., 2016). In the human studies reporting anti-hyperglycemic effects and lipid-lowering effects, mulberry leaf extract was administered via the oral route for 3 g per day for 30 days (Andallu et al., 2001), or 1 g per day repeated in 1 week (Mudra et al., 2007), or 1 g per day for 12 weeks (Shin et al., 2016), etc. The recommended daily amounts of mulberry leaf extracts commercially available in Europe, Canada and America range from 1 g to 4.5 g per day as dietary supplement. These applications may be attributed to various bioactive compounds that mulberry leaves contain, including polyhydroxy alkaloids, stilbenoids, flavonoids, and of particular interest medicinally, iminosugars (Gryn-Rynko et al., 2016; Ou-yang et al., 2013; Yang et al., 2014). Concerning the evidence of their bioactive properties (Jeszka-Skowron et al., 2014; Rebai et al., 2017; Sánchez-Salcedo et al., 2015), especially antidiabetic activity and antioxidative activity (Hunyadi et al., 2013; Katsube et al., 2010; Kim and Jang, 2011; Kojima et al., 2010; Shin et al., 2016), mulberry leaves and their constituents are gaining more interest in recent years.

Although mulberry leaves has been consumed in China traditionally, there are still relative few safety studies on the mulberry leaf extract (MLE), which is composed of concentrated particular active substances. It was considered prudent to conduct a series of studies to demonstrate the safety of such a product for possible use in food. What's more, the absence of lethal effects cannot demonstrate the safety of plant preparations with respect to genotoxicity. In view of this, the current study describes a battery of genotoxicity studies, an acute toxicity and a 30-day toxicity study conducted in rats to evaluate the toxicity of mulberry leaf extract.

2. Materials and methods

All aspects in this project involving animal care, use and welfare were performed in compliance with the Food and Drug Administration (FDA) principles of Good Laboratory Practice (GLP) and in accordance with the FDA Guidance for Industry and Other Stakeholders, “Toxicological Principles for the Safety Assessment of Food Ingredients Redbook 2000” (FDA, 2000) and Chinese National food safety standard “The procedures and methods of the toxicological assessment for food safety” (GB 15193-2003). All animal study protocols have been approved by the Office of Laboratory Animal Welfare, China National Center for Food Safety Risk Assessment (Beijing, China). The protocol for the animal study was reviewed and approved by the Institutional Animal Care and Use Committee before animal receipt.

2.1. Test substance

Mulberry Leaf Extract (MLE, Batch No. 20140801) was prepared by Botanic Century (Beijing) Co. Ltd. Dried leaves were extracted by water. The extraction solution was filtered with a 10KD membrane and then ultra-filtered with a 3KD membrane. The filtrate was concentrated and spray dried to obtain MLE. It was a yellowish brown powder and was stored at room temperature for testing. MLE was considered stable for a period of 24 months.

2.2. Animals and housing conditions

Healthy Kunming mice (SPF grade) and SD rats (SPF grade) were supplied by Beijing HFK Bioscience Co. Ltd, and Beijing Vital River Laboratory Animal Technology Co. Ltd with the license number SCXK (Jing) 2014-0004 and SCXK (Jing) 2012-0001, respectively.

Animals were reared in the Animal Laboratory of Beijing Stomatology Hospital, Capital Medical University, iPhase Biosciences (Beijing) and Institute of Laboratory Animal Sciences, CAMS & PUMC, with license No. SYXK (Beijing) 2013-0018, SYXK (Jing) 2014-0022 and SYXK (Jing) 2013-0014, respectively. Animal rooms were maintained well-ventilated at temperature of 20-23°C, relative humidity of 40-55%, with a 12-h light/dark cycle. Animals had free access to solid feed and water during the experiments.

Animal feeds were sterilized with Co-60 irradiation supplied by both Beijing HFK Bioscience Co. Ltd and Experiment Animal Centre, China Academy of Military Medical Sciences with the licenses number SCXK (Jing) 2014-0008 and SCXK (Jing) 2012-0003 respectively.

2.3. Genotoxicity studies

2.3.1. Bacterial reverse mutation assay (Ames test)

The tester strains of auxotrophic *Salmonella typhimurium* TA97, TA98, TA100 and TA102 (MOLECULAR TOXICOLOGY INC., NC, USA) was employed, in the presence and absence of the metabolism activation system (S9, iPhase Biosciences, Beijing). Based on the results of a preliminary toxicity dose-range test, five dose levels were set as 62, 185, 556, 1667 and 5000 µg/dish in the present study, with three plates for each dose. The solvent control was sterile water and positive controls were standard mutagens as follows, sodium azide (NaN₃, 1.5 µg/plate) for TA100 without S9, 2-aminofluorene (2-AF, 10 µg/plate) for TA97, TA98, and TA100 with S9, 4-nitro-o-phenylenediamine (4-NOPD, 20 µg/plate) for TA97 and TA98 without S9, mitomycin (MMC, 2.5 µg/plate) for TA102 with S9, and 8-dihydroxyanthraquinone (DHAQ, 50 µg/plate) for TA102 without S9.

