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ORIGINAL ARTICLE

Extraction, Purification, Content Analysis and Hypoglycemic Effect of Mulberry marc Anthocyanin

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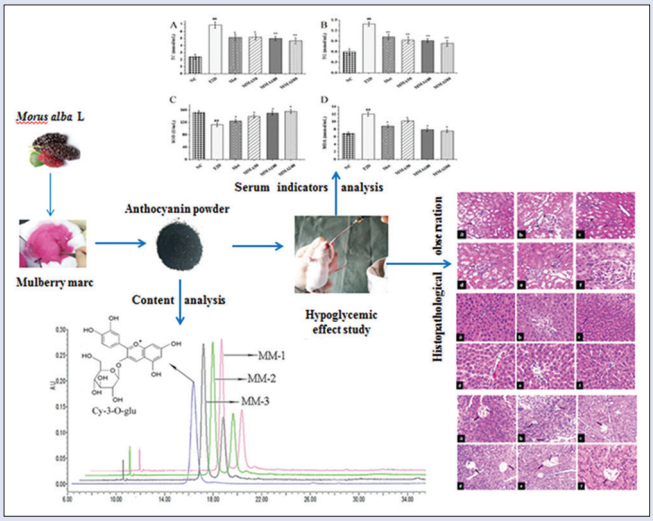
ABSTRACT

Background: Mulberry marc (MM) which as a wasted residue contains many available resources and one of the valuable nutrients is anthocyanin. In this study, the extraction, purification, content analysis, and hypoglycemic effect of anthocyanin from MM were studied. **Materials and Methods:** The total anthocyanin and cyanidin-3-O-glucoside of the purified anthocyanin of mulberry marc (MMA) were determined using pH differential method and high-performance liquid chromatography. The hypoglycemic effects were studied by streptozotocin and high-fat diet-induced type 2 diabetic rats. The rats were given MMA for 4 weeks and at the end of experiment; the blood was collected for determination serum indexes; the tissue of liver, kidney, pancreas, and spleen was removed for weighting and observing pathological changes. **Results:** The content of total anthocyanin and cyanidin-3-O-glucoside of MMA was 39.72% and 18.63%, respectively. The fasting blood glucose of rats in the MMA treatment groups was decreased. The lipid metabolites (triglycerides and total cholesterol) and malondialdehyde were decreased and superoxide dismutase was increased of type 2 diabetes rats after MMA treated; the pathological changes were alleviated in various degrees. **Conclusion:** The content of anthocyanin in mulberry marc is higher than mulberry juice, and the MMA has a good hypoglycemic effect.

Key words: Anthocyanins, extraction, mulberry marc, pathological, type 2 diabetic

SUMMARY

- Mulberry marc (MM) can be used as a resource for anthocyanin extraction
- The total anthocyanin of MM is much higher than that of mulberry juice
- Purified anthocyanin of MM has a good effect of hypoglycemia and improving type 2 diabetes.



Abbreviations used: MMA: Purified anthocyanin of mulberry marc; STZ: Streptozotocin; T2D: Type 2 diabetes; ROS: Reactive oxygen species; RNS: Active nitrogen species; MM: Mulberry marc; Cy-3-O-glu: Cyanidin-3-O-glucoside; HPLC: High-performance liquid chromatography; NC: Normal; Met: Metformin; FBG: Fasting blood glucose; OGTT: Oral glucose tolerance test; SOD: Superoxide dismutase; MDA: Malondialdehyde; TC: Total cholesterol; TG: Triglyceride.

