SAFETY EVALUATION OF MULBERRY POWDER: ASSESSMENT OF SUBCHRONIC TOXICITY IN RATS

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Obesity has reached epidemic proportions globally, with at least 2.8 million people dying each year due to being overweight or obese. Because of the botamical's chemical diversity and the ability to act on various biological targets, plant products have long been a thriving source for discovering new compounds to treat obesity. MULBERRY powder was a herbal-derived product combined with Amorphophallus konjac K. Koch starch and mulberry (Morus alba L.) leaves extract; these plants have long been used in Asia as food sources and as traditional medicines. So far, the safety of this product has not been reported yet in the world and Vietnam. Thus, this study was designed to assess the subchronic toxicity of MULBERRY powder in Wistar rats. The method used in this study followed the guidance of the World Health Organization and Organization for Economic Co-operation and Development in rats with two oral doses of 1.2 g/kg b.w/day and 3.6 g/kg b.w/day for 12 consecutive weeks. As a result, MULBERRY powder at both doses caused no significant changes in body weight, hematological indexes, functions, and microscopic images of livers and kidneys, although MULBERRY powder at 3.6 g/kg b.w/day caused diarrhea at about 20% of rats. In conclusion, MULBERRY powder did not cause subchronic toxicity in experimental rats. However, this partly revealed the safety of MULBERRY powder in clinical practice.

Keywords: MULBERRY powder, Amorphophallus konjac K. Koch, Morus alba L., subchronic toxicity, rats.

I. INTRODUCTION

Obesity is a worldwide epidemic characterized by excess adipose tissue and contributes to numerous chronic diseases and early mortality. This epidemic has received both national and international attention because of obesity's detrimental impact on health, the enormous economic burden it imposes, and its increasing prevalence. The adverse health consequences associated with obesity include cardiovascular disease, stroke, type 2 diabetes mellitus, hypertension, dyslipidemia, cancers of the breast, endometrium, prostate, colon cancer, gallbladder disease, osteoarthritis, respiratory problems, asthma and sleep apnea and perhaps depression.[1] According to WHO, obesity has reached epidemic proportions worldwide, with approximately 1.9 billion overweight and 650 million obese adults.[2] NICE currently recommends pharmacological treatment for weight loss maintenance in addition to a reduced-calorie diet and optimal physical exercise, such as orlistat, liraglutide, naltrexone/bupropion. However, these synthetic drugs caused undesirable effects such as nausea, headaches, and constipation.[3] Therefore, one of the most urgent research missions was the discovery of novel drugs derived from herbs which not only exhibited anti-obesity effect but also limited side effects. Toxicity refers to unwanted effects on biological systems. In order to evaluate biological toxicity, it is imperative to choose the

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correct system since no effects may otherwise be seen. Toxicity of a substance can be impacted by many factors, such as the route of exposure (skin absorption, ingestion, inhalation, or injection); the time of exposure (a brief, acute, subchronic, or chronic exposure); the number of exposures (a single dose or multiple doses over time); the physical form of the toxin (solid, liquid, or gas); the organ system involved (cardiovascular, nephro-, hemo-, nervous-, or hematopoietic-system); and even the genetic makeup and robustness of the target cells or organisms.[4] Subchronic systemic toxicity is defined as adverse effects occurring after a test sample's was administered repeatly or continuously for up to 90 days or not exceeding 10% of the animal's lifespan.[5]

Amorphophallus konjac K. Koch and mulberry (*Morus alba* L.) were the main components of MULBERRY powder. These plants have long been used in Asia as food sources and as traditional medicines. Glucomannan (GM), a soluble fiber derived from *Amorphophallus konjac*, is marketed for weight loss.[6] The leaves, roots, and branches of Morus species are used to treat fevers, hepatic protection, vision improvement, strengthen joints, reduce blood pressure, and control blood glucose levels.[7] However, there have been no report available on the toxicity of the combination of these components in the world and Vietnam so far. Therefore, in this study, we aimed to assess the subchronic toxicity of MULBERRY powder in experimental animals.