In the test, 0.1 mL of the test sample (or control), 0.1 mL of the bacterial suspension and 0.5 mL of either the S9 mixture or the phosphate buffer (pH 7.4) were added to 2.0 mL of top agar. After mixing thoroughly, the mixture was poured onto minimal glucose agar plates. After 48 h incubation at 37°C, the number of revertant colonies was counted manually. A positive result was determined where the revertant colony counts were greater than 2-fold those of the solvent control and a clear dose-response relationship was observed.

2.3.2. In vivo mouse bone marrow micronucleus assay

Fifty healthy Kunming mice (SPF grade), weighing 28-30 g, were randomized by body weight into 5 groups (5 male and 5 female for each group). Mice were treated by oral gavage (20 mL/kg bw) with either distilled water (negative control), or MLE solution at a dose of 2.5, 5.0 or 10.0 g/kg bw. The highest dose level was prepared with 50.0 g MLE dissolved in distilled water to make 100 mL. Lower doses were prepared through 2-fold serial dilution with

distilled water. Cyclophosphamide (40 mg/kg bw) was used as positive control. All groups were treated by oral gavage twice, and the second treatment was precisely 24 h after the first gavage.

Six hours after the second gavage treatment, animals were euthanized and sternums were aseptically removed. The contents of the spinal canal were squeezed out, suspended with calf serum, and then smeared onto slides. The slides were fixed with methanol and stained with Giemsa. Red blood cells (RBC) and polychromatic erythrocytes (PCE) were observed under microscopy. The number of PCE was counted from 200 RBC for each animal and the ratio of PCE/RBC was calculated. For each animal, 1000 PCE were examined to determine the incidence of micronucleus.

2.3.3. *Mouse sperm malformation assay*

Sexually mature male healthy Kunming mice (SPF grade), weighing 28-30g, were randomized into 5 groups. The 5 groups included a negative control group (distilled water), a positive control group (40 mg/kg bw cyclophosphamide), and 3 MLE treatment groups (2.5, 5.0 and 10.0 g/kg bw). 10 g MLE was dissolved in 20 mL distilled water to make the MLE solution for the high-dose group, which was double diluted with distilled water to make the MLE solution for the middle-dose and low-dose group. All the mice were treated by oral gavage with volume of 20mL/kg bw, once daily for 5 successive days. Thirty days after the last gavage, the mice were sacrificed by cervical dislocation. The bilateral epididymides were harvested and adipose tissue was removed. The epididymides were then cut into pieces in normal saline, and then centrifuged at 1000 r/min for 7 min. The supernatant was disposed and sperm suspension was applied on a slide, dried in air, fixed with methanol and stained with 1.5% eosin. The slides were examined with microscopy. One thousand sperms per mouse were screened and classified into normal and different abnormal types based on similar criteria described by Wyrobek and Bruce (Wyrobek and Bruce, 1975).

2.4. *Rat studies*

2.4.1. *Acute toxicity study*

Twenty healthy SD rats, half males and half females, weighing between 186.2 g and 212.8 g were used. The animals were fasted for 16 hours with free water intake before test. To make a 0.5 g/mL MLE suspension, 37.5 g of test sample was mixed with 75 ml of distilled water. The volume administered by oral gavage was 15.0 ml/kg bw, twice a

day, which is equivalent to the acute toxicity dose of 15.0 g/kg bw. The animals were observed on a daily base throughout 14 days after treatment and the animal's general behaviour and any deaths due to toxicity were monitored and recorded.

2.4.2. Subacute oral toxicity study

After acclimating to the laboratory environment for one week, 80 weaning SD rats weighing approximately 76 to 113 grams were assigned randomly to 4 groups consisting of 10 males and 10 females in each group. The 4 groups included 1 control group and 3 treatment groups dosed at 1.88, 3.75 and 7.5 g/kg bw/d (equivalent to 75-, 150- and 300-fold of the recommended human dose). The formulation was calculated on the basis of daily intake/body weight ratio (10%) for Sprague-Dawley rats. To make the diet for the treatment group, normal diet was blended with 375 g, 750 g and 1500 g of MLE and 83 g, 165 g and 330 g of casein, respectively, followed by mixing well and quantifying to 20 kg. Animals in the control group were fed on normal diet, and MLE was administered by treating groups in constant dietary concentrations of 1.88%, 3.75%, and 7.5%, respectively, for 30 days.

2.4.2.1. In-life observations

Throughout the experimental study, each animal was kept in a single cage with free access to water. General clinical observations were recorded daily. Body weight change and food consumption was measured and recorded weekly.

2.4.2.2. Hematology and clinical chemistry

At the end of 30-day treatment periods, the rats were fasted of feed for 16-18 h with free drinking water. The rats were then anesthetized with 3% sodium pentobarbital solution and blood was collected from the inner canthus vein. The whole blood stabilized by the anticoagulant ethylene diamine tetraacetic acid (EDTA) was analyzed using Coulter Diff Hematology Analyzer (Beckman Coulter Corporation). The parameters evaluated include red blood cell counts (RBC), hemoglobin (HG), platelet counts (PLT), white blood cells counts (WBC), and leukocyte differential counts. Clinical chemistry was analyzed with an automatic clinical analyzer (Hitachi 7080, Hitachi High-Technologies Corporation) to determine serum alanine aminotransferase (ALT), aspartate aminotransferase (AST),

alkalinephosphatase (ALP), total protein, albumin, glucose, blood ureanitrogen, creatinine, cholesterol, and triglyceride.