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INTRODUCTION

There were 425 million in the world suffered from diabetes according to the public health data.[1] Moreover, the prevalence of diabetes is predicted to increase to 592 million by 2035.[2] It is the main disease that causes rising mortality and is a global health problem of which type 2 diabetes (T2D) accounting for 90%.[3] It characterized by hyperglycemia will cause a serious metabolic disorder, such as glucose, lipid, and protein.[4-6] Furthermore, T2D is also associated with many complications such as nephropathy and cardiovascular and cerebrovascular diseases.[7] Most diabetics have hyperlipidemia, which suggested that lipid metabolism may contribute to insulin resistance.[8] Moreover, numerous documents reported that oxidative stress is closely related to the occurrence of diabetes. Hyperglycemia-generated reactive oxygen species (ROS) and active nitrogen species will damage the pancreatic islet β-cell and compromises the quantity and quality of

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insulin secreted.[9,10] Insulin resistance and islet β-cell dysfunction are the fundamental mechanisms of diabetes, and reducing oxidative stress to protection islet β-cells may be an effective method for treatment of T2D.[11-14] At present, drug therapy combined with diet has been a conventional strategy against diabetes, specific components of some functional foods have proven to be of important health value, and dietary intervention may be a significant method to prevention and therapy of T2D.[15,16]

Anthocyanin is a kind of water-soluble natural pigment widely existed in plants, which belongs to flavonoids that can show different colors with the changes of season and cell fluid composition.[17] Moreover, its molecular structure is small, which is easy to be absorbed. It has reported that anthocyanin has various functions including antioxidant, anti-inflammatory, antibacterial, antiaging, anticancer, and protective effects on the liver, cardiovascular and cerebrovascular, and vision.[18] Mulberry is a kind of fruit that sets in both nutrition and medicine and has the reputation "the best health product of the 21st century," which has multiple nutritional ingredients such as flavonoid, polysaccharide, and vitamin.[19] To our knowledge, the utilization rate of the mulberry marc (MM) is very low after the mulberry juice is squeezed. It is mainly wasted or a small amount used for feed.[20] However, MM has multiple nutrient substances and will be deodorized rotten quickly when discarded in the environment, which not only wastes resources but also causes environmental pollution. Therefore, it is particularly important to study how to improve the utilization value of MM. In this paper, we extracted and purified anthocyanin of Mulberry marc (MMA) and then studied it's hypoglycemic effect and mechanism to provide the theoretical basis for improving the utilization value of MM.

MATERIALS AND METHODS

Materials

Mulberry (*Morus alba L.*) from dashi breed was collected from Zhejiang, China, and identified by Zhi-Qiang Lv from Zhejiang Academy of Agricultural Sciences. Cyanidin-3-O-glucoside (Cy-3-O-glu) standard and streptozotocin (STZ) were purchased from Sigma Chemical Co. (Shanghai, China). Glucose, triglycerides, total cholesterol, malondialdehyde, and superoxide dismutase kits were purchased from Nanjing Jiancheng Co., Ltd. (Nanjing, China). Metformin was purchased from Yunnan Kunming Co., Ltd (Kunming, China).

Preparation of purified anthocyanin of Mulberry marc

Five kilograms of fresh fruit of mulberry was squeezed and filtered, and the obtained MM was ultrasonically extracted with 50% ethanol solution (pH=2.0 ± 0.1) for three times (40 min/time). The extract was concentrated by flash evaporation concentration method until there was no ethanol and the MM concentrate was obtained. The concentrate was passed through disposed resin columns (Diaion HP 2MGL) and eluted with water and then with acidified 60% ethanol (pH=3.0 ± 0.1) until there was no color in the eluent. The eluent was concentrated on a rotary evaporator (RV3V, Staufen, Germany) under reduced pressure at 60°C to get MMA purification power. The flowchart is shown in Figure 1.

Determination of total anthocyanin in MM

Potassium chloride and hydrochloric acid buffer with pH 1.0 and hydrochloric acid and sodium acetate buffer with pH 4.5 were prepared. The purified anthocyanins from MM and mulberry juice were dissolved with 10% diluted alcohol. They were immobilized in the buffer solutions of pH 1.0 and pH 4.5. After incubation at dark for 1 h, the absorbance value was determined using a UV-2102 PCS UV spectrophotometer (Shanghai UNICO Co., Ltd., Shanghai, China), and the total anthocyanin was calculated using equation (1).

$$TA\ (w/w) = (A \times M \times DF \times V)/(\varepsilon \times L \times Wt)$$

Where TA is total anthocyanin, A = (A510 nm pH 1.0 – A700 nm pH 1.0) – (A510 nm pH 4.5– A700 nm pH 4.5), M is the molecular weight, DF is the dilution factor, V is the volume, ε is the extinction coefficient, L is the optical path, and Wt is the fruit weight.