II. METHODS

1. The preparation of MULBERRY powder

MULBERRY was manufactured by Ngoc Thien Trading and Pharmaceutical Joint Stock Company. This product was prepared and offered in the form of sachets. Each sachet contained 3.860 mg *Amorphophallus konjac* K. Koch starch, and 1.140 mg mulberry (*Morus alba* L.) leaves extract. The recommended dosage in patients was two sachets per day. MULBERRY powder was prepared daily by dissolving with distilled water before giving to rats. Rats were orally administered this mixture immediately after dissolving, the remaining product was removed.

2. Chemicals and laboratory equipments

Kits for testing enzymes and metabolites in blood: ALT (alanin aminotransferase), AST (aspartat aminotransferase), total bilirubin, albumin, total cholesterol, creatinine kits from Hospitex Diagnostics (Italy) và and DIALAB GmbH (Austria) were used for Screen Master machine of Hospitex Diagnostics (Italy). Blood-testing solutions ABX Minidil LMG of ABX Diagnostics were used for Vet abcTM Animal Blood Counter. Chemicals for tests and histopathological examination.

3. Experimental animals

Healthy *Wistar* rats (180 ± 20 g) were used in this study. The animals were housed in cages (groups of ten rats/cage) under the standard conditions (temperature $25^0C \pm 2^0C$ and relative humidity 80% ± 10%), 12 hours dark/light time. We fed the rats with standard animal feed and allowed free access to water. They were acclimated to housing for at least one week prior to the study at the Department of Pharmacology, Hanoi Medical University.

4. Subchronic toxicity study

Subchronic toxicity studies were carried out according to WHO Guidance and OECD guidelines.[8,9]

The study was carried out in a continuous 12-week period. *Wistar* rats were divided into three groups of ten animals:

- Group 1 (control) was the distilled water control group. Each rat was given 1 ml distilled

water/100 g b.w/day orally.

- Group 2 was given orally MULBERRY powder at the dose of 1.2 g/kg b.w/day (equivalent to the human recommended dose, conversion ratio 6);

- Group 3 was given orally MULBERRY powder at the dose of 3.6 g/kg b.w/day (3 times as high as the dose at group 2).

Animals were treated once daily in the morning for 12 consecutive weeks, and observed once daily to detect signs of toxicity.

The signs and indexes checked during the study are:

- General condition consisting of mortality and clinical signs.

- Body weight changes.

- Hematopoietic function test: red blood cells (RBC), hemoglobin (HGB), hematocrit, total white blood cells (WBC), WBC differentials, platelet count (PLT).

- Serum biochemistry: aspartate amino-transferase (AST), alanine aminotransferase (ALT), total bilirubin, albumin, total cholesterol, and creatinine levels.

The parameters were checked before treatment, four weeks after treatment, eight weeks after treatment, and twelve weeks after treatment. All animals were subjected to a complete gross necropsy at the end of the experiment. Liver and kidney were removed for histopathology examinations in 30% pf rats in each group. The micro-histological examination was carried out at the Center for Research and Early Detection of Cancer (CREDCA). Assoc. Prof. Le Dinh Roanh, Director of CREDCA, gave results of pathological image analysis.

5. Statistical analysis

Data were analyzed using Microsoft Excel software version 2010. The levels of significance between the experimental groups and the control group were made using the student's t-test. Data are shown as mean ± standard deviation. All data were considered significant at $p < 0.05$.

III. RESULTS

1. General condition

None of the animals showed any macroscopic or gross pathological changes compared with the control group. In general, animals in groups 1 and 2 had normal locomotor activities and good feedings. However, in group 3, after one week of oral administration of MULBERRY powder at the dose of 3.6 g/kg b.w/day, about 20% of rats had diarrhea, loose stool, and wet anus. This condition was reduced and resolved entirely for two weeks after stopping the treatment.

