

A 28-Day Repeated Dose Toxicological Study of an Aqueous Extract of *Morus Alba* L.

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Abstract

Morus alba L. (white mulberry) leaves are one of the oldest recognized traditional Chinese medicines. More recently, *M alba* leaves and their constituents, particularly iminosugars (or azasugars), have garnered attention for their ability to maintain normal blood glucose concentrations, an effect identified in both animal studies and human clinical trials. Reducose (Phynova Group Limited) is a commercial water-soluble extract of *M alba* leaves standardized to 5% 1-deoxynojirimycin (DNJ), an iminosugar with α -glucosidase inhibition properties. Although there is an extensive history of consumption of *M alba* leaves by humans and animals worldwide, suggesting that the leaves and their extracts have a relatively good safety profile, we are unaware of safety assessments on an extract containing a higher amount of DNJ than that occurs naturally. The current 28-day repeated dose oral toxicity study in rats, conducted according to Organisation for Economic Co-operation and Development guidelines, was carried out to assess the safety of Reducose. Male and female Hsd.Han Wistar rats (4 groups of 10 animals/sex) were administered Reducose via gavage at doses of 0, 1,000, 2,000 and 4,000 mg/kg body weight (bw)/d. No treatment-related mortality or adverse effects (per clinical observations, body weight/weight gain, food consumption, ophthalmoscopy, clinical pathology, gross pathology, organ weights, or histopathology) were observed, and no target organs were identified. The no observed adverse effect level was determined to be 4,000 mg/kg bw/d for both male and female rats, the highest dose tested.

Keywords

Morus Alba Linn., Moraceae, safety assessment, toxicity, Reducose, NOAEL, white mulberry, iminosugar, azasugar, GLP

Introduction

Morus alba L., commonly known as white mulberry, is an agroforestry tree of the Moraceae family, native to Asia and widely distributed in tropical, subtropical, and temperate regions.¹ Most notably, *M alba* is recognized for its vital role in sericulture, having been domesticated thousands of years ago for this purpose.² *Morus alba* leaves are used as the sole nutritional source for the silkworm (*Bombyx mori*) and are known to enhance the growth and development of the caterpillar and increase the quality of their cocoons, thereby improving silk production.^{3,4} In addition to their role in sericulture, *M alba* leaves are utilized worldwide as feed for livestock, including cattle, sheep, goats, pigs, hens, and rabbits (including pregnant animals), at levels up to 75% of the diet without safety concerns.^{2,5-7}

Also one of the world's oldest medicinal plants, its use first described in Divine Husbandman's Materia Medica (the earliest known Chinese pharmacopoeia).⁸ *Morus alba* (particularly the leaves) is a traditional medicine of China. Various ethnomedicinal uses for the leaves have been documented in Japan, Chile, Spain, Turkey, Yugoslavia, Peru, France, and South Asia.⁸⁻¹⁰ In recent years, *M alba* leaves and their constituents are garnering more interest, primarily due to the evidence of their antihyperglycemic properties in humans.¹¹⁻¹⁶

Dried leaves of *M alba* are composed mainly of fiber, carbohydrates, protein, and ash and are rich in β -carotene, ascorbic acid, iron, calcium, and zinc.¹⁷ Constituents also include various polyhydroxy alkaloids, stilbenoids (such as resveratrol and oxyresveratrol), flavonoids (including quercetin and kaempferol), and of particular interest medicinally, iminosugars (also known as azasugars).^{18,19} Iminosugars are carbohydrate mimetics, or sugar analogues, possessing an atom of nitrogen rather than oxygen in the ring system template of carbohydrates or their hydrolysis transition states. These small polar molecules are chemically similar to mono- and disaccharides but possess special attributes, including chemical stability and potentially a wide range of biological targets that have intrigued those involved with therapeutic development.²⁰

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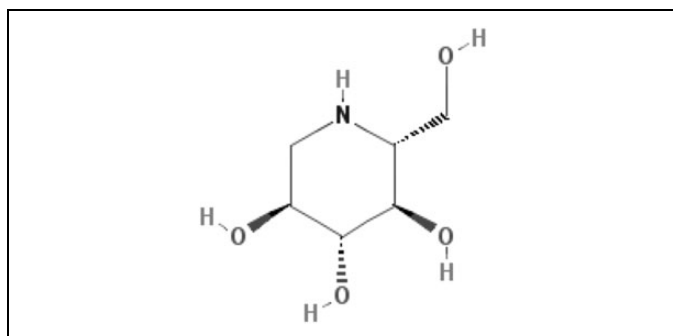


Figure 1. Chemical Structure of 1-deoxyjirimycin (DNJ)

The most predominant iminosugar in *M alba* is the piperidine alkaloid 1-deoxyjirimycin (DNJ) (CAS# 19130-96-2), a D-glucose analogue with an amine group replacing the oxygen on the pyranose ring (see Figure 1).^{21,22} As an α -glucosidase inhibitor, DNJ is thought to lower blood glucose levels by interacting with carbohydrate-processing enzymes.¹⁹ However, the amount of naturally occurring DNJ in *M alba* is relatively low with dried mulberry species leaves containing approximately 1.4 to 3.5 mg/g DNJ.¹⁹

Reduceose 5% (formerly IminoNorm 5%), produced by Phynova Group Limited, is an iminosugar-rich aqueous extract of *M alba* with a concentrated DNJ amount of 5% by weight. A previous acute oral toxicity study conducted on Reduceose 5% (unpublished data) determined that the LD₅₀ of the water extract was greater than 5 g/kg body weight (bw) in ICR mice. A similar *M alba* extract containing 1% DNJ (Reduceose 1%), also produced by Phynova Group Limited, was assessed in a battery of good laboratory practice (GLP) toxicological studies conducted in a China National Centre for Food Safety Risk Assessment. This unpublished assessment was comprised of genotoxicity (bacterial reverse mutation, bone marrow micronucleus, and sperm deformity tests), 14-day repeated dose (gavage), and 30-day repeated dose (dietary admix) oral toxicity studies. It was concluded that the test article was not mutagenic or genotoxic in a bacterial reverse mutation (levels up to 5,000 μ g/plate in 4 strains of *Salmonella typhimurium* with and without metabolic activation), bone marrow mouse micronucleus (Kunming SPF mice; oral dose of up to 10 g/kg bw twice at an interval of 24 hours), and sperm deformity (Kunming SPF mice; oral dose of up to 10 g/kg bw/d for 5 days) studies. The 14-day repeated dose oral toxicity study conducted in Sprague Dawley rats concluded that the maximum tolerated dose (MTD) was greater than 15 g/kg bw. The 30-day repeated dose oral toxicity study in Sprague Dawley rats utilized levels of 1.88, 3.75 and 7.5 g/kg bw/d, and although slight blood chemistry and organ weight changes were noted, these changes were not considered to be test article related. Therefore, the NOAEL was determined to be 7.5 g/kg bw/d, the highest dose tested. Numerous other published studies on various *M alba* leaf extracts, including genotoxicity, mutagenicity, and acute oral toxicity studies, as well as a subchronic repeated dose oral toxicity study on silkworm powder extract (containing 1.25%