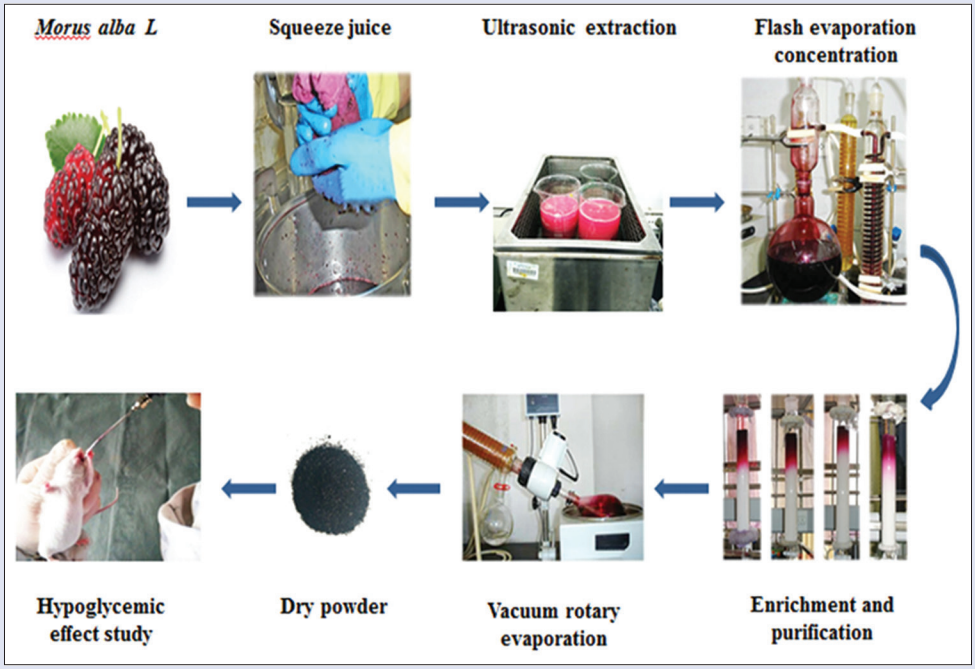

Figure 1: The experimental flowchart

Quantitation of cyanidin-3-O-glucoside in mulberry marc by high-performance liquid chromatography

The sample solution and the standard solution were prepared by dissolving the MMA (0.01 g) and Cy-3-O-glu standard sample (0.002 g) in methanol solution of 2% hydrochloric acid (pH = 3) and volume to 10 mL, respectively. Moreover, the standard solution was then diluted with methanol solution of 2% hydrochloric acid to prepare standard solutions with concentrations of 0.001, 0.002, 0.003, 0.004, 0.005, and 0.006 mg/mL, respectively. Moreover, the standard curve was prepared with the peak area as the ordinate and the mixed standard solution concentration as the abscissa. The content of Cy-3-O-glu in MMA was determined using the prepared standard curve. The peak area of samples was determined by high-performance liquid chromatography (HPLC) (Waters 2695 Milford, MA, USA), which includes Waters 2695 separation unit, type 2996 diode array detector, evaporative light scattering detector 2420, and chromatographic management workstation. Sample analysis was performed with SunFire-C18 column (4.6 mm × 250 mm, 5 μm). The mobile phase is 2% hydrochloric acid methanol (A) and water/methanol/acetonitrile/acetic acid at a ratio of 160:90:90:40 (v/v/v/v) (B). Using an isocratic elution with the ratio of A:B as 93:7 at a flow rate of 1.0 mL/min, detection wavelength was set at 530 nm. The measurements were performed in triplicate.