2. Body weight changes

Table 1. The effect of MULBERRY powder on body weight

Time		Body weight (g)		
		Group 1	Group 2	Group 3
Before treatment		196.0 ± 50.4	197.0 ± 35.0	203.0 ± 41.4
After treatment (weeks)	4	239.0 ± 58.6	240.0 ± 38.6$^{\Delta}$	230.0 ± 45.9
	8	262.0 ± 48.9$^{\Delta\Delta}$	258.0 ± 35.8$^{\Delta\Delta}$	230.0 ± 50.3
	12	265.0 ± 50.2$^{\Delta\Delta}$	255.0 ± 36.0$^{\Delta\Delta}$	238.0 ± 45.4

$^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}$ $p < 0.01$, $^{\Delta\Delta\Delta}$ $p < 0.001$ compared with the time point "Before treatment"

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Group 1

Table 1 showed that after four weeks, eight weeks, and twelve weeks of treatment, body weight of rats in groups 1 and 2 increased substantially compared with body weight "Before treatment". In group 3, there was no statistical change in the body weight of rats as compared with the time point "Before treatment". No significant differences were observed between groups treated with MULBERRY powder and the control group (group 1) ($p > 0.05$).

3. Effect on hematological examination

Table 2. Effect of MULBERRY powder on hematopoietic function

Parameters	Group	Before treatment	After treatment (weeks)		
			4	8	12
Red blood cells count (T/L)	Group 1	10.58 ± 1.19	9.56 ± 1.11	9.89 ± 1.02	10.34 ± 1.12
	Group 2	9.24 ± 1.86	10.42 ± 0.75	10.08 ± 0.93	9.84 ± 0.80
	Group 3	9.32 ± 1.69	9.90 ± 1.35	10.34 ± 0.94	10.48 ± 0.53
Hemoglobin level (g/dL)	Group 1	14.58 ± 1.56	12.73 ± 2.38	13.20 ± 1.44	13.28 ± 1.24
	Group 2	13.58 ± 1.14	13.97 ± 1.31	13.02 ± 1.13	12.98 ± 0.87
	Group 3	13.79 ± 1.32	13.50 ± 1.78	13.54 ± 1.19	13.41 ± 0.81
Hematocrit (%)	Group 1	58.77 ± 7.09	51.93 ± 7.73	52.10 ± 7.54	53.55 ± 6.18
	Group 2	53.93 ± 3.05	56.05 ± 4.63	50.76 ± 4.43	50.65 ± 4.73
	Group 3	54.79 ± 5.11	52.63 ± 7.55	52.74 ± 4.88	53.36 ± 4.88
MCV (fL)	Group 1	55.50 ± 1.58	52.10 ± 5.15	51.60 ± 5.80	52.50 ± 4.28
	Group 2	53.60 ± 2.55	53.00 ± 2.71	51.00 ± 3.33	51.10 ± 3.28
	Group 3	53.90 ± 2.92	53.10 ± 1.37	51.80 ± 2.39	52.00 ± 2.26
Platelet count (G/L)	Group 1	475.9 ± 136.6	592.7 ± 147.8	603.0 ± 158.6	627.8 ± 184.1
	Group 2	524.9 ± 73.3	614.9 ± 130.5	616.8 ± 120.0	598.1 ± 247.4
	Group 3	575.5 ± 80.0	686.0 ± 145.9	669.6 ± 129.7	545.3 ± 156.7

MCV: Mean corpuscular volume

$^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}$ $p < 0.001$ compared with the time point "Before treatment"

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Group 1

There was no significant difference in red blood cells count, hematocrit, hemoglobin level, MCV and platelet count between groups treated MULBERRY powder and group 1 ($p > 0.05$) (Table 2).