2.4.2.3. Necropsy and histopathology

At study termination, all animals were weighed and euthanized for complete gross necropsy. The organs such as liver, kidney, spleen, testes (or ovary) were collected and weighed. Organ-to-bodyweight ratios (relative organ weight) were calculated as $(\text{organ/body weight}) \times 100$. Gross examination of the liver, spleen, kidneys, stomach, duodenum, testes or ovaries of animals in each dose groups was carried out. Organs and tissues from each animal were fixed in 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin. Histopathological examinations were first conducted on liver, spleen, kidneys, stomach, duodenum, testes or ovaries of animals only in the high-dose and control groups. If there were pathological changes observed, histological examination of the corresponding organs were also carried out in the mid-dose and low-dose groups.

2.5. Statistical analysis

Statistical analysis was performed using SPSS software. If the variance in each group was homogeneous, one-way ANOVA was carried out, and differences were considered significant at $p < 0.05$. The mean was compared between each dose group and control group using S-N-K(S) test. If homogeneity of variance was not met, other statistical methods such as Brown-Forsythe test or Welch test were employed. The X^2 -test was used for numeration data.

The data from the *in vivo* mouse micronucleus assay were analyzed using Poisson's distribution. Data from the mouse sperm abnormality assay were processed using the Wilcoxon rank sum test.

3. Results

3.1. Genotoxicity tests

3.1.1. Bacterial reverse mutation test

181 As shown in Table 1, the positive controls induced increases in the number of revertant colonies as compared with
182 the negative controls. However, MLE did not increase the number of revertants in any tester strains in the absence or
183 presence of S9 metabolic activation, and no dose-related changes were observed either.

Table 1 Bacterial reverse mutation test conducted with mulberry leaf extract (confirmatory assay). Data are given as mean \pm SD revertants/plate for three replicates for each concentration in each experiment.

Treatment	Dose ($\mu\text{g}/\text{dish}$)	TA97		TA98		TA100		TA102	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
MLE	62	140.3 \pm 8.0	121.3 \pm 14.0	49.7 \pm 5.7	49.3 \pm 3.2	124.3 \pm 12.7	115.7 \pm 10.0	286.7 \pm 5.9	307.7 \pm 13.1
	185	140.0 \pm 13.1	147.3 \pm 8.5	47.3 \pm 2.1	42.0 \pm 6.6	141.7 \pm 13.1	145.0 \pm 12.0	293.0 \pm 4.6	273.0 \pm 12.1
	556	135.7 \pm 15.9	120.7 \pm 8.7	48.7 \pm 8.6	37.7 \pm 2.3	136.0 \pm 15.9	127.3 \pm 18.0	290.0 \pm 24.3	287.3 \pm 23.5
	1667	125.3 \pm 14.2	142.7 \pm 25.1	53.0 \pm 3.5	41.7 \pm 6.7	121.7 \pm 13.0	136.3 \pm 23.5	292.3 \pm 17.2	281.7 \pm 11.8
	5000	131.7 \pm 22.5	157.0 \pm 17.1	49.7 \pm 6.7	47.3 \pm 7.4	149.7 \pm 37.9	128.0 \pm 17.1	295.7 \pm 28.3	285.0 \pm 3.0
Untreated control		135.3 \pm 16.4	136.3 \pm 15.0	36.7 \pm 1.2	39.7 \pm 4.0	124.7 \pm 16.7	134.7 \pm 20.5	281.3 \pm 18.8	282.0 \pm 15.7
Solvent control		132.7 \pm 23.7	148.3 \pm 5.9	41.0 \pm 4.4	37.7 \pm 4.0	128.3 \pm 8.5	126.0 \pm 14.2	282.7 \pm 2.5	271.3 \pm 15.3
Positive controls		-	-	-	-	-	-	-	-
NaN ₃	1.5	-	-	-	-	1579.3 \pm 119.0*	-	-	-
2-AF	10	-	1312.0 \pm 149.0*	-	2967.3 \pm 213.8*	-	1536.0 \pm 228.1*	-	-
4-NO	20	1065.3 \pm 205.2*	-	1817.3 \pm 151.5*	-	-	-	-	-
MMC	2.5	-	-	-	-	-	-	1638.7 \pm 57.5*	-
1,8-DHAQ	50	-	-	-	-	-	-	-	670.0 \pm 84.9*

* Statistically significant difference from control values ($P < 0.05$)

Abbreviations: -S9 = without metabolic activation; +S9 = with metabolic activation; NaN₃= sodium azide; 2-AF= 2-aminofluorene; 4-NO= 4-nitro-O-phenylenediamine ; MMC= mitomycin C; 1,8-DHAQ= 1,8-dihydroxyanthraquinone

3.1.2. *In vivo mammalian cell micronucleus test*

As shown in Table 2, there was no significant change in PCE/RBC ratios in the MLE treatment groups compared with the control group, indicating that MLE was not cytotoxic to bone marrow through oral exposure. The micronucleus frequency in the positive control groups was significantly higher than that in the negative control group ($p < 0.05$) for both male and female animals. By contrast, no significant differences were encountered in micronucleus frequency between all MLE groups and the negative control groups ($p > 0.05$). The results indicated that MLE was not mutagenic to somatic chromosomes in mouse.