DNJ) have also demonstrated a general lack of safety concerns at doses of up to 5,000 mg/kg bw/d in rodents.²³⁻³¹

Although available toxicological data suggest that *M alba* leaves and leaf extracts have a relatively good safety profile and that *M alba* leaves are recognized as a highly digestible, highly palatable, nutrient-rich feed for various animal species without toxic effects, until now, we are unaware of any safety assessments on an extract containing a more concentrated DNJ content.²

Material and Methods

Test Article

The test article was Reduceose 5%, a commercial aqueous extract of the leaves of *M alba* Linn. standardized to 4.5% to 5.5% DNJ as determined by high-performance liquid chromatography. The raw leaf material for the extract is sourced from China, a geographical location known to produce high levels of iminosugars. The raw material is analyzed for DNJ content, heavy metals, pesticide residues, yeast, and molds and is then air-dried by the raw material supplier. The dried mulberry leaf then undergoes a water extraction and ion exchange chromatography to enrich the alkaloid components. The eluent is reduced under vacuum to allow optimum spray drying. The remaining components of Reduceose 5% include additional iminosugars (2%-3%), carbohydrates (27%-29%), amino acids (14%-15%), and maltodextrin as the carrier material (48%-52%).

Reduceose 5% (batch number IM150522), a water-soluble, light brown powder with a characteristic odor and slightly sour taste, containing 4.65% DNJ was provided to Toxi-Coop Zrt (Budapest, Hungary) along with specifications, a certificate of analysis, and MSDS by the sponsor of the studies (Phynova Group Limited, Oxford, United Kingdom).

Twenty Eight-Day Repeated Dose Oral Toxicity Study in Rats

This 28-day repeated dose oral toxicity study was conducted to characterize the toxicity profile of Reduceose 5%, the test article. The study was conducted in compliance with GLP and internationally accepted guidelines Organisation for Economic Co-operation and Development (OECD) 407³² and *US FDA Redbook 2000*, IV.C.3.a.,³³ and the care and use of study animals was in compliance with laboratory standard operating procedures under the permission of the laboratory's Institutional Animal Care and Use Committee, the National Research Council Guide for Care and Use of Laboratory Animals,³⁴ and the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act 1998 XXVIII) regulating animal protection.

Test article doses were prepared daily as a solution by dissolving Reduceose 5% in distilled water (vehicle) to achieve concentrations of 100, 200, and 400 mg/mL (approximately 5, 10 and 20 mg/mL DNJ) in order to provide a constant dosing volume of 10 mL/kg bw. Stability data conducted by the

manufacturer have indicated that DNJ is stable in an aqueous (water) solution for up to 3 months. Doses were prepared by careful weight measurement and administered within 4 hours of preparation. The negative control groups received the same volume of the distilled water vehicle only.

Specific pathogen-free male and female Hsd.Han Wistar rats (Toxi-Coop) were housed individually with a 12-hour light–dark cycle at $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and 30% to 70% relative humidity in type II polypropylene/polycarbonate cages with Lignocel certified laboratory wood bedding (J. Rettenmaier & Sohne GmbH+Co.KG; D-73494 Rosenberg). Animals received ssniff SM R/M-Z+H complete diet for rats and mice (ssniff Spezialdiäten GmbH, Soest, Germany) and potable tap water ad libitum.

At the start of the experimental period, animals were 37 to 40 days old and weighed 131 to 162 g (males) and 103 to 121 g (females). Eighty rats were stratified by weight and randomly assigned to 4 groups of 10 rats/sex/group. The test article was administered by gavage in doses of 0 (vehicle-control), 1,000, 2,000, and 4,000 mg/kg bw/d (approximately 50, 100 and 200 mg/kg bw/d of DNJ) for 28 days (males) or 29 days (females).

Because the available literature on *M alba* and the test article itself does not specifically suggest toxicity of the extract and taking into account the conclusion of the 14-day repeated dose oral toxicity study of an MTD >15 g/kg bw/d, the high dose for the 28-day study was set at 4,000 mg/kg bw/d. Additional doses were selected using a graduated declining dose schedule to include 3 treatment groups in order to encompass the broadest reasonable range of doses. Doses were selected with the aim of inducing toxic effects but no mortality or life-shortening toxicity at the highest dose and a NOAEL at the lowest dose.

Animals were observed twice daily for morbidity and mortality, and general cage-side observations for clinical signs were made twice during the acclimation period and once daily after administration of the test article. Detailed clinical observations were conducted on the day prior to the first test article administration and once weekly during the experimental period. A functional observational battery was performed during the final week to assess parameters such as general physical condition and behavior, sensory reactions to various stimuli, grip strength, and motor activity using a modified Irwin test.³⁵ Individual body weights were recorded twice during the acclimation period, on the first experimental day prior to treatment, twice weekly during weeks 1 to 4, and immediately prior to sacrifice. Food intake was determined and food efficiency calculated once weekly (body weight gain/food consumption; calculated using SPSS PC+ statistical software). Ophthalmological examination (using Humapent 5 mg/mL eye drops; TEVA Pharmaceutical Works Private Ltd. Co, Gödöllő, Hungary) was carried out on all animals prior to the experimental period and prior to the study termination in control and high-dose group animals.

After an overnight fast (approximately 16 hours) following final administration of the test article, blood samples were collected from the retro-orbital venous plexus under Isoflurane

CP anesthesia (CP-Pharma Hadneltsgesellschaft GmbH, Ostlandring, Burgdorf, Germany), after which the animals were euthanized by exsanguination. Blood samples were analyzed for hematologic, blood coagulation, and clinical chemistry parameters. Gross pathological examinations were conducted on male animals on day 28 and on female animals on day 29, and absolute and relative organ weights were determined on all animals. Full histopathological examinations were conducted on preserved organs (as specified by OECD guidelines) of all animals of the control and high-dose groups.

Statistical Analyses

Statistical analyses were conducted using SPSS PC+ software (version 4; SPSS, Inc, Chicago, Illinois). Bartlett homogeneity of variance test was used to assess heterogeneity of variance between groups. One-way analysis of variance (ANOVA) was performed if no significant heterogeneity was detected, and Duncan multiple range test was used to assess the significance of intergroup differences if a positive ANOVA result was obtained. Where significant heterogeneity was detected by Bartlett test, the Kolmogorov-Smirnov test was performed to examine normally distributed data. Kruskal-Wallis nonparametric 1-way ANOVA, followed by the Mann-Whitney *U* test for intergroup comparisons of positive results, was used in the case of a nonnormal distribution. A *P* value of $<.05$ was considered statistically significant, and statistically significant results were reported at the $P < .05$ and $P < .01$ levels.

