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Mulberry seeds perform high hypoglycaemic effect partially by inhibition of α-glucosidase activity.

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Abstract

Mulberry is one of the Chinese traditional medicines. But few studies focused on the hypoglycemic effect of Mulberry seed and the possible mechanisms. We identified that mulberry seed is rich in DNJ, the content of which is 0.6015%. Both mulberry seeds powder and its aqueous extract were used to detect the hypoglycemic effect of mulberry seeds. The results indicated that mulberry seeds powder could reduce the FBG, PBG and HbA1c, and abate the organ injuries such as liver, kidney and spleen of diabetic mice. The results also showed that aqueous extract of mulberry seed powder orally to diabetic mice significantly improved the glucose tolerance of mice, and the mulberry seeds aqueous extract significantly inhibited the activity of sucrase and maltase *in vitro*. These suggested that Mulberry seeds perform high hypoglycemic effect by inhibition of α-glucosidase activity.

Keywords: Mulberry seed, Hypoglycemic effect, DNJ, α-glucosidase.

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Introduction

Morus alba L. was used as Chinese traditional medicine for hundreds of years and its fruit, mulberry, has been proved to have high nutritional value and perfect healthy function [1]. The content of mulberry seeds was about 3%-5% in fresh mulberry. Studies showed that mulberry seeds contain about 29%-33% oil including not less than 80% unsaturated fatty acid, 21%-29% crude protein, 31%-37% dietary fiber, 6%-11% carbohydrate, and 0.03%-0.1% total flavonoids. Besides, there are many kinds of nutrient substances and biological active compounds in mulberry seed, such as vitamin E, Morin, polyphenol and many microelements [2]. The mulberry seed had the function of reducing blood lipid, anti-atherosclerosis and anti-oxidants [3].

The bioactivity of DNJ attracted more and more attention. Predominant sericultural resources such as mulberry leaves and silkworm contain many kinds of alkaloidals [4] such as DNJ, flavonoid [5] and polysaccharides [6], which could decrease blood glucose through inhibiting activity of α-glucosidase [7], repairing islet cells and stimulating insulin secretion [8], promoting the synthesis of liver glycogen [9] and promoting the uptake of glucose into body cells [10]. While the hypoglycemic effect of mulberry seeds have not been reported up to now.

Our study determined the content of DNJ and other biological active compounds in mulberry seeds and its hypoglycemic effect, which could provide reference to developing hypoglycemic products from mulberry seeds.

Materials and Methods

Chemicals

The reagents were purchased from the following suppliers: 1-Deoxynojirimycin-HCl (DNJ-HCl), alloxan from Sigma Chemical Co. (St. Louis, MO, USA); 9-Fluorenyl-Methyl Chloroformate (FMOC-Cl) from Fluca (St. Gallen, Switzerland); Methyl Cyanides (chromatographic pure) from Kaitong Chemicals Ltd. (Tianjin, China); acarbose from Bayer Healthcare Ltd. (Leverkusen, Germany); Glucose Detection Kit (GOD-POD) from Dongou Biologicals Ltd. (Wenzhou, Zhejiang, China); liver glycogen Detection Kit and heparin from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China); rutin from Mingrui natural product Ltd. (Xi'an, Shanxi, China).

Determination of the content of active compounds in mulberry seeds

The mulberry seeds were obtained from a hybrid variety sha 2×lunjiao 109. The fresh dry seeds were shattered by medicinal

cracker, then sieved by 100 screen opening and preserved in 4°C. Some other mulberry seeds were germinated and sowed. The sprouted seeds, whole plant with cotyledon and the mulberry leave grown 15 d and 30 d respectively, were sampled respectively, and then dried under 60°C, shattered by medicinal cracker, passed through 100 mesh sieve and preserved in 4°C.

The DNJ content was detected by Ultraviolet-reverse-phase high-performance liquid chromatography (Dionex 500E High Performance Liquid Chromatograph, Dionex Co., Sunnyvale, CA, USA) [7]. Polysaccharides were detected by sulphate-anthrone method. Flavonoid was detected by Liuli's method [11-13].

Detection of hypoglycemic effect of mulberry seeds

Inducing of diabetic mice: Healthy SPF Kunming mice (21-25 g) purchased from Taibang Biologicals Ltd. (Taian, Shandong, China) (certification number: SCXK (Lu) 2009-0608) were used in the study. The mice were maintained in a sanitary environment at a room temperature of 23-26°C and under a 12 h light/dark cycle. This study was approved by the ethics committee of Shandong Agricultural University.