Table 2 *In vivo* mouse bone marrow micronucleus assay conducted with mulberry leaf extract.

Sex	Dose (g/kg bw)	PCE analysis		Micronucleus analysis	
		PCE counts (counts / each)	PCE / RBC (%)	Micronucleus counts (counts / each)	Micronucleus frequency (%)
Female	0	108.2 \pm 6.1	54.1	1.6 \pm 1.1	1.6 \pm 1.1
	2.5	107.4 \pm 4.5	53.7	2.0 \pm 1.0	2.0 \pm 1.0
	5	108.2 \pm 4.0	54.1	1.8 \pm 1.5	1.8 \pm 1.5
	10	109.2 \pm 4.8	54.6	1.4 \pm 1.5	1.4 \pm 1.5
	Cyclophosphamide ^a 40mg/kg bw	92.4 \pm 7.3	46.2	15.6 \pm 4.6	15.6 \pm 4.6**
Male	0	107.8 \pm 6.1	53.9	1.4 \pm 1.1	1.4 \pm 1.1
	2.5	109.0 \pm 6.5	54.5	1.6 \pm 0.9	1.6 \pm 0.9
	5	107.8 \pm 6.0	53.9	1.4 \pm 1.1	1.4 \pm 1.1
	10	108.0 \pm 3.5	54.0	2.0 \pm 1.6	2.0 \pm 1.6
	Cyclophosphamide ^a 40 mg/kg bw	94.6 \pm 7.8	47.3	16.2 \pm 6.1	16.2 \pm 6.1**

** Statistically significant difference from control values ($P < 0.01$)

Abbreviations: PCE= Polychromatic Erythrocytes; RBC= Red Blood Cells

^a positive control

3.1.3. *Mouse sperm malformation assay*

As shown in Table 3, the sperm malformation rate in the positive control group was significantly higher than that in the negative control group ($p < 0.05$). The differences in sperm malformation rate between each MLE group and negative control group, however, showed no statistical significance ($p > 0.05$) (Table 3), indicating that MLE had no adverse effect on spermatozoa generation in mouse. Likewise, there was no indication of any specific sperm abnormality under MLE treatment.

Table 3 Mouse sperm abnormality test conducted with mulberry leaf extract.

Dose (g/kg bw)	Number of various types of sperm malformation (% of total malformations)					Total number of malformation	Malformation incidence (%)
	Amorphous	Hooklets	Big head	Banana-shaped head	Other		
H ₂ O	39(45.9)	27(31.8)	17(20.0)	2(2.3)	0(0.0)	17.0 ± 3.7	1.70
2.5	35(43.2)	26(32.1)	19(23.5)	1(1.2)	0(0.0)	16.2 ± 3.1	1.62
5	40(44.4)	31(34.4)	17(18.9)	2(2.3)	0(0.0)	18.0 ± 2.9	1.80
10	38(43.7)	27(31.0)	19(21.8)	3(3.5)	0(0.0)	17.4 ± 2.1	1.74
CP 40mg/kg bw ^a	85(43.4)	65(33.2)	36(18.4)	7(3.6)	3(1.4)	39.2 ± 3.1	3.92**

** Statistically significant difference from control values ($P < 0.01$)

^a positive control

Taken together, the results of the *in vivo* micronucleus and sperm abnormality assays showed no evidence of genotoxic activity of MLE either in somatic or germ cells.

3.2. Rat studies

3.2.1. Acute toxicity

Animals did not show any signs of toxicity-induced symptoms during daily monitoring and no mortality was recorded. Body weights before and after 14 days test were showed in Table 4. The test result indicated that the LD₅₀ of the mulberry leaf extract was greater than 15.0 g/kg bw.

Table 4 Weights of rats treated orally with mulberry leaf extract in the acute toxicity study.

Sex	Weight before Test (g)	Weight after Test (g)	LD ₅₀ (g/kg bw)
Female	192.8±5.1	230.2±6.6	>15.0
Male	204.6±9.1	286.2±16.1	>15.0

Values are the Mean ± SD (n = 10).

3.2.2. Thirty-day feeding study

No death or treatment related toxic signs were detected during the 30-day feeding study. As shown in Table 6, the decrease in feeds intake in the females treated with 3.75 g/kg bw MLE was significant compared with that in the control group during the second week of the test ($P < 0.05$). This value was within the historical records of the testing lab

and was considered as having no biological significance. The rest of the animals (both sexes) in the test groups showed no significant difference in body weight, food intake and efficiency of food utilization compared with the control group ($P > 0.05$) as showed in Table 5-8.