Results

No mortality was observed in any group throughout the study. Daily and weekly detailed clinical and functional observations of the animals did not reveal any toxicologically relevant abnormalities. Slight salivation occurred in 3 female rats in the 4,000 mg/kg bw/d group appearing shortly after administration and ceasing approximately half an hour thereafter. Scarring and alopecia on the neck were noted for a single male in the 2,000 mg/kg bw/d group from day 18 until the end of the observational period.

No test item–related changes were noted in body weight (see Supplemental Figures 1a and 1b), body weight gain, daily food consumption, or feed efficiency in male or female rats at any dose level. A statistically significant decrease was noted in mean body weight gain in female animals at 4,000 mg/kg bw/d compared to controls between days 18 and 21. Mean daily food consumption was slightly but significantly lower compared to controls in female animals in the 4,000 mg/kg bw/d group during the first week of the study, and a slightly higher feed efficiency was noted in the same animals during week 2. Additionally, a lower feed efficiency was noted in female and male animals in the 4,000 mg/kg bw/d group during weeks 3 and 4, respectively.

No alterations were noted in ophthalmoscopic examinations. Slight, statistically significant changes were noted in various hematology and clinical chemistry parameters in both

Table 1. Summary of Hematological Findings.^a

Group (mg/kg bw/d)	WBC × 10 ⁹ /L	NEU %	LYM %	MONO %	EOS %	BASO %	RBC × 10 ¹² /L	HGB g/L	HCT L/L	MCV fL	MCH pg	MCHC g/L	PLT × 10 ⁹ /L	RET %	PT Seconds	APTT Seconds
Male (n = 10/group)																
Control	11.26 ± 2.46	10.24 ± 2.80	86.78 ± 3.54	2.26 ± 0.80	0.64 ± 0.37	0.08 ± 0.04	8.97 ± 0.24	167.90 ± 4.82	0.457 ± 0.015	51.00 ± 1.84	18.74 ± 0.64	367.30 ± 3.02	922.00 ± 96.32	4.83 ± 0.53	22.11 ± 1.15	18.63 ± 2.85
1,000	9.63 ± 1.87	13.13 ± 3.04	84.04 ± 3.15	1.96 ± 0.44	0.81 ± 0.32	0.06 ± 0.05	8.88 ± 0.49	169.40 ± 4.20	0.459 ± 0.011	51.75 ± 2.64	19.11 ± 0.92	369.20 ± 2.49	984.50 ± 45.56	4.21 ± 0.85	21.48 ± 1.41	19.14 ± 1.83
2,000	9.84 ± 1.45	12.44 ± 5.06	84.98 ± 5.39	1.83 ± 0.48	0.69 ± 0.32	0.06 ± 0.05	8.97 ± 0.25	170.60 ± 5.78	0.467 ± 0.013	52.14 ± 1.68	19.04 ± 0.57	365.10 ± 5.20	970.00 ± 127.94	4.03 ± 0.54	21.80 ± 1.61	20.47 ± 2.27
4,000	9.50 ± 1.91	15.11 ± 4.93 ^b	81.79 ± 5.02 ^b	2.15 ± 0.46	0.88 ± 0.59	0.07 ± 0.05	8.81 ± 0.44	170.50 ± 6.06	0.464 ± 0.015	52.77 ± 1.64	19.37 ± 0.51	367.30 ± 3.53	966.60 ± 99.33	3.94 ± 0.83 ^b	21.44 ± 1.19	20.59 ± 2.07
Historical range ^d	6.59-18.37	3.4-30.3	66.9-95.7	0.5-4.9	0.0-1.1	0.0-0.4	7.4-9.9	142-184	0.39-0.52	47.8-57.6	17.8-20.3	350-375	478-1,119	3.52-7.97	18.9-25.8	14.2-22.2
Female (n = 10/group)																
Control	7.55 ± 1.25	9.22 ± 4.76	87.75 ± 4.90	2.06 ± 0.49	0.94 ± 0.28	0.03 ± 0.07	8.66 ± 0.42	160.40 ± 7.81	0.455 ± 0.021	52.52 ± 1.37	18.54 ± 0.60	352.90 ± 4.48	806.00 ± 53.89	4.88 ± 0.90	20.33 ± 0.82	17.93 ± 0.99
1,000	6.11 ± 1.39 ^b	12.21 ± 2.64 ^b	84.91 ± 2.94	1.81 ± 0.42	1.04 ± 0.40	0.03 ± 0.07	8.31 ± 0.77	156.80 ± 14.16	0.439 ± 0.037	52.95 ± 2.02	18.89 ± 0.55	356.80 ± 6.09	785.70 ± 86.51	4.06 ± 0.43 ^b	19.81 ± 1.17	18.79 ± 2.24
2,000	6.34 ± 1.43	15.04 ± 4.91 ^c	81.72 ± 5.22 ^c	2.12 ± 0.31	1.12 ± 0.37	0.00 ± 0.00	8.43 ± 0.44	156.70 ± 6.48	0.442 ± 0.014	52.50 ± 1.31	18.61 ± 0.40	354.30 ± 4.14	778.10 ± 56.54	4.56 ± 0.79	20.83 ± 0.95	20.62 ± 1.82 ^c
4,000	7.33 ± 1.14	13.97 ± 2.12 ^c	83.05 ± 2.32 ^b	1.87 ± 0.59	1.09 ± 0.24	0.02 ± 0.06	8.46 ± 0.33	161.00 ± 5.19	0.451 ± 0.017	53.30 ± 2.43	19.06 ± 0.66	357.60 ± 6.04	793.50 ± 88.42	4.28 ± 0.41	20.28 ± 1.11	22.13 ± 3.42 ^c
Historical range ^c	3.54-12.73	4.9-44.2	47.7-93.9	0.5-7.3	0.3-1.9	0.0-0.2	5.0-9.1	98-169	0.27-0.46	48.9-59.1	18.2-20.6	346-376	609-1,096	3.33-6.19	15.3-23.9	14.6-22.8

Abbreviations: APTT, activated partial thromboplastin time; BASO, basophils; EOS, eosinophils; GLUC, glucose; HCT, hematocrit; HGB, hemoglobin; LYM, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; NEU, neutrophils; PLT, platelet; PT, prothrombin time; RBC, red blood cell; RET, reticulocyte; TE, total erythrocytes; WBC, white blood cell.

^aData represent the mean values and the standard deviation.