Table 3. Effects of MULBERRY powder on total WBC count and WBC differentials

Parameters	Group	Before treatment	After treatment (weeks)		
			4	8	12
Total WBC count (G/L)	Group 1	10.64 ± 3.55	9.54 ± 3.86	10.47 ± 2.39	11.61 ± 3.36
	Group 2	8.53 ± 1.94	9.97 ± 1.89	11.36 ± 3.87	10.68 ± 3.41
	Group 3	9.92 ± 2.92	12.06 ± 2.74	10.59 ± 3.10	10.83 ± 1.75
Lymphocytes (G/L)	Group 1	7.6 ± 2.8	6.6 ± 2.8	7.4 ± 1.8	8.5 ± 2.7
	Group 2	6.1 ± 1.6	6.7 ± 1.2	7.0 ± 1.6	7.2 ± 2.7
	Group 3	6.8 ± 1.8	8.6 ± 1.7	7.5 ± 2.8	7.2 ± 1.8
Neutrophils (G/L)	Group 1	1.6 ± 0.4	1.4 ± 0.6	1.8 ± 0.9	1.4 ± 0.2
	Group 2	1.4 ± 0.5	1.7 ± 0.6	1.8 ± 1.0	2.3 ± 1.6
	Group 3	1.6 ± 0.5	1.7 ± 0.8	1.6 ± 0.5	1.8 ± 0.8

WBC: white blood cells

$^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}$ $p < 0.001$ compared with the time point "Before treatment"

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Group 1

Table 3 demonstrated that at all time points, there was no significant difference in total WBC count, lymphocytes, and neutrophils at groups treated MULBERRY powder compared with group 1 and the time point "before treatment" ($p > 0.05$).

4. Effect on liver parameters

There were no significant differences in aspartate aminotransferase (AST) level, and alanine aminotransferase (ALT) level, total bilirubin, albumin concentration, and total cholesterol concentration between groups treated MULBERRY powder and group 1 ($p > 0.05$). The results are shown in Table 4.

Table 4. Effects of MULBERRY powder on liver parameters.

Parameters	Group	Before treatment	After treatment (weeks)		
			4	8	12
AST level (UI/L)	Group 1	79.4 ± 17.0	78.6 ± 18.6	79.2 ± 28.8	76.7 ± 12.9
	Group 2	95.6 ± 24.8	101.4 ± 30.6	93.6 ± 26.8	77.7 ± 15.6
	Group 3	97.2 ± 24.0	101.0 ± 30.1	91.6 ± 28.1	89.2 ± 18.0
ALT level (UI/L)	Group 1	46.5 ± 9.6	36.7 ± 12.4	36.0 ± 13.5	37.1 ± 10.5
	Group 2	43.7 ± 7.9	47.3 ± 19.8	37.2 ± 11.1	36.7 ± 10.6
	Group 3	47.6 ± 10.7	38.2 ± 10.6	40.8 ± 9.7	38.9 ± 8.9

Parameters	Group	Before treatment	After treatment (weeks)		
			4	8	12
Total bilirubin (mmol/L)	Group 1	13.33 ± 0.55	13.44 ± 0.37	13.45 ± 0.48	13.44 ± 0.29
	Group 2	13.01 ± 0.76	13.46 ± 0.23	13.53 ± 0.39	13.38 ± 0.63
	Group 3	13.16 ± 0.76	13.62 ± 0.26	13.69 ± 0.48	13.55 ± 0.54
Albumin concentration (g/dL)	Group 1	3.22 ± 0.31	3.10 ± 0.23	3.27 ± 0.34	3.14 ± 0.48
	Group 2	3.28 ± 0.39	3.31 ± 0.34	3.12 ± 0.22	3.16 ± 0.25
	Group 3	3.19 ± 0.41	3.32 ± 0.36	3.23 ± 0.42	3.34 ± 0.33
Total cholesterol concentration (mmol/L)	Group 1	1.25 ± 0.25	1.22 ± 0.19	1.12 ± 0.13	1.24 ± 0.25
	Group 2	1.42 ± 0.25	1.38 ± 0.22	1.20 ± 0.23	1.23 ± 0.14
	Group 3	1.42 ± 0.27	1.40 ± 0.22	1.34 ± 0.18	1.34 ± 0.18

$^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}$ $p < 0.001$ compared with the time point "Before treatment".

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Group 1.

5. Effect on kidney function

Table 5 illustrated that MULBERRY powder caused no significant differences in serum creatinine level between groups treated MULBERRY powder and group 1 ($p > 0.05$).