Table 5 Effects of MLE on body weight gain of rats treated orally with mulberry leaf extract for 30 days.

Sex	Dose (g/kg bw/d)	Initial Weight (g)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)
Female	0	97.0 ± 10.7	128.9 ± 6.7	159.0 ± 8.8	181.9 ± 11.8	203.6 ± 15.3
	1.88	92.8 ± 8.1	125.8 ± 7.6	154.3 ± 9.4	177.3 ± 13.5	197.6 ± 16.4
	3.75	96.2 ± 7.4	128.3 ± 5.4	159.2 ± 10.2	179.9 ± 11.4	204.0 ± 13.3
	7.5	95.8 ± 4.6	128.1 ± 11.6	160.2 ± 15.4	180.2 ± 18.7	201.2 ± 22.7
Male	0	89.6 ± 6.4	159.0 ± 18.9	224.1 ± 22.6	282.2 ± 26.0	326.5 ± 24.7
	1.88	88.9 ± 5.6	151.3 ± 13.1	215.1 ± 15.4	269.5 ± 31.8	315.2 ± 39.1
	3.75	89.0 ± 6.5	153.0 ± 14.0	218.7 ± 14.1	273.7 ± 17.6	321.9 ± 24.2
	7.5	89.4 ± 5.8	148.8 ± 6.4	210.0 ± 10.2	267.1 ± 12.0	314.8 ± 15.4

Values are the Mean ± SD (n = 10).

Table 6 Effects of MLE on food consumption of rats treated orally with mulberry leaf extract for 30 days.

Sex	Dose (g/kg bw/d)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)
Female	0	115.2 ± 9.0	129.6 ± 10.2	125.9 ± 11.7	132.7 ± 16.3
	1.88	109.3 ± 10.6	119.3 ± 10.0	120.3 ± 9.4	125.1 ± 9.0
	3.75	107.0 ± 9.9	115.4 ± 11.8*	116.7 ± 8.8	123.7 ± 9.0
	7.5	105.4 ± 13.0	119.7 ± 12.4	115.4 ± 9.9	119.7 ± 12.1
Male	0	127.2 ± 15.5	172.1 ± 16.3	184.8 ± 14.2	193.5 ± 10.1
	1.88	126.9 ± 13.4	158.4 ± 20.9	179.6 ± 20.6	182.7 ± 23.5
	3.75	128.7 ± 11.6	163.0 ± 13.1	172.4 ± 35.2	188.2 ± 15.0
	7.5	119.7 ± 9.4	155.4 ± 14.6	173.5 ± 10.8	179.6 ± 13.9

Values are the Mean ± SD (n = 10).

* Statistically significant difference from control values ($P < 0.05$).

Table 7 Effects of MLE on food weekly feed consumption of rats treated orally with mulberry leaf extract for 30 days

Sex	Dose (g/kg bw/d)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)
Female	0	27.1 ± 11.6	23.2 ± 4.0	18.1 ± 3.6	16.2 ± 3.3
	1.88	30.1 ± 8.9	24.0 ± 6.0	18.9 ± 4.9	16.2 ± 2.7
	3.75	30.2 ± 4.3	26.5 ± 3.9	17.8 ± 3.3	19.4 ± 2.6
	7.5	30.4 ± 5.8	26.7 ± 3.9	17.1 ± 5.6	17.4 ± 3.7
Male	0	54.3 ± 11.2	37.9 ± 2.4	31.4 ± 2.8	23.1 ± 3.2
	1.88	48.8 ± 6.8	41.0 ± 9.4	29.5 ± 10.7	24.9 ± 6.0
	3.75	49.4 ± 4.6	40.4 ± 4.3	32.2 ± 3.0	25.7 ± 5.3
	7.5	49.5 ± 4.7	39.5 ± 3.0	32.9 ± 1.5	26.5 ± 5.2

Values are the Mean ± SD (n = 10).

Table 8 Effects of MLE on overall food utilization efficiency of rats treated orally with mulberry leaf extract for 30 days

Sex	Dose (g/kg bw/d)	Body weight gain (g)	Total food intake (g)	Total food utilization rate (weight gain as % of total food intake)
Female	0	106.6 ± 24.7	503.4 ± 42.9	21.0 ± 3.7
	1.88	104.8 ± 18.9	474.0 ± 26.6	22.1 ± 3.5
	3.75	107.8 ± 13.4	462.8 ± 37.8	23.3 ± 2.1
	7.5	105.5 ± 20.0	460.3 ± 42.0	22.8 ± 2.5
Male	0	237.0 ± 23.0	677.6 ± 49.4	35.0 ± 2.2
	1.88	226.2 ± 39.8	647.6 ± 65.7	34.7 ± 3.5
	3.75	232.9 ± 23.0	652.3 ± 51.6	35.7 ± 1.8
	7.5	225.3 ± 15.6	628.2 ± 42.3	35.9 ± 1.4

Values are the Mean ± SD (n = 10).