^bP < 0.05.

^cP < 0.01.

^dMinimum and maximum levels reported as the range of historical control values.

Table 2. Summary of Clinical Chemistry Findings.^a

Group (mg/kg bw/d)	ALT U/L	AST U/L	GGT U/L	ALP U/L	TBIL μmol/L	CREA μmol/L	UREA mmol/L	GLUC mmol/L	CHOL mmol/L	Pi mmol/L	Ca ⁺⁺ mmol/L	Na ⁺ mmol/L	K ⁺ mmol/L	Cl ⁻ mmol/L	ALB g/L	TPROT g/L	A/G
Males (n = 10/group)																	
Control	39.54 ± 6.08	95.20 ± 12.16	-	143.3 ± 28.4	2.03 ± 0.23	23.13 ± 2.14	7.57 ± 0.92	6.03 ± 0.68	1.93 ± 0.25	2.65 ± 0.14	2.66 ± 0.05	139.8 ± 0.9	4.12 ± 0.19	102.53 ± 0.69	33.49 ± 1.08	57.91 ± 1.43	1.37 ± 0.08
1,000	43.51 ± 5.00	92.53 ± 6.92	-	159.7 ± 26.1	1.73 ± 0.41	22.53 ± 2.41	7.76 ± 0.99	6.16 ± 0.63	1.75 ± 0.15	2.71 ± 0.24	2.72 ± 0.08	139.3 ± 0.7	4.37 ± 0.29 ^b	102.64 ± 0.92	34.61 ± 1.40	61.26 ± 3.07 ^c	1.31 ± 0.10
2,000	44.42 ± 9.07	94.77 ± 12.07	-	166.7 ± 26.4	1.51 ± 0.45 ^c	20.97 ± 1.32 ^b	8.25 ± 1.26	6.80 ± 0.91 ^b	1.87 ± 0.37	2.52 ± 0.20	2.71 ± 0.08	138.3 ± 0.5 ^c	4.49 ± 0.18 ^c	102.34 ± 0.87	34.18 ± 0.76	58.58 ± 1.89	1.42 ± 0.08
4,000	50.96 ± 8.29 ^c	106.26 ± 12.26 ^b	-	146.8 ± 17.5	1.85 ± 0.32	20.79 ± 1.31 ^b	8.02 ± 1.30	5.91 ± 0.47	1.72 ± 0.25	2.71 ± 0.24	2.68 ± 0.04	138.5 ± 1.4 ^b	4.26 ± 0.22	102.64 ± 0.64	34.13 ± 0.58	58.94 ± 3.01	1.39 ± 0.16
Historical range ^d	42.4-76.7	68.3-144.8	-	112-321	0.64-2.76	17.7-30.3	5.27-11.12	4.66-7.69	1.32-2.74	2.11-3.23	2.49-2.89	132.0-143.0	3.66-4.94	95.1-102.2	31.5-35.8	51.4-65.4	1.1-1.8
Female (n = 10/group)																	
Control	46.42 ± 6.77	97.78 ± 10.30	-	97.40 ± 27.62	1.91 ± 0.39	26.12 ± 1.57	7.31 ± 1.00	5.59 ± 0.68	1.95 ± 0.35	1.99 ± 0.34	2.62 ± 0.06	140.40 ± 0.97	3.90 ± 0.21	104.10 ± 1.19	34.45 ± 1.34	57.22 ± 2.76	1.53 ± 0.09
1,000	45.16 ± 5.22	92.90 ± 8.10	-	105.30 ± 24.53	1.70 ± 0.33	24.54 ± 2.03	7.23 ± 1.30	5.35 ± 0.94	2.03 ± 0.30	1.72 ± 0.23	2.55 ± 0.06	139.10 ± 1.29 ^b	4.02 ± 0.27	104.42 ± 1.05	34.29 ± 0.70	56.83 ± 2.52	1.53 ± 0.13
2,000	44.41 ± 8.51	95.59 ± 8.31	-	109.40 ± 13.74	1.57 ± 0.45	24.71 ± 2.21	7.71 ± 0.85	5.87 ± 0.64	2.00 ± 0.32	1.90 ± 0.39	2.54 ± 0.07 ^b	139.70 ± 1.06	3.99 ± 0.30	104.58 ± 0.69	34.00 ± 1.09	56.56 ± 1.80	1.53 ± 0.09
4,000	46.13 ± 8.65	93.95 ± 8.20	-	102.90 ± 21.06	1.72 ± 0.23	24.54 ± 1.29	7.79 ± 1.40	5.54 ± 1.09	1.72 ± 0.19	1.99 ± 0.20	2.58 ± 0.04 ^b	138.20 ± 1.03 ^c	4.07 ± 0.29	104.00 ± 1.04	34.21 ± 0.80	56.95 ± 1.36	1.51 ± 0.07
Historical range ^d	36.8-86.4	76.8-272.1	-	56-192	0.59-2.86	18.3-31.1	4.67-10.94	3.40-7.68	1.03-2.57	1.73-2.89	2.36-2.87	136.0-149.0	3.04-5.36	95.8-103.9	32.3-38.4	55.2-65.2	1.2-1.7

Abbreviations: ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; A/G, albumin/globulin ratio; BUN, blood urea nitrogen; Ca⁺⁺, calcium; CHOL, cholesterol; Cl⁻, chloride; CREA, creatinine; GGT, gamma glutamyl transferase; K⁺, potassium; Na⁺, sodium; Pi, inorganic phosphate; TBIL, total bilirubin; TPROT, total protein.

^aData represent the mean values and the standard deviation.

^bP < 0.05.

^cP < 0.01.

^dMinimum and maximum levels reported as the range of historical control values.