Experimental animals

Standard deviation (SD) male rats (180–200 g) were provided by the experimental animal center, Zhejiang Academy of Medical Science, China (SCXK 2014-0001). They were adaptive feeding with 23°C ± 1°C and a humidity of 50% ± 5% with a 12/12-h light–dark cycle for 1 week before starting experiments. All experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of Zhejiang and were approved by the Committee on the Ethics of Animal Experiments at Animal Center of Zhejiang Agriculture and Forestry University (Ethics Certificate No. 2014001812184).

Experimental design

All rats were divided into two groups: normal (NC) group (n = 10) and STZ-injected group (n = 50). The NC group was fed with chow diet consisting of 20% casein, 70% corn starch, and 10% corn oil and milk fat. The STZ-injected group was administrated with high-fat diet which contains 20% casein, 20% corn starch, and 60% corn oil and milk fat[21] and 25% sucrose water for 4 weeks. Then, the rats in the STZ-injected group were injected intraperitoneally with STZ (30 mg/kg).[22] After STZ injection, the STZ-induced rats were randomly divided into five groups (n = 10): type 2 diabetic (T2D), metformin (Met), MMA 50 mg/kg, MMA 100 mg/kg, and MMA 200 mg/kg groups. The rats of the NC and T2D groups were intragastrically (ig) equivalent volume of normal saline, Met group was ig metformin (100 mg/kg), and MMA 50, 100, and 200 groups were ig 50, 100, and 200 mg/kg total anthocyanin of MMA for 4 weeks. At the end of experiment, the rats were sacrificed under deep anesthesia (ip. chloral hydrate) after fasting overnight, and blood was collected and then centrifuged immediately. The livers, kidneys, pancreas, and spleen were collected, washed with cold phosphate-buffered saline, and weighed and conserved in 10% neutral formaldehyde for further study.

Fasting blood glucose measurement and oral glucose tolerance test

Fasting blood glucose (FBG) was measured by glucose kit according to the commercial instruction. The oral glucose tolerance test (OGTT) was performed after rats were fasted overnight in the 3rd week of treatment. All animals received a load of 1.5 g of glucose/kg body weight. Blood was sampled from the tail vessels of conscious animals before load t = 0, 30, 60, and 120 min after glucose administration. Glucose was determined by a glucose kit.

Biochemical measurements

The superoxide dismutase (SOD) malonyldialdehyde (MDA), total cholesterol (TC) and triglycerides (TG) of serum were respectively measured using commercially available kits according to the commercial instruction.

Histopathological examinations

Liver, kidney, and pancreas were excised from the rats at the end of the experiment and fixed in 10% neutral formaldehyde for 24 h and then paraffin-embedded. Paraffin sections of 5–7 μm thickness were carved up and stained with hematoxylin and eosin. Histopathological changes were observed under a light microscope (BX20, Olympus, Tokyo, Japan).

Statistical analysis

All data were expressed as mean ± SD and analyzed by the SPSS statistical software (SPSS 19.0 Inc., Chicago, IL, USA). One-way ANOVA with Duncan's test was used for inter-group comparison. $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered extremely significant.

RESULTS

The content of total anthocyanin and cyanidin-3-O-glucoside in mulberry marc

Mulberry is a kind of fruit that is rich in anthocyanin. The total anthocyanin from purified sample of MM was determined as 385.31 ± 4.73 mg/g which is higher than mulberry juice (152.26 ± 2.25 mg/g). The content of total anthocyanin could reach 39.72% of MMA. The determined regression equation of Cy-3-O-glu was y = 3958554x-782711.3, R^2 = 0.9992, and the content of Cy-3-O-glu was measured as 186.3 mg/g, which was about 50% of total anthocyanin. It is supposed that Cy-3-O-glu is the major component of MMA. The HPLC chromatogram of Cy-3-O-glu and MMA is shown in Figure 2.