Result of hematology parameters (Table 9) showed that there was no significant change at all treatment doses of MLE on both sexes when compared with their respective control group ($P > 0.05$).

As showed in Table 9, ALT levels in female rats of the 7.5 g/kg bw dose group were significantly lower than those in the control group ($P < 0.05$). This statistically significant difference in clinical chemistry parameters fell within the historical control range of the testing laboratory and showed no clear-dose response relationships. Other hematology parameters showed no differences.

Table 9 Effects of MLE on blood biochemistry and hematological parameters in rats following 30 days of treatment.

Parameter	Group (Female)				Group (Male)			
	Control	1.88 g/kg bw/d	3.75 g/kg bw/d	7.5 g/kg bw/d	Control	1.88 g/kg bw/d	3.75 g/kg bw/d	7.5 g/kg bw/d
WBC ($10^9/L$)	9.38 \pm 1.98	10.27 \pm 3.08	10.35 \pm 2.01	12.00 \pm 2.38	12.48 \pm 4.36	12.42 \pm 3.17	11.49 \pm 3.73	12.47 \pm 3.15
RBC ($10^{12}/L$)	7.66 \pm 0.51	7.71 \pm 0.54	7.70 \pm 0.55	7.80 \pm 0.43	7.69 \pm 0.55	8.13 \pm 0.53	7.78 \pm 0.36	7.84 \pm 0.14
Hemoglobin (g/L)	153.10 \pm 6.87	151.70 \pm 7.33	149.80 \pm 7.02	153.50 \pm 6.50	149.80 \pm 8.40	154.90 \pm 7.77	150.70 \pm 4.16	152.90 \pm 4.04
Lymphocytes (%)	83.74 \pm 3.30	80.58 \pm 6.01	80.97 \pm 5.44	82.33 \pm 6.88	83.25 \pm 3.04	84.22 \pm 5.58	82.10 \pm 3.78	85.32 \pm 2.93
Other cells (%)	5.43 \pm 0.69	5.36 \pm 1.81	5.52 \pm 1.46	4.29 \pm 0.81	6.16 \pm 1.35	7.43 \pm 1.83	7.57 \pm 1.86	6.10 \pm 2.06
Neutrophils (%)	10.10 \pm 2.28	11.99 \pm 4.55	11.46 \pm 3.93	11.57 \pm 5.46	11.32 \pm 2.78	10.42 \pm 4.09	12.38 \pm 3.45	10.39 \pm 2.61
ALT (U/L)	50.80 \pm 5.05	47.60 \pm 6.50	51.40 \pm 7.41	43.60 \pm 3.47*	53.30 \pm 7.09	47.90 \pm 8.95	46.40 \pm 8.10	46.50 \pm 5.28
AST(U/L)	287.70 \pm 57.50	272.10 \pm 54.92	284.60 \pm 37.19	258.90 \pm 18.68	310.30 \pm 51.28	280.10 \pm 59.44	306.50 \pm 51.51	259.60 \pm 44.57
BUN (mmol/L)	6.63 \pm 0.67	6.57 \pm 0.46	6.69 \pm 0.52	6.59 \pm 0.51	6.58 \pm 0.53	6.28 \pm 0.37	6.70 \pm 0.91	6.36 \pm 1.15
CRE (μ mol/L)	75.76 \pm 5.79	70.54 \pm 8.31	69.39 \pm 7.84	67.38 \pm 7.71	63.72 \pm 6.36	61.78 \pm 5.97	63.13 \pm 8.45	60.54 \pm 5.71
CHOL (mmol/L)	2.42 \pm 0.75	2.36 \pm 0.59	2.48 \pm 0.59	2.52 \pm 0.43	2.07 \pm 0.29	2.25 \pm 0.31	2.18 \pm 0.38	2.13 \pm 0.25
TG (mmol/L)	0.74 \pm 0.28	0.59 \pm 0.13	0.82 \pm 0.22	0.84 \pm 0.26	0.59 \pm 0.12	0.66 \pm 0.18	0.57 \pm 0.16	0.67 \pm 0.22
BG (mmol/L)	2.41 \pm 0.74	2.24 \pm 0.79	1.92 \pm 0.45	2.02 \pm 0.48	1.92 \pm 0.46	2.04 \pm 1.00	1.74 \pm 0.42	2.02 \pm 0.59
TPROT (g/L)	69.73 \pm 5.20	69.97 \pm 7.95	72.70 \pm 8.68	69.88 \pm 7.90	69.54 \pm 4.08	66.32 \pm 6.48	70.83 \pm 6.00	71.80 \pm 7.91
ALB (g/L)	40.55 \pm 3.41	40.93 \pm 5.88	42.52 \pm 6.03	40.03 \pm 4.74	37.35 \pm 2.54	36.30 \pm 4.52	38.90 \pm 5.78	38.52 \pm 3.47

Values are the Mean \pm SD (n = 10).

* Statistically significant difference from control values (P < 0.05).