Table 3. Summary of Organ weights (g).^a

Group (mg/kg bw/d)	Body weight	Brain	Liver	Kidneys	Heart	Thymus	Spleen	Testes	Epididymides	Adrenals
Males (n = 10/group)										
Control	273.7 ± 20.07	1.95 ± 0.12	7.83 ± 0.74	1.90 ± 0.15	0.89 ± 0.09	0.58 ± 0.08	0.58 ± 0.09	3.04 ± 0.23	1.10 ± 0.11	0.080 ± 0.013
1,000	273.5 ± 23.02	2.03 ± 0.07	8.50 ± 1.10	1.92 ± 0.20	0.91 ± 0.09	0.52 ± 0.08	0.58 ± 0.06	3.11 ± 0.14	1.11 ± 0.15	0.079 ± 0.007
2,000	273.6 ± 12.15	1.90 ± 0.09	8.66 ± 0.76	1.92 ± 0.10	0.88 ± 0.08	0.54 ± 0.08	0.50 ± 0.07 ^b	3.06 ± 0.37	0.99 ± 0.11 ^b	0.080 ± 0.011
4,000	260.7 ± 17.38	2.00 ± 0.06	8.58 ± 0.73	1.98 ± 0.16	0.83 ± 0.11	0.42 ± 0.07 ^c	0.49 ± 0.05 ^c	3.00 ± 0.13	0.95 ± 0.07 ^c	0.075 ± 0.008
Historical range ^d	241-348	1.80-2.18	6.11-11.34	1.44-2.50	0.75-1.22	0.25-0.80	0.46-0.99	2.29-3.72	0.56-1.47	0.053-0.100
Females (n = 10/group)										
Control	177.3 ± 8.15	1.92 ± 0.06	5.33 ± 0.53	1.31 ± 0.12	0.67 ± 0.05	0.47 ± 0.09	0.46 ± 0.06	0.63 ± 0.24	0.146 ± 0.018	0.081 ± 0.012
1,000	173.0 ± 7.57	1.84 ± 0.08	5.35 ± 0.49	1.28 ± 0.12	0.62 ± 0.07	0.43 ± 0.07	0.39 ± 0.04 ^b	0.51 ± 0.15	0.123 ± 0.016 ^b	0.078 ± 0.008
2,000	174.4 ± 10.76	1.83 ± 0.10 ^b	5.66 ± 0.71	1.31 ± 0.11	0.63 ± 0.07	0.46 ± 0.07	0.42 ± 0.06	0.56 ± 0.14	0.120 ± 0.033 ^b	0.080 ± 0.013
4,000	174.1 ± 5.40	1.81 ± 0.10 ^b	5.51 ± 0.31	1.31 ± 0.09	0.62 ± 0.05	0.40 ± 0.08	0.39 ± 0.06 ^b	0.63 ± 0.27	0.107 ± 0.019 ^c	0.080 ± 0.013
Historical range ^d	155.0-203.0	1.67-2.07	4.69-6.76	1.08-1.52	0.52-0.82	0.26-0.54	0.34-0.75	0.26-1.09	0.058-0.180	0.055-0.116

^aData represent the mean values and the standard deviation.^bp < 0.05.^cp < 0.01.^dMinimum and maximum levels reported as the range of historical control values.**Table 4.** Summary of Organ Weights Relative to Body Weight (%).^a

Group (mg/kg bw/d)	Brain	Liver	Kidneys	Heart	Thymus	Spleen	Testes	Epididymides	Adrenals
Males (n = 10/group)									
Control	0.713 ± 0.052	2.859 ± 0.143	0.694 ± 0.026	0.324 ± 0.025	0.212 ± 0.019	0.209 ± 0.018	1.110 ± 0.064	0.404 ± 0.045	0.029 ± 0.004
1,000	0.745 ± 0.072	3.098 ± 0.159 ^b	0.701 ± 0.045	0.333 ± 0.026	0.190 ± 0.018 ^c	0.214 ± 0.022	1.143 ± 0.113	0.407 ± 0.063	0.029 ± 0.003
2,000	0.695 ± 0.035	3.163 ± 0.168 ^b	0.697 ± 0.041	0.320 ± 0.022	0.196 ± 0.026	0.184 ± 0.024 ^c	1.118 ± 0.134	0.363 ± 0.033	0.029 ± 0.004
4,000	0.769 ± 0.045 ^c	3.290 ± 0.130 ^b	0.759 ± 0.052 ^b	0.320 ± 0.038	0.161 ± 0.023 ^b	0.187 ± 0.019 ^c	1.158 ± 0.109	0.367 ± 0.043	0.029 ± 0.003
Historical range ^d	0.600-0.851	2.314-3.481	0.545-0.788	0.263-0.399	0.095-0.306	0.171-0.355	0.722-1.227	0.224-0.473	0.0190-0.0357
Females (n = 10/group)									
Control	1.082 ± 0.049	3.003 ± 0.211	0.741 ± 0.063	0.377 ± 0.031	0.262 ± 0.045	0.256 ± 0.032	0.357 ± 0.137	0.0824 ± 0.0092	0.0456 ± 0.0050
1,000	1.068 ± 0.071	3.089 ± 0.177	0.740 ± 0.057	0.360 ± 0.037	0.247 ± 0.036	0.225 ± 0.016 ^c	0.295 ± 0.096	0.0708 ± 0.0088	0.0448 ± 0.0041
2,000	1.049 ± 0.046	3.239 ± 0.239 ^c	0.749 ± 0.056	0.361 ± 0.040	0.266 ± 0.043	0.243 ± 0.031	0.320 ± 0.082	0.0689 ± 0.0190 ^c	0.0458 ± 0.0063
4,000	1.039 ± 0.055	3.168 ± 0.216	0.750 ± 0.046	0.355 ± 0.034	0.232 ± 0.048	0.226 ± 0.033 ^c	0.360 ± 0.154	0.0614 ± 0.0119 ^b	0.0460 ± 0.0072
Historical range ^d	0.865-1.174	2.672-3.406	0.590-0.843	0.306-0.437	0.141-0.308	0.191-0.426	0.142-0.661	0.034-0.102	0.031-0.074

^aData represent the mean values and the standard deviation.^bp < 0.01.^cp < 0.05.^dMinimum and maximum levels reported as the range of historical control values.