Diabetic mice were induced using Alloxan. The model was made successfully if the Fasting Blood Glucose (FBG) was up to 11-25 mmol/L. Based on FBG the mice were divided into different groups among which the difference was not more than 2 mmol/L.

Preparation of mouse feed

Basal feed was made. High sugar and high fat feed was composed of 15% sucrose, 15% lard and 70% basal feed. The mulberry seed feed was composed of 96% high sugar and high fat feed and 4% mulberry seeds powder. And 0.016% Acarbose was added to high sugar and high fat feed to make the Acarbose feed.

Impact of mulberry seed powder on FBG and physiological index of diabetic mice

Four groups with 10 mice in each were set. Each group were fed with one of the following diet: Normal Group (NG), normal mice fed on basal diet; diabetes model group (MGP), diabetic mice fed on high sugar and high fat feed; mulberry seed powder group (MSP), diabetic mice fed on mulberry seed feed; Acarbose group (ACP), diabetic mice fed on Acarbose feed. The Acarbose dose was equal to the conventional intake dose of diabetic patients.

During the test period, the appearance, activities, body weight, food intake and water intake of each group were observed.

After feeding for four weeks, the mice then fasted for 12 h. Tail blood of the mice were collected to measure FBG and glycosylated haemoglobin (HbAlc). Two hours after feeding carotid blood of mice was taken to determine the Postprandial Blood Glucose (PBG). The mice were sacrificed and the weight of liver, kidney and spleen were measured immediately. Then the liver was used to determine the liver glycogen content. The organ index was calculated according to the following formula:

Organ index=organ weight (mg)/body weight (g)

The blood glucose concentration was measured by Glucose-Oxidase (GOD-PAP) method. HbAlc was measured using affinity chromatography micro-column method in clinical laboratory of Taian Central Hospital. The liver glycogen was determined by oxidase method.

Preparation of the aqueous extract of mulberry seed powder

Quantitative mulberry seed powder was weighed out and 10 times the volume of distilled water was added into it. The mixture was extracted for 24 h at 90°C in the backflow device then ultrasonic processed for 40 min and filtered to obtain the supernatant. The residue was extracted following the previous procedure. The supernatants were mixed, then concentrated by decompress and vacuum freeze-dried. The yield was 10.16% and the extract was stored in 4°C.

Glucose tolerance test

Five treatment groups were set: Normal Group (NG), Model Group (MG), Acarbose group (AC), Low-dose aqueous extract of Mulberry seed powder group (LMS) and High-dose aqueous extract of Mulberry seed powder group (HMS) with 10 mice in each group. The four groups were diabetic mice except NG.

In the first 3 days, the mice were given different solution of the same volume (0.2 ml/mouse) by gavage every day. NG and MG were given physiological saline , AC was given Acarbose solution (6.25 mg/kg body weight), LMS and HMS were given low or high concentration solution of aqueous extract of mulberry seed powder respectively (LMS: equivalent to 100 mg crude drug/kg body weight. HMS: equivalent to 400 mg crude drug/kg body weight). After 3 days, all the mice were fasted 5 h. Then NG and MG were fed glucose with 2.0 g/kg body weight by gavage; AC, LMS and HMS were given the same amount of glucose and the corresponding ingredients, AC: 6.25 mg/kg body weight Acarbose, LMS: aqueous extract of mulberry seed equivalent to 100 mg crude drug/kg body weight, HMS: aqueous extract of mulberry seed equivalent to 400 mg crude drug/kg body weight. Blood glucose of each group was measured 0 min (A0), 30 min (A30) and 120 min (A120) after gavage respectively. The area under curve of the blood glucose was calculated using the following formula:

$GluAUC=0.25 \times (A0+4 \times A30+3 \times A120)$.

Starch tolerance test

The test method is basically the same as glucose tolerance test except that the starch was given to the mice with a dose of 4.0 g/kg body weight instead of glucose.

Inhibit activity of mulberry seed aqueous extract against α-glucosidase in vitro

The mulberry seed aqueous extract was dissolved in phosphate buffer (0.05 mol/L, pH 7.4, PBS) with the concentration equivalent to crude drugs of 0 g/L (blank), 10 g/L, 20 g/L, 40 g/L, 80 g/L, 120 g/L, 140 g/L, 160 g/L, 180 g/L, 200 g/L, 250 g/L and 300 g/L. Mulberry seed aqueous extract of each concentration was used to detect the inhibition effect on α-glucosidase. In the determination the concentration of these samples was diluted 10 times. The half Inhibitory Concentration value (IC_{50}) was calculated.