Abbreviations: WBC = White Blood Cells; RBC = Red Blood Cells; ALT = Alanine Aminotransferase; AST = Aspartate Aminotransferase; BUN = Urea Nitrogen; CRE = Creatinine; CHOL = Cholesterol; TG = Triglycerides; BGL = Blood Glucose; TPROT = Total Proteins; ALB = Albumin.

253 As showed in Table 10, the kidney weight and the organ to body weight ratio in female rats of the 7.5 g/kg bw
254 dose group were significantly higher than those in the control group ($P<0.05$), the liver to body weight ratio in the male
255 rats of the 3.75 and 7.5 g/kg bw dose groups were significantly higher than those of the control group ($P<0.05$) and the
256 spleen weight and the spleen to body weight ratio in male rats of the 1.88, 3.75 and 7.5 g/kg bw dose groups were
257 significantly lower than those of the control group ($P<0.05$). But these changes were non-dose-dependent, and were all
258 within the range of the historical records of the testing lab, so these changes were not considered as biological
259 significant. Compared with the control group, there were no significant differences in body weight before sacrifice,
260 liver, kidney, spleen and testis weight as well as those organ to body weight ratio ($P>0.05$).

Table 10 Organ weights and relative organ weights (g/100 g of body weight) of rats treated orally with MLE.

Parameter	Group (Female)				Group (Male)			
	Control	1.88 g/kg bw/d	3.75 g/kg bw/d	7.5 g/kg bw/d	Control	1.88 g/kg bw/d	3.75 g/kg bw/d	7.5 g/kg bw/d
Weight when killed (g)	186.3 ± 11.7	181.7 ± 15.5	188.7 ± 13.4	186.7 ± 21.7	299.6 ± 26.1	290.4 ± 32.3	285.6 ± 24.9	289.8 ± 17.5
Liver								
g	6.22 ± 0.63	5.90 ± 0.69	6.36 ± 0.58	5.93 ± 0.98	9.07 ± 0.79	8.85 ± 0.92	9.23 ± 0.89	9.44 ± 0.93
g/100g	3.34 ± 0.29	3.24 ± 0.19	3.37 ± 0.20	3.18 ± 0.39	3.04 ± 0.22	3.05 ± 0.11	3.23 ± 0.10*	3.26 ± 0.22*
Spleen								
g	0.48 ± 0.08	0.50 ± 0.11	0.45 ± 0.05	0.43 ± 0.10	0.73 ± 0.09	0.57 ± 0.10*	0.61 ± 0.08*	0.61 ± 0.05*
g/100g	0.26 ± 0.04	0.28 ± 0.06	0.24 ± 0.03	0.23 ± 0.03	0.24 ± 0.02	0.20 ± 0.03*	0.22 ± 0.04*	0.21 ± 0.02*
Kidney								
g	1.55 ± 0.09	1.54 ± 0.14	1.64 ± 0.15	1.73 ± 0.21*	2.55 ± 0.24	2.50 ± 0.28	2.55 ± 0.29	2.50 ± 0.17
g/100g	0.83 ± 0.05	0.85 ± 0.05	0.87 ± 0.05	0.93 ± 0.05*	0.85 ± 0.05	0.86 ± 0.06	0.89 ± 0.06	0.86 ± 0.04
Testis								
g	-	-	-	-	3.06 ± 0.30	3.05 ± 0.25	2.80 ± 0.24	2.96 ± 0.22
g/100g	-	-	-	-	1.02 ± 0.08	1.06 ± 0.11	0.98 ± 0.05	1.02 ± 0.08

Values are the Mean ± SD (n = 10).

* Statistically significant difference from control values (P < 0.05).

Gross necropsy and histopathological examinations showed no abnormal changes during the macroscopic analysis of the organs and tissues. The samples of liver, kidney, spleen, stomach, duodenum and testis or ovary tissues, obtained from 10 males and 10 females of the control and high-dose groups, revealed no changes associated with MLE treatment. Only a few animals showed spotty necrosis of the hepatocytes (1/20 cases in control group, 1/20 cases in high-dose group) and focal necrosis of the liver cells (2/20 cases in control group, 0/20 cases in high-dose group). For kidneys, some animals showed renal tubular calcium deposits (1/20 cases in the control, 0/20 cases in high-dose group). Spleen from some animals showed splenic sinus with slight dilation and congestion. Given the fact that the above pathological changes in liver, kidneys and spleen are common lesions in these animals, and the differences between high-dose group and control group had no statistical significance, these changes were considered to be unrelated to MLE treatment. There were no significant pathological changes induced by MLE observed in the liver, kidney, spleen, stomach, duodenum, testis and ovary.

4. Discussion

Mulberry leaf has been reported in the literature for its anti-oxidative, antiobesity, anti-cancerous, and anti-inflammatory activity, especially for its effect to reduce the risk of type 2 diabetes (Lim et al., 2013; Riviere et al., 2014; Shin et al., 2016; Zhang et al., 2017). However, literature is sparse on regulatory toxicological evaluation on mulberry leaf extracts. The pioneer of the toxicology studies either used extracts prepared with ethanol, different in the amount of certain active ingredients, or adopted lower doses tested (Marx et al., 2016; Oliveira et al., 2016) compared with this study. In the current study, we conducted comprehensive investigation of the toxicity of mulberry leaf extracts both *in vivo* and *in vitro*. The results demonstrated that, the aqueous extract of mulberry leaves have no adverse effects in the genotoxicity study, acute and subacute oral toxicity study. These data provided supportive evidence for the safety of mulberry leaves that may be used in the form of food or dietary supplements.