Table 5. Summary of Body Weight and Organ Weight Relative to Brain Weight (%).^a

Group (mg/kg bw/d)	Body weight	Liver	Kidneys	Heart	Thymus	Spleen	Testes	Epididymides	Adrenals
Males (n = 10/group)									
Control	14,095.6 ± 987.66	403.44 ± 39.55	97.82 ± 7.12	45.70 ± 4.78	29.82 ± 3.15	29.53 ± 3.69	156.28 ± 10.90	56.78 ± 5.60	4.15 ± 0.75
1,000	13,523.2 ± 1,258.46	420.24 ± 55.87	94.69 ± 9.53	44.88 ± 4.08	25.75 ± 4.10 ^b	28.80 ± 2.87	153.48 ± 7.27	54.60 ± 7.05	3.90 ± 0.33
2,000	14,423.2 ± 666.83	456.59 ± 37.73 ^b	100.51 ± 7.94	46.12 ± 3.44	28.26 ± 4.39	26.57 ± 4.22	161.27 ± 21.65	52.38 ± 5.50	4.19 ± 0.46
4,000	13,039.5 ± 751.36 ^b	429.14 ± 33.25	98.87 ± 6.46	41.63 ± 4.85	21.02 ± 3.30 ^c	24.34 ± 2.38 ^c	150.34 ± 8.00	47.60 ± 3.77 ^c	3.75 ± 0.34
Historical range ^d	11,756.1-16,666.7	316.6-532.4	80.0-124.4	38.7-61.7	13.0-38.3	24.1-46.6	113.4-178.2	28.9-68.1	2.69-4.78
Females (n = 10/group)									
Control	9,261.4 ± 399.47	278.33 ± 25.25	68.60 ± 6.38	34.87 ± 2.91	24.29 ± 4.63	23.72 ± 2.95	33.06 ± 12.66	7.62 ± 0.86	4.23 ± 0.52
1,000	9,400.7 ± 629.26	290.89 ± 31.17	69.45 ± 5.93	33.85 ± 3.79	23.29 ± 3.97	21.21 ± 2.58	27.39 ± 7.88	6.63 ± 0.67 ^b	4.22 ± 0.59
2,000	9,550.7 ± 421.90	309.41 ± 27.12 ^b	71.45 ± 4.46	34.37 ± 3.37	25.44 ± 4.52	23.25 ± 3.38	30.68 ± 8.57	6.58 ± 1.80	4.37 ± 0.60
4,000	9,645.9 ± 510.57	305.12 ± 19.86 ^b	72.24 ± 3.94	34.17 ± 3.12	22.40 ± 4.84	21.71 ± 2.40	34.99 ± 15.90	5.90 ± 1.04 ^c	4.42 ± 0.52
Historical range ^d	8,516.5-11,556.9	248.9-361.5	56.5-84.0	28.6-45.6	13.8-30.9	18.7-39.3	14.8-62.6	3.3-9.8	3.0-6.5

^aData represent the mean values and the standard deviation.^bp < 0.05.^cp < 0.01.^dMinimum and maximum levels reported as the range of historical control values.

sexes compared to control animals (see Tables 1 and 2). Among these changes was a significant increase in neutrophils (NEU) in all treated females. In females of the 2,000 and 4,000 mg/kg bw/d groups, a significant increase in activated partial thromboplastin time (APTT) and a significant decrease in lymphocytes (LYMs) were also noted. Significant increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were noted in male animals in the 4,000 mg/kg bw/d group and appeared to be dose dependent.

Similarly, slight, statistically significant differences compared to controls were found in various absolute and relative organ weights in both sexes (see Tables 3-5). A few of these changes appeared possibly dose dependent: a decrease in spleen (absolute and relative to body weight) and epididymides (absolute) weights in males of the 2,000 and 4,000 mg/kg bw/d groups, a decrease in epididymides weights (relative to brain weights) in the 4,000 mg/kg bw/d group, and an increase in liver weights (relative to body weight) in all treated males. Additionally, although a dose dependency was not noted, thymus weights (relative to body weight and brain weight) were significantly lower in males of the 1,000 and 4,000 mg/kg bw/d groups, and a significant decrease in thymus weights (absolute) in the 4,000 mg/kg bw/d group was observed.

In all treated female animals, ovarian weights (absolute) were significantly and dose dependently decreased, and a significant decrease in ovarian weights (relative to body weight) was observed in the 2,000 and 4,000 mg/kg bw/d groups. A significant decrease in ovarian weights (relative to brain weight) in the 1,000 and 4,000 mg/kg bw/d groups was also observed. Brain weights (absolute) were also significantly decreased in the 2,000 and 4,000 mg/kg bw/d groups. Lastly, although dose dependency was not clear, liver weights (relative to brain weight) were significantly higher in females of the 2,000 and 4,000 mg/kg bw/d groups and spleen weights (absolute and relative to body weight) were significantly decreased in females of the 1,000 and 4,000 mg/kg bw/d groups.

Upon gross examination, pyelectasis (dilatation of the renal pelvis) was seen unilaterally in 1 male in the 4,000 mg/kg bw/d group. An elevated percentage of NEU and decreased percentage of LYMs were detected in this animal; however, no correlating clinical or histopathological findings were discovered. In female animals, hydrometra (dilated and fluid-filled uterine horns) was detected in all groups including control upon macroscopic and microscopic evaluations (see Table 6). Mild alveolar emphysema and mild hyperplasia of bronchus-associated lymphoid tissue (BALT) were noted in both control and treated animals with similar incidence. No other histopathological findings were observed in the examined organs and tissues.

Discussion

No toxicologically relevant abnormalities were observed in the clinical and functional observational examinations conducted throughout the study. The salivation in the three 4,000 mg/kg bw/d females was not considered toxicologically

Table 6. Summary of Notable Histopathology Findings.^a

		Incidence of observations per group dose groups (mg/kd bw/d)			
Organs	Observations	Control	1,000	2,000	4,000
Male					
Kidneys					
Pyelectasis		0/10	/	/	1/10
Alveolar emphysema		2/10	/	/	2/10
Lungs					
Hyperplasia of BALT		1/10	/	/	1/10
Female					
Lungs					
Alveolar emphysema		2/10	/	/	1/10
Uterus					
Dilatation		1/10	/	/	2/10

Abbreviations: BALT, bronchus associated lymphoid tissue; /, not examined.

^aData represent the number of animals with observation per number of animals observed. Full histopathological examinations were conducted on the preserved adrenals, aorta, femur bone marrow, cerebrum, cerebellum, pons and medulla oblongata, esophagus, lacrimal and Harderian glands, female mammary glands, testes with epididymides, ovaries, uterus, vagina, heart, kidneys, cecum, colon, rectum, Peyer's patches, liver, main stem bronchi, submandibular and mesenteric lymph nodes, quadriceps muscle, nasal turbinates, pancreas, pituitary, prostate, submandibular salivary glands, sciatic nerve, seminal vesicle with coagulating gland, skin, duodenum, ileum, jejunum, cervical, midthoracic and lumbar spinal cord, spleen, sternum, stomach, thymus, thyroid, parathyroid, trachea, and urinary bladder of all animals of the control and high-dose groups.

relevant due to its transient nature (occurring at least 1 day and up to 16 days, depending on the animal) and may have also been due to large doses of the test article. The scarring and alopecia findings in the single male in the 2,000 mg/kg bw/d group were also not considered toxicologically relevant because they are known to be common dermal changes in experimental rats.³⁶

The changes noted in body weight gain, daily food consumption, and feed efficiency were not considered to be of toxicological relevance due to their transient nature, the small degree of change, and because they had no significant influence on overall body weight or in body weight gain. In hematological examinations, changes in APTT were primarily due to a relatively low mean control value (with respect to historical control values) and were not related to any histopathological or clinical abnormalities. The changes noted in NEU and LYM were not dose dependent and remained well within historical controls. The significant increases in ALT and AST in male animals in the 4,000 mg/kg bw/d group were slight in magnitude, fell well within the historical control ranges, and lacked related histopathological changes. All of the remaining statistically significant changes in hematology and clinical chemistry parameters remained within historical controls and did not correlate with any clinical or histopathological findings and, therefore, were not considered to be of toxicological relevance.