Statistical analysis

The results were presented as mean ± SD values. Tukey's test was used for multiple comparisons. P values of ≤ 0.05 were considered statistically significant. All statistical analyses were completed with SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Contents of hypoglycemic active material in mulberry seed and mulberry sapling

The results were showed in Table 1. There was a high concentration of DNJ in mulberry seeds (0.6015 ± 0.0055%). With the development of mulberry seed and mulberry sapling, its DNJ contents were gradually reduced, and basically stabilized 15 days after sprouting.

In addition, the content of DNJ in aqueous extract of mulberry seed was 2.7239 ± 0.0842%; the content of flavonoids and polysaccharides of mulberry seeds were 0.0345 ± 0.0079 % and 0.0023 ± 0.0006 % respectively.

Table 1. Change of DNJ content in development of mulberry seed and seeding.

Developmental stages	No accelerating germination mulberry seed	Accelerating germination mulberry seed	Sapling	Leave of sapling after 15 days development	Leave of sapling after 30 days development
DNJ content (%)	0.6015 ± 0.0055	0.4943 ± 0.0595	0.4517 ± 0.0091	0.1491 ± 0.0011	0.1395 ± 0.0011

The effect of mulberry seeds on blood glucose of diabetic mice

The test results were showed in Table 2. The FBG, PBG and HbA1c of MSP were significantly higher than NG but were significantly lower than MGP (decreased by 37.23%, 12.95% and 23.33% respectively). And the results of MSP had no significant difference with Acarbose group. The results indicted mulberry seed had significant hypoglycemic effect, and the hypoglycemic effect of feed adding 4% mulberry seed powder was close to routine dose of Acarbose. The liver glycogen contents of MSP, ACP and MGP had no difference between each other but were all significantly lower than NG, which showed mulberry seed did not promote the synthesis of liver glycogen.

Table 2. Effect of mulberry seed on sugar level of model mice.

Group	FBG (mmol/L)	PBG (mmol/L)	HbA1c (%)	Liver glycogen (mg/g)
Normal group (NG)	5.25 ± 0.76	9.03 ± 0.78	3.80 ± 0.27	16.22 ± 2.51
Model group (MGP)	14.21 ± 1.50[a]	17.14 ± 0.73[a]	9.00 ± 0.36[a]	7.02 ± 1.26[a]
Acarbose group (ACP)	7.93 ± 1.18[ab]	13.25 ± 2.67[ab]	7.20 ± 0.29[ab]	9.17 ± 1.32[a]
Mulberry seed group (MSP)	8.92 ± 2.37[ab]	14.92 ± 1.89[ab]	6.90 ± 0.21[ab]	8.85 ± 1.16[a]

Results are expressed as mean ± SD values of 10 individuals. [a]Statistically significant difference from the Normal Group (NG) at p ≤ 0.05. [b]Statistically significant difference from the model group (MGP) at p ≤ 0.05.

The effect of mulberry seed on intake, drinking amount and organs indexes of diabetic mice

In all test groups the mice of NG showed the best state, they had shiny white fur, showed an active state and had the lest drinking amount and urination volume; while MGP was the worst in the previous aspects. The mice of ACP and MSP showed whiter fur, had less drinking amount and urination volume and showed more active state than MGP, which suggested that the mulberry seed could improve the behavior state, metal status and physiological state of diabetic mice.

The results of intake showed that with the increase of test days the intakes of mice in MGP and MSP gradually increased (the increase of MSP was lower than MGP), while the intake of ACP decreased. The intake of ACP and MSP were respectively decreased by 45.39% and 17.02% than MGP at 28th day in this test. This showed mulberry seed had ability to reduce intake of diabetic mice.

The results showed that body weight of NG, ACP and MSP gradually increased in the test. The body weight of MGP slightly increased in earlier stage and then decreased. The body weight of NG, MSP, ACP and MGP respectively increased

13.5 g, 10.9 g, 9.6 g and 2.6 g by the 28th day in test. This showed mulberry seed could improve the symptom of weight loss of diabetic mice.

We got the liver, kidney and spleen weight of mice in all groups by anatomy at 28th day, and got their viscera indexes (Table 3). All the results of MSP had no difference with ACP. The liver weight, kidney weight, liver index and kidney index of MSP decreased by 13.35%, 13.20%, 36.56% and 35.86% respectively compared with than MGP, and the spleen weight increased by 31.74%. This showed mulberry seed could alleviate the injury of liver, kidney and spleen caused by diabetes.