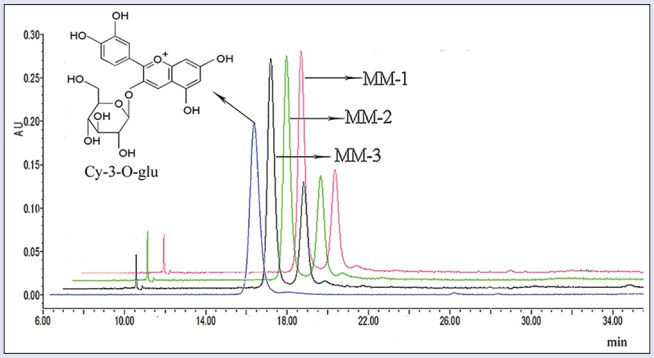

Figure 2: High-performance liquid chromatography chromatogram of Cy-3-O-glu in standard liquid and purified anthocyanin of mulberry marc. The MM-1, MM-2, and MM-3 standing for the purified anthocyanin of mulberry marc was repeated measured for three times

Effects of purified anthocyanin of mulberry marc on body and organ weight of rats

As shown in Figure 3a, the body weight of rats was significantly ($P < 0.01$) lower after STZ induced compared with the NC group. With the duration of the medication, the weight of the Met and MMA treatment groups increased continually, and after 4 weeks of administration, they were significantly ($P < 0.01$) higher than that of the T2D group. Moreover, the result of organ weight was showed in Figure 3b, compared with the NC group; the weight of liver, kidney, and spleen was significantly ($P < 0.01$) increased in the T2D group, whereas the Met and MMA treatment groups were decreased when compared with the T2D group. Moreover, there is a dose-dependent in the MMA treatment groups.

Effects of purified anthocyanin of mulberry marc on oral glucose tolerance test and fasting blood glucose of rats

As shown in Figure 4b, the FBG was significantly ($P < 0.01$) increased in the T2D group when compared with the NC group, while significantly decreased in the Met and MMA treatment groups when compared with the T2D group. OGTT is used to detect abnormal glucose metabolism in patients.[23] The result of OGTT is shown in Figure 4a; the glucose level of the T2D group was significantly ($P < 0.01$) higher than the NC group in at 0, 30, 60, and 120 min; it indicated that insulin resistance may have been occurred.[24] The glucose level in the Met and MMA treatment groups was lowed in a degree at 0, 30, 60, and 120 min compared with the T2D group, and there is a dose-dependent in the MMA treatment groups.

Effects of purified anthocyanin of mulberry marc on total cholesterol, triglycerides, superoxide dismutase, and malondialdehyde of rats

As shown in Figure 5, compared with the NC group, the content of TG, TC, and MDA in serum was significantly ($P < 0.01$) increased in the T2D group, while the content of SOD in serum was significantly ($P < 0.01$) decreased. Conversely, the SOD in the Met and MMA treatment groups was significantly ($P < 0.05$) increased and TG, TC, and MDA were significantly ($P < 0.05$) decreased compared with the T2D group, and there is a dose-dependent.

Effect of purified anthocyanin of mulberry marc on histopathological changes of rat liver

The histopathological changes of the liver are showed in Figure 6; the liver tissue structure of NC group was intact and hepatocytes were arranged radially around the central venous center. Compared with the NC group, the hepatocyte cytoplasm was found lipid droplets and hepatic cord disorder of rats in the T2D group; there were obviously necrosis, degeneration, and infiltration of inflammatory cells. Compared with the T2D group, liver pathological changes of rats in the MMA treatment groups were improved in varying degrees.