Although there was some indication of dose dependency in certain organ weight changes, they were slight in magnitude, the values remained within historical control ranges, and there were no correlating clinical or histopathological findings. The degree of increase in the renal pelvic diameter noted in 1 high-dose male animal was slight and lacked medullar or cortical atrophy, inflammatory infiltrates, hemorrhage, hemosiderin, and degenerative or fibrotic lesions; therefore, it was

considered an individual disorder without toxicological significance. The presence of hydrometra was not dose dependent and is considered a common neurohormonal phenomenon in connection with the proestrus phase of the female sexual cycle, a frequent observation in experimental animals.^{37,38} The mild alveolar emphysema and mild hyperplasia of BALT were considered a consequence of exsanguination and a physiological immunomorphological phenomenon, respectively.³⁹⁻⁴²

All of the significant differences noted above were slight in magnitude and remained well within the laboratory's historical control ranges for the species of animal used. No clinical or histopathological correlates were found with respect to hematological and clinical chemistry findings or organ weight changes; therefore, these findings were not considered to be toxicologically relevant.

Conclusions

In the present study, Reducose 5%, a commercial aqueous extract of *M alba*, did not cause toxic effects in male or female Hsd.Han Wistar rats after a 28-day repeated oral administration of 1,000, 2,000 or 4,000 mg/kg bw/d. The NOAEL was concluded as 4,000 mg/kg bw/d, the highest dose group tested, in both male and female animals.

The safety of *M alba* is corroborated by its use as a component of traditional medicines in China and the broad use of mulberry leaves as animal feed worldwide without safety concerns. Several other published toxicological and human clinical studies on various *M alba* extracts, as well as the previously described unpublished toxicological studies on Reducose 1% and 5%, also support the conclusion of this study.^{12,31,43} The present study reinforces this safety profile and also supports the safety of *M alba* extracts with a higher DNJ content (5%) than what is found naturally.

Authors Contributions

Tennille K. Marx contributed to acquisition, analysis or interpretation of data, drafted the manuscript, and critically revised the manuscript for important intellectual content. Róbert Glávits contributed to acquisition, analysis or interpretation of data, and critically revised the manuscript for important intellectual content. John R. Endres substantially contributed to conception or design, contributed to acquisition, analysis, or interpretation of data, and critically revised the manuscript for important intellectual content. Philip A. Palmer contributed to acquisition, analysis, or interpretation of data, drafted the manuscript, and critically revised the manuscript for important intellectual content. Amy E. Clewell contributed to acquisition, analysis, or interpretation of data and critically revised the manuscript for important intellectual content. Timothy S. Murbach contributed to acquisition, analysis, or interpretation of data and critically revised the manuscript for important intellectual content. Gábor Hirka substantially contributed to conception or design, contributed to acquisition, analysis, or interpretation of data, and critically revised the manuscript for important intellectual content. Ilona Pasicz contributed to conception or design, contributed to acquisition, analysis, or interpretation of data, and critically revised the manuscript for important intellectual content. All authors gave final approval and agree to be accountable for all aspects of the work in ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: AIBMR Life Sciences, Inc. was contracted by the study sponsor, as an independent third party, to determine appropriate study protocols and dose selections, place the study, approve the study plan, monitor the toxicological study described above, as well as to analyze and interpret the resulting data and prepare the manuscript. Toxi-Coop Zrt was contracted by AIBMR to develop the study plan and conduct, analyze and interpret, and report the results of the toxicological study described above.

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Supplemental Material

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References

1. Yuan Q, Xie Y, Wang W, et al. Extraction optimization, characterization and antioxidant activity in vitro of polysaccharides from mulberry (*Morus alba* L.) leaves. *Carbohydr Polym*. 2015; 128:52-62.
2. Food and Agriculture Organization. Mulberry for Animal Production: *Proceedings of an Electronic Conference Carried Out Between May and August 2000*. Animal Production and Health Paper 147. Viale delle Terme di Caracalla, Rome, Italy: FAO; 2002.
3. Kanafi R, Ebadi R, Mirhosseini S, Seidavi A, Zolfaghari M, Etebari K. A review on nutritive effect of mulberry leaves enrichment with vitamins on economic traits and biological parameters of silkworm *Bombyx mori* L. *Invert Surviv J*. 2007;4(2):86-91.
4. Ramesha C, Lakshmi H, Kumari SS, Anuradha CM, Kumar CS. Nutrigenetic screening strains of the mulberry silkworm, *Bombyx mori*, for nutritional efficiency. *J Insect Sci*. 2012;12:15.
5. Prasad P, Reddy D, Reddy M, Reddy G. Effect of feeding mulberry (*Morus alba*) hay in the rations to pregnant ewes. *Indian J Anim Nutr*. 1995;12(2):109-111.
6. Prasad P, Reddy M. Nutritive value of mulberry (*Morus alba*) leaves in goats and sheep. *Indian J Anim Nutr*. 1991;8(4): 295-296.
7. Kandyli K, Hadjigeorgiou I, Harizanis P. The nutritive value of mulberry leaves (*Morus alba*) as a feed supplement for sheep. *Trop Anim Health Prod*. 2009;41(1):17-24.
8. Bensky D, Gamble A, *Morus alba* L. *Chinese Herbal Medicine. Materia Medica. Revised Edition*. Seattle, WA: Eastland Press; 1993:56-57.
9. Abbasi AM, Khan MA, Khan N, Shah MH. Ethnobotanical survey of medicinally important wild edible fruits species used by tribal communities of Lesser Himalayas-Pakistan. *J Ethnopharmacol*. 2013;148(2):528-536.
10. da Silva Almeida J, da Cruz Araújo E, Silva F, et al. Chapter 11. Medicinal plants and natural compounds from the genus *Morus* (moraceae) with hypoglycemic activity: A review. In: *Glucose Tolerance*. INTECH Open Access Publisher; 2012.
11. Andallu B, Suryakantham V, Lakshmi Srikanthi B, Reddy GK. Effect of mulberry (*Morus indica* L.) therapy on plasma and erythrocyte membrane lipids in patients with type 2 diabetes. *Clin Chim Acta*. 2001;314(1-2):47-53.
12. Kimura T, Nakagawa K, Kubota H, et al. Food-grade mulberry powder enriched with 1-deoxynojirimycin suppresses the elevation of postprandial blood glucose in humans. *J Agric Food Chem*. 2007;55(14):5869-5874.
13. Asai A, Nakagawa K, Higuchi O, et al. Effect of mulberry leaf extract with enriched 1-deoxynojirimycin content on postprandial glycemic control in subjects with impaired glucose metabolism. *J Diabetes Investig*. 2011;2(4):318-323.
14. Kojima Y, Kimura T, Nakagawa K, et al. Effects of mulberry leaf extract rich in 1-deoxynojirimycin on blood lipid profiles in humans. *J Clin Biochem Nutr*. 2010;47(2):155-161.
15. Miyahara C, Miyazawa M, Satoh S, Sakai A, Mizusaki S. Inhibitory effects of mulberry leaf extract on postprandial hyperglycemia in normal rats. *J Nutr Sci Vitaminol (Tokyo)*. 2004;50(3): 161-164.