Table 5. Effects of MULBERRY powder on serum creatinine level

Days		Creatinine level (mg/dl)		
		Group 1	Group 2	Group 3
Before treatment		0.86 ± 0.20	0.88 ± 0.09	0.87 ± 0.14
After treatment (weeks)	4	0.78 ± 0.11	0.78 ± 0.15	0.79 ± 0.14
	8	0.79 ± 0.17	0.76 ± 0.16	0.84 ± 0.17
	12	0.85 ± 0.14	0.80 ± 0.14	0.90 ± 0.19

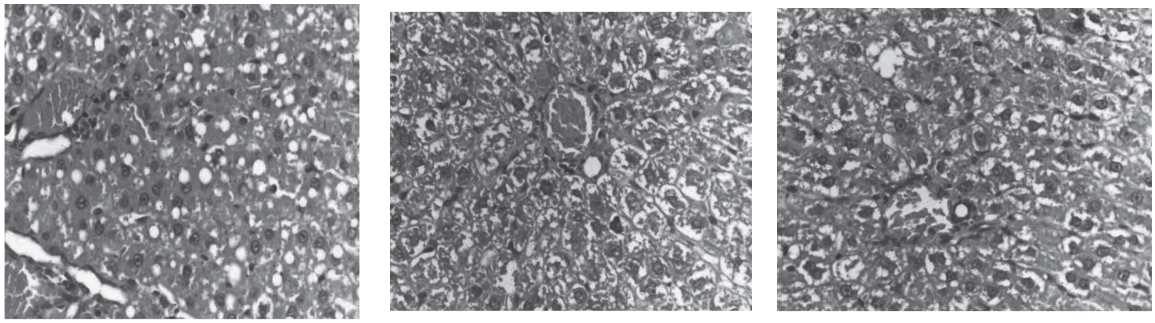
$^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}$ $p < 0.001$ compared with the time point "Before treatment"

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Group 1

6. Histopathological examination

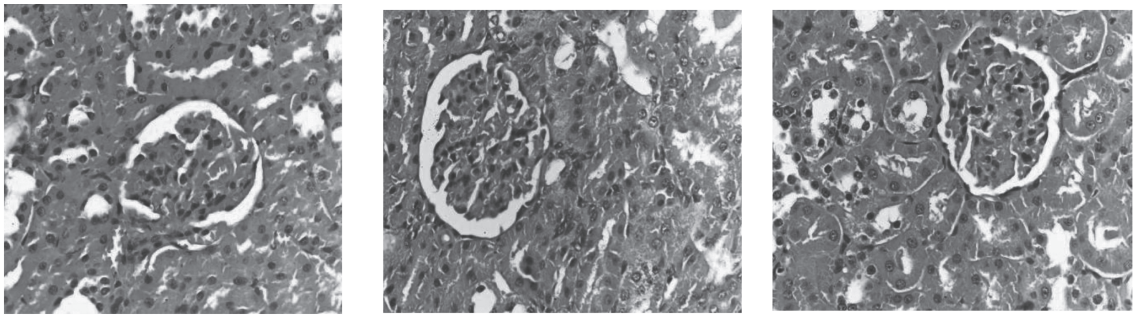
No gross lesions or changes in size were examined in the hearts, livers, lungs, kidneys, and abdominal cavities when all experimental rats were subjected to a complete gross necropsy.

There was no significant difference in histopathological examination of livers and kidneys between groups treated MULBERRY powder and the control group after 12 weeks of treatment (Figures 1 and 2).