Effect of purified anthocyanin of mulberry marc on histopathological changes of rat kidney

The histopathological changes of the kidney are shown in Figure 7; the kidney structure was clear and the glomerular structure was regular of rats in the NC group. Compared with the NC group, the glomerular volume of rats in the T2D group was enlarging and the base membrane

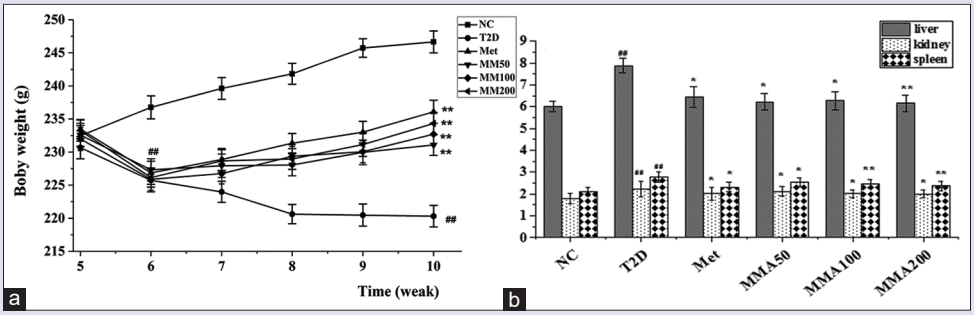

Figure 3: Effect of purified anthocyanin of mulberry marc on body weight (a) and organ weight of rats (b). The data were expressed as mean ± standard deviation ($n = 10$), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus NC group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus type 2 diabetes group

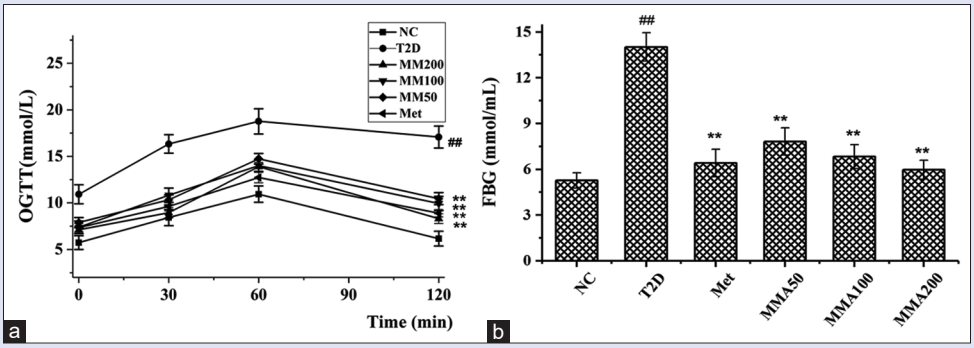

Figure 4: The effect of purified anthocyanin of mulberry marc anthocyanin on oral glucose tolerance test (a) and fasting blood glucose (b) of rats. The data were expressed as mean ± standard deviation ($n = 10$), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus NC group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus type 2 diabetes group