1-Deoxynojirimycin (DNJ) is one of the major active compounds in mulberry leaf extracts (Kim et al., 2010; Kimura et al., 2007; Tao et al., 2010). DNJ is an alkaloid azasugar or iminosugar which has been proved to possess antidiabetic (Do et al., 2015; Hu et al., 2017), anti-obesity, and antiviral features (Gao et al., 2016). It is proved that mulberry leaf water extracts contained more DNJ and fewer phytochemicals than ethanol extracts, and water extracts

showed higher inhibitory activity of glucose uptake (Ji et al., 2013). Currently, available information concerning the toxicity of DNJ is very rare. It is only reported that iminosugars are toxic to some insects (Hori et al., 2014). In dried mulberry leaves, the DNJ concentration is about 0.1% (Gao et al., 2016), and in MLE we tested its concentration is up to 1%. Though the natural mulberry leaf has a long history of consumption, indicating mulberry leaf was relatively safe for human use, we are still unaware of safety profile of the extract containing higher amount of DNJ. In the present study, the DNJ-rich extract did not exhibited genotoxicity or subacute toxicity.

The extract of mulberry leaf with 50% ethanol was studied toxicologically in male and female SD rats for 90 days (Miyazawa et al., 2003), and the results indicate that dietary intake of 1% mulberry leaf extract for 90 days (884.5 mg/kg/d for males and 995.7 mg/kg/d for females) causes no toxicological change in rats. The extracts were made with ethanol, so the ingredients may be different from the current study using water. A 28-day repeated dose toxicological study of an aqueous extract of mulberry leaves was conducted, and no toxicologically relevant abnormalities were observed at doses up to 4000 mg/kg (bw)/d (Marx et al., 2016). In the present study, the DNJ content is twice as much as the extract discussed above, and higher doses were tested, which is meaningful for the safety assessment. The studies above proved the safety use of mulberry leaf extracts prepared in various methods, and the current study act as a vital complementary addition for the mulberry leaf products. In this study, MLE was subject to an acute oral toxicity test, and results demonstrated that the LD₅₀ of MLE in rats was greater than 15 g/kg bw. The results of subacute toxicity experiment showed no evidence of any untoward effects of dietary exposure to MLE at doses of up to 7.5 g/kg bw/d for 30 days. Several statistically significant changes in haematology and clinical chemistry were not considered to be of toxicological relevance since the changes were all small in nature and fell within the historical control range of the testing laboratory and/or showed no clear dose-response relationship. A few statistically significant differences in absolute and/or relative organ weight values were recorded, but all again were of minor magnitude, generally limited to a single sex, within the historical control ranges, and there were no histological or clinical pathology correlates. The results of the 30-day study provided no indication of any safety concerns of MLE. The no-observable-adverse-effect level (NOAEL) was considered to be 7.5 g/kg bw/d, the highest dietary dose tested.

An ethanol extract of *Morus alba* L. (Moraceae) with highly inhibitory effect against acute inflammation was evaluated for acute toxicity and genotoxicity in mice (Oliveira et al., 2016). The result showed that oral administration

of the extract did not result in genotoxicity, but intraperitoneal injection caused several forms of damage to the mice. Though the extract made with ethanol may contain different ingredients compared with the water extract, the results of the oral test are still consistent with the negative results obtained in the current study of the Ames assay and in the *in vivo* mouse micronucleus and sperm abnormality assays. The results of the present series of genotoxicity studies on MLE showed no evidence of mutagenic or clastogenic effects in either somatic or germ cells. The Ames test was negative in all tester strains to the limit dose of 5 mg/plate. There was no indication of micronucleus induction in the bone marrow cells of mice treated at up to 10 g/kg bw dose level, and there were no effects on the incidence of sperm abnormalities in mice treated at up to 10 g/kg bw. What's more, *Morus alba* and *Morus nigra* leaf extracts, and their mixtures were reported to have genoprotective properties, since they decreased the mutability level induced with chemical mutagens, gamma-rays and aging in the plants and rats cells (Kesuma and Norio, 2012).

In summary, the negative results of the acute toxicity study and a 30 day feeding study together with the negative genotoxicity results for MLE, support the safety use of this product for potential dietary consumption by humans, either in the form of food or dietary supplements.

Conflict of interest

The authors declare that there are no conflicts of interest.

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- ▶ MLE showed no acute toxicity ($LD_{50} > 15.0$ g/kg bw)
- ▶ MLE showed no subacute toxicity (NOALE=7.50 g/kg bw/d)
- ▶ MLE showed no genotoxicity activity
- ▶ Aqueous extract of mulberry leaf was considered safe for dietary consumption