16. Ren C, Zhang Y, Cui W, et al. A polysaccharide extract of mulberry leaf ameliorates hepatic glucose metabolism and insulin signaling in rats with type 2 diabetes induced by high fat-diet and streptozotocin. *Int J Biol Macromol*. 2015;72:951-959.
17. Srivastava S, Kapoor R, Thathola A, Srivastava RP. Nutritional quality of leaves of some genotypes of mulberry (*Morus alba*). *Int J Food Sci Nutr*. 2006;57(5-6):305-313.
18. Kim SY, Gao JJ, Lee WC, Ryu KS, Lee KR, Kim YC. Antioxidative flavonoids from the leaves of *Morus alba*. *Arch Pharm Res*. 1999;22(1):81-85.
19. Song W, Wang HJ, Bucheli P, Zhang PF, Wei DZ, Lu YH. Phytochemical profiles of different mulberry (*Morus* sp.) species from China. *J Agric Food Chem*. 2009;57(19):9133-9140.
20. Horne G, Wilson FX, Tinsley J, Williams DH, Storer R. Iminosugars past, present and future: medicines for tomorrow. *Drug Discov Today*. 2011;16(3-4):107-118.
21. Kimura T, Nakagawa K, Saito Y, et al. Determination of 1-deoxynojirimycin in mulberry leaves using hydrophilic interaction chromatography with evaporative light scattering detection. *J Agric Food Chem*. 2004;52(6):1415-1418.
22. Kim JY, Kwon HJ, Jung JY, et al. Comparison of absorption of 1-deoxynojirimycin from mulberry water extract in rats. *J Agric Food Chem*. 2010;58(11):6666-6671.
23. Kim YC, Takaya Y, Chung SK. Tyrosinase inhibition and mutagenicity of phenolic compounds from mulberry leaves. *J Food Sci Nutr*. 2007;12:119-121.
24. Chichioco-Hernandez C, Wudarski J, Gevaert L, Verschaeve L. Evaluation of cytotoxicity and genotoxicity of some Philippine medicinal plants. *Pharmacogn Mag*. 2011;7(26):171-175.
25. Agabeyli R. Antimutagenic activities extracts from leaves of the *Morus alba*, *Morus nigra* and their mixtures. *Int J Biol*. 2012;4(2):166-172.
26. Yadav AV, Kawale LA, Nade VS. Effect of *Morus alba* L. (mulberry) leaves on anxiety in mice. *Indian J Pharmacol*. 2008;40(1):32-36.
27. Abdulla M, Ali H, Ahmed K, Noor S, Ismail S. Evaluation of the anti-ulcer activities of *Morus alba* extracts in experimentally-induced gastric ulcer in rats. *Biomed Res*. 2009;20(1):35-39.
28. Laddha G, Vidyasagar G. Anti-psychotic effect of aqueous leaves extract of *Morus alba* in animal models. *Int J Pharm*. 2012;2(3):513-519.
29. Aditya Rao S, Ramesh C, Basavaraj P, Jamuna K. Evaluation of anti-inflammatory and analgesic activity in three *Morus* species. *Res J Pharm Biol Chem Sci*. 2013;4(3):822-830.
30. Zhang Y, Ren C, Lu G, et al. Anti-diabetic effect of mulberry leaf polysaccharide by inhibiting pancreatic islet cell apoptosis and ameliorating insulin secretory capacity in diabetic rats. *Int immunopharmacol*. 2014;22(1):248-257.
31. Heo HS, Choi JH, Oh JJ, et al. Evaluation of general toxicity and genotoxicity of the silkworm extract powder. *Toxicol Res*. 2013;29(4):263-278.
32. Organisation for Economic Co-operation and Development. *Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section 4*. Paris, France: OECD Publishing; 2008.
33. Food and Drug Administration (FDA). Redbook 2000. Toxicological principles for the safety assessment of food ingredients. IV. C.3.a. Short-term toxicity studies with rodents. 2003.
34. National Research Council. Washington, DC: Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, Division on Earth and Life Studies, National Research Council; 2011.
35. Irwin S. Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. *Psychopharmacologia*. 1968;13(3):222-257.
36. Mecklenburg L, Kusewitt D, Kolly C, et al. Proliferative and non-proliferative lesions of the rat and mouse integument. *J Toxicol Pathol*. 2013;26(3 suppl):27S-57S.
37. Vidal JD, Mirsky ML, Colman K, et al. Chapter 18. Reproductive system and mammary gland. In: Sahota PS, Popp JA, Hardisty JF, Gopinath C, eds. *Toxicologic Pathology. Nonclinical Safety Assessment*. Boca Raton: CRC Press; 2013:717-830.
38. Dixon D, Heider K, Elwell MR. Incidence of nonneoplastic lesions in historical control male and female Fischer-344 rats from 90-day toxicity studies. *Toxicol Pathol*. 1995;23(3):338-348.
39. Vandenberghe J. *Life-Span Data and Historical Data in Carcinogenicity Testing in Wistar Rats Crl:(WI) BR. Addendum 3.2*. Beerse, Belgium: Janssen Research Foundation, Department of Toxicology, Charles River Deutschland; 1990.
40. Johnson R, Spaet R, Potenta D. Chapter 8. Spontaneous lesions in control animals used in toxicity studies. In: *Toxicologic Pathology. Nonclinical Safety Assessment*. Boca Raton, FL: CRC Press; 2013:209-254.
41. Boorman G, Eustis S, Lung II. Normal lung B. Anatomy—4. Bronchial-associated lymphoid tissue. In: Boorman G, Eustis S, Elwell M, MacKenzie W, eds. *Pathology of the Fischer Rat: Reference and Atlas*. San Diego, CA: Academic Press; 1990:343-344.
42. Haschek W, Rousseaux C, Wallig M. Chapter 6. Respiratory system. Structure and cell biology. Physiology and functional considerations—lymphoid tissue. In: *Fundamentals of Toxicologic Pathology*. Amsterdam, Netherlands: Elsevier; 2009:98.
43. Miyazawa M, Miyahara C, Satoh S, Sakai A. Ninety-day dietary toxicity study of mulberry leaf extract in rats [in Japanese]. *Shokuhin Eiseigaku Zasshi*. 2003;44(4):191-197.