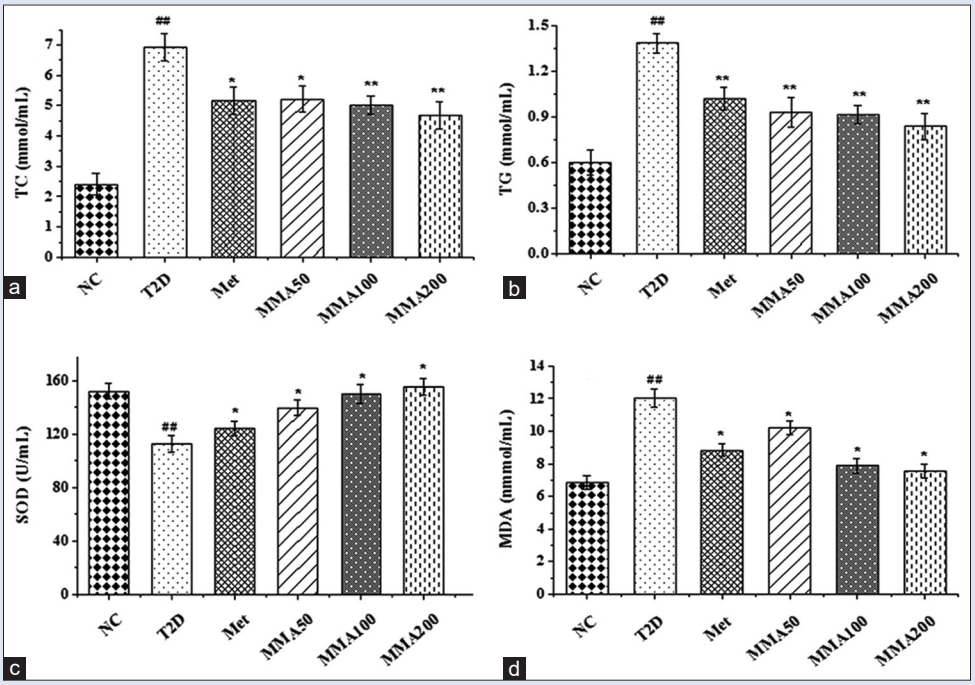

Figure 5: The effect of purified anthocyanin of mulberry marc on total cholesterol (a), triglycerides (b), superoxide dismutase (c) and malondialdehyde (d) of rats. The data were expressed as mean ± standard deviation ($n = 10$), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus NC group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus type 2 diabetes group

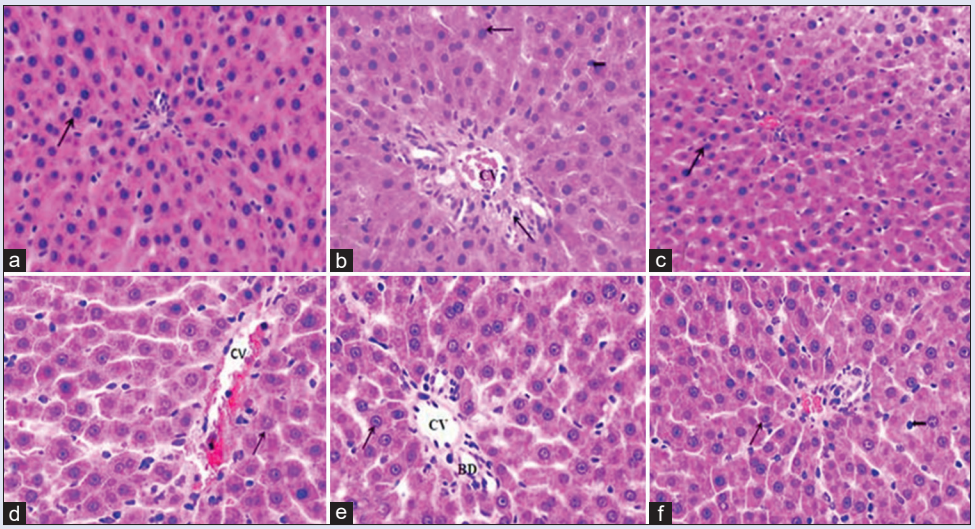

Figure 6: Effect of purified anthocyanin of mulberry marc on histopathological of the liver. Histological observation (a, c × 200, b, d × 400), (a) NC group, (b) type 2 diabetes group, (c) Met group, (d) 50 mg/kg group, (e) 100 mg/kg group, (f) 200 mg/kg group, (↑): Hepatocyte; (↑): Inflammatory cell; CV: Central venous; BD: Bile ductile

thickening; the lumen was expanded, and the epithelial cells were vacuolated. Compared with the T2D group, the glomerular volume was decreased of rats in the MMA treatment groups, the number of mesangial cells decreased, and the mesangial matrix was mild hyperplasia.

Effect of purified anthocyanin of mulberry marc on histopathological changes of rat pancreas

The histopathological changes of the kidney are shown in Figure 8; the morphology of islet in the NC group was complete, the boundary between the islet and exocrine glands is clear, the number of cells in the islet is very large, and the arrangement is closely uniform. Compared with the NC group, pancreatic islet was an obvious modification of rats in the T2D group, volume was smaller, and the number of cells in the islet decreased; the boundary was vague and the structure is unclear. Compared with the T2D group, the changes of islet tissue in each MMA treatment groups were improved in various degrees.

DISCUSSION

T2D is an endocrine and metabolic disease based on pathological of insulin resistance insulin secretion defects. STZ is a hydrophilic