Table 3. *Effect of mulberry seed on organ weight and index of model mice.*

Group	Liver weight (mg)	Renal weight (mg)	Spleen weight (mg)	Liver index ($mg.g^{-1}$)	Renal index ($mg.g^{-1}$)	Spleen index ($mg.g^{-1}$)
Normal Group (NG)	1180.6 ± 55.8	396.9 ± 23.0	129.0 ± 13.7	32.2 ± 1.5	10.8 ± 0.6	3.5 ± 0.3
Model Group (MGP)	1611.8 ± 53.5[a]	497.9 ± 11.7[a]	87.6 ± 8.6[a]	64.0 ± 2.1[a]	19.8 ± 0.5[a]	3.4 ± 0.3
Acarbose group (ACP)	1322.4 ± 54.4[ab]	426.2 ± 8.9[b]	112.4 ± 9.3[b]	41.7 ± 1.7[ab]	13.5 ± 0.3[ab]	3.6 ± 0.3
Mulberry seed group (MSP)	1396.6 ± 20.1[ab]	434.2 ± 10.6[ab]	115.4 ± 9.9[b]	40.6 ± 0.6[ab]	12.7 ± 0.3[ab]	3.4 ± 0.3

Results are expressed as mean ± SD values of 10 individuals. [a]Statistically significant difference from the Normal Group (NG) at $p \leq 0.05$. [b]Statistically significant difference from the model group (MGP) at $p \leq 0.05$.

Table 4. *Effect of mulberry seed on glucose tolerance of model mice.*

Group	Postprandial time (min)	Blood sugar value (mmol/L)	Area under the curve
Normal group (NG)	0	4.44 ± 0.50	17.08 ± 2.50
	30	11.13 ± 2.0[c]	
	120	6.45 ± 0.74[c]	
Model group (MG)	0	11.70 ± 2.44[a]	47.13 ± 11.43[a]
	30	28.00 ± 6.75[a]	
	120	21.61 ± 5.61[a]	
Acarbose group (AC)	0	11.20 ± 1.84[a]	36.91 ± 7.10[ab]
	30	22.58 ± 4.97[a]	
	120	15.37 ± 2.60[abc]	
Low-dose mulberry seed group (LMS)	0	11.10 ± 1.27[a]	38.15 ± 10.09[ab]
	30	22.49 ± 6.17[a]	
	120	17.18 ± 5.06[abc]	
High-dose mulberry seed group (HMS)	0	12.17 ± 2.17[a]	35.00 ± 9.01[ab]
	30	20.08 ± 5.40[bc]	
	120	15.83 ± 4.34[abc]	

Results are expressed as mean ± SD values of 10 individuals. [a]Statistically significant difference from the Normal Group (NG) at $p \leq 0.05$. [b]Statistically significant difference from the model group (MGP) at $p \leq 0.05$. [c]Statistically significant difference from the value of the same group at 0 m at $p \leq 0.05$.

Table 5. *Mulberry seed effect on starch tolerance of model mice.*

Group	Postprandial time (min)	Blood sugar value (mmol/L)	Area under the curve
Normal group (NG)	0	3.84 ± 0.56	20.38 ± 2.81
	30	12.15 ± 1.85[d]	
	120	9.68 ± 1.32[d]	
Model group (MG)	0	11.74 ± 1.20[a]	51.81 ± 10.24[a]
	30	30.48 ± 6.20[a]	
	120	24.52 ± 5.08[a]	
Acarbose group (AC)	0	11.43 ± 0.47[a]	33.06 ± 3.01[ab]
	30	19.37 ± 1.85[abd]	
	120	14.45 ± 1.99[abd]	
Low-dose mulberry seed group (LMS)	0	11.19 ± 1.26[a]	39.24 ± 6.28[abc]
	30	22.78 ± 4.04[abcd]	

	120	18.65 ± 2.81^{abcd}	
High-dose mulberry seed group (HMS)	0	11.03 ± 1.10^{a}	33.22 ± 4.21^{ab}
	30	19.88 ± 2.50^{abd}	
	120	14.12 ± 2.03^{abd}	

The effect of mulberry seed on glucose and starch tolerances of diabetic mice

The results of glucose tolerance were shown in Table 4. At 30 m after gavage the blood glucose level of HMS was significantly lower than MG. At 120 m after gavage the blood glucose level of AC, LMS and HMS were all significantly higher than NG, and lower than MG. The areas under the curve of blood glucose of AC, LMS and HMS were all significantly lower than MG. This showed mulberry seed could increase the glucose tolerance of diabetic mice.