Group 1 **Group 2** **Group 3**

Figure 1. Histopathological images of livers (HE × 400)



Group 1 **Group 2** **Group 3**

Figure 2. Histopathological images of kidneys (HE × 400)

IV. DISCUSSION

Subchronic toxicity of MULBERRY powder

Toxicity is the degree to which a substance can harm humans or animals. Toxicity can refer to the effect on a cell (cytotoxicity), an organ (e.g., renal or liver toxicity), or the whole organism. In order to determine the safety of drugs and plant products for human use, toxicological evaluation is carried out in various experimental animal models to predict the toxicity and provide guidelines for selecting 'safe' therapeutic doses in humans. A subchronic toxicity study provides information on the effects of repeated oral exposure and can indicate the need for further longer-term studies.[8,11] Subchronic studies assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds over a portion of the average life span of experimental animals, such as rodents. Specifically, they provide information on target organ toxicity.[12]

In general, during the experiment, rats in groups 1 and 2 showed normal conditions (normal locomotor activities and good feedings). However, in group 3, about 20% of rats administrated MULBERRY powder at the dose of *3.6 g/kg b.w/day* had diarrhea, loose stool, and wet anus. No significant change was observed in all hematological and biochemical parameters and histopathological results.

The body weight changes serve as a sensitive indicator of the general health status of

animals.[12] Weights were observed in all animals treated with MULBERRY powder. However, body weight gain at the dose of 3.6 g/kg b.w/ day decreased compared with groups 1 and 2, but no significant difference was observed compared with the *control group (group 1)*. It can be concluded that *MULBERRY powder* did not interfere with the normal metabolism of animals, as corroborated by the nonsignificant difference from animals in the control group.

The hematopoietic system is one of the most sensitive targets of toxic compounds and is an essential index of physiological and pathological status in men and animals.[8,11] After four weeks, eight weeks, and twelve weeks of the treatment, there were no significant differences in total red blood cells, hematocrit, hemoglobin level, platelet count, total WBC count, and WBC differentials between groups treated MULBERRY powder and control group. It could be concluded that the administration of MULBERRY powder did not affect the hematological profile and blood formation products. Furthermore, such analysis is relevant to risk evaluation as changes in the hematological system have higher predictive value for human toxicity when the data are translated from animal studies.

Analysis of kidney and liver function is critical in the toxicity evaluation of drugs and plant extracts as they are both necessary for the survival of an organism. The clinical biochemistry analyses were carried out to evaluate the plant products' possible alterations in hepatic and renal functions.[13] The liver releases AST, ALT, and an elevation in plasma concentration indicates liver damage.[8] There was no substantial change in AST level and ALT level between the group treated MULBERRY powder and the control group. These results indicated that MULBERRY powder had no deleterious effect on liver function.

Creatinine levels can be used in describing the function of the kidneys.[11] No significant difference was observed in blood biochemical parameters between the control group and groups treated MULBERRY powder at various dose levels ($p > 0.05$). Thus, MULBERRY powder did not affect liver and kidney function.

The histopathological examination revealed the alteration in cell structure when viewed under the light microscope. The further histological study could furnish more information regarding the hepatotoxicity and nephrotoxicity of MULBERRY powder. Our study showed no significant difference in histopathological examination of the liver and kidney between groups treated with MULBERRY powder and the control group.

Overall, the findings of this study indicated that no significant differences were observed in blood profile, biochemistry parameters, and histopathological observations of liver and kidney tissues between groups treated with MULBERRY powder and the control group.

Previous reports about the toxicity of *Amorphophallus konjac* K. Koch and *Morus alba* L. were still limited globally. A few clinical trials proved the safety of glucomannan (a soluble fiber derived from *Amorphophallus konjac*) in adults.[14] According to the study of Atiya sabsung, the oral administration of *Morus alba* L. leaf extract at doses 1.2 g/kg/day and 3.0 g/kg/day for 60 days did not significantly affect blood chemistry, hematologic values, and microscopic examination of *Wistar* rats.[15] Another report about the safety of *Morus alba* L. showed that no treatment-related mortality or adverse effects were observed at group treated M alba leaves extract at the highest dose of 4.000 mg/kg bw/d for both male and female rats.[16]

V. CONCLUSION

MULBERRY powder doses of 1.2 g/kg b.w/day and 3.6 g/kg b.w/day administered orally for 12 weeks did not cause toxic signs or evident symptoms of subchronic toxicity rats. About 20% of rats administrated MULBERRY powder at the dose of 3.6 g/kg b.w/day had diarrhea. However, no significant change was observed in all hematological and biochemical parameters and histopathological results.

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