The results of starch tolerance were shown in Table 5. At 30 m and 120 m after starch gavage, the blood glucose levels of AC, HMS and LMS were all significantly lower than MG, and the former two had no difference between them, and were both lower than LMS. The blood glucose level of HMS was decreased by 34.87% and 42.41% respectively at 30 m and 120 m than MG. The area under the curve showed the same pattern. This showed mulberry seed intake could effectively increase the starch tolerance of diabetic mice and its effect had relation with the dose.

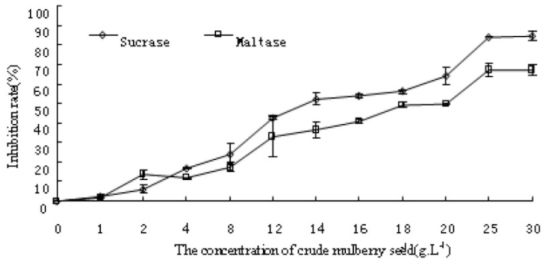

Figure 1. *The inhibition effect of the aqueous extract of mulberry seed on sucrase and maltase.*

The inhibition effect of aqueous extract of mulberry seed on sucrase and maltase

The results were indicated in Figure 1. The inhibition abilities of aqueous extract of mulberry seed were gradually increased with the increasing of concentration within a certain range, and its inhibition ability on sucrase was higher than maltase. The highest inhibition rate of both enzyme activities were 84.02% and 67.08% respectively at the concentration of aqueous extract equivalent to 25 g/L crude drug concentration. And there was a significant dose-effect relationship between concentration and the inhibition abilities. For sucrase the IC_{50} of crude drug concentration was 14.356 g/L (namely the aqueous extract concentration of 1459 mg/L) and good curve fitting ($p=0.104>0.05$). And for maltase the IC_{50} of crude drug concentration was 20.752 g/L (namely the aqueous extract concentration of 2109 mg/L) and good curve fitting ($p=0.151>0.05$).

Discussion

Mulberry seed was determined to the best hypoglycemic effect than other parts of mulberry [14,15]. This suggested that adding 4% mulberry seed powder to the fed had equivalent hypoglycemic effect to Acarbose of normal dose on chemical diabetic mice, which could improve glucose tolerance and three-in-more and one-in-less symptoms of diabetic mice significantly, and reduce the injury degree of liver, kidney and spleen caused by diabetes mellitus.

The hypoglycemic substances of mulberry now known mainly include DNJ and other alkaloids, flavonoids and active polysaccharides [16]. In the results, the content of DNJ in mulberry seed was the most among all parts of mulberry tree, it was approximately 3-6 times than that in mature mulberry leaves, and about 1.5-2 times than that in mulberry stalks skin or silkworm powder [14]. The results showed that the mulberry seed extract significantly inhibited the sucrase and maltase with a dose-effect relationship. At the same time mulberry seed didn't show obvious effect on promoting glycogen synthesis. All these results indicated that the hypoglycemic effect was related to DNJ with the high inhibiting activity on α-glucosidase.

Our study showed that mulberry seeds could improve the glucose tolerance of diabetic mice, which also indicated that the hypoglycemic effect of mulberry seed depend on the inhibit activity on α-glucosidase.

However, we found in another experiment that the skim mulberry seed powder using n-hexane and its aqueous extract showed significantly lower hypoglycemic effect than non-skim mulberry seed powder and its aqueous extract [14]. But DNJ is insoluble in n-hexane, therefore degreasing by n-hexane would not cause the loss of DNJ in mulberry seed powder, which suggested there might be hypoglycemic ingredients present in the fat-soluble ingredients of mulberry seed. Mentang et al. reported that silkworm chrysalis oil (mainly unsaturated fatty acids) had hypoglycemic effect [17,18]. Mulberry seed also contain large amounts of fats and unsaturated fatty acids, and whether they have hypoglycemic effect remains to be further studied. In addition, we cannot rule out the possibility of other unknown hypoglycemic substances in mulberry seeds.

Conclusions

In summary, Mulberry seed not only has a high DNJ content and more significantly hypoglycemic effect than the mulberry leaf tea, silkworm powder, silkworm sand granules, but also is more secure. It could be made into a variety of food and health products so that the mulberry seed have a high value of use and development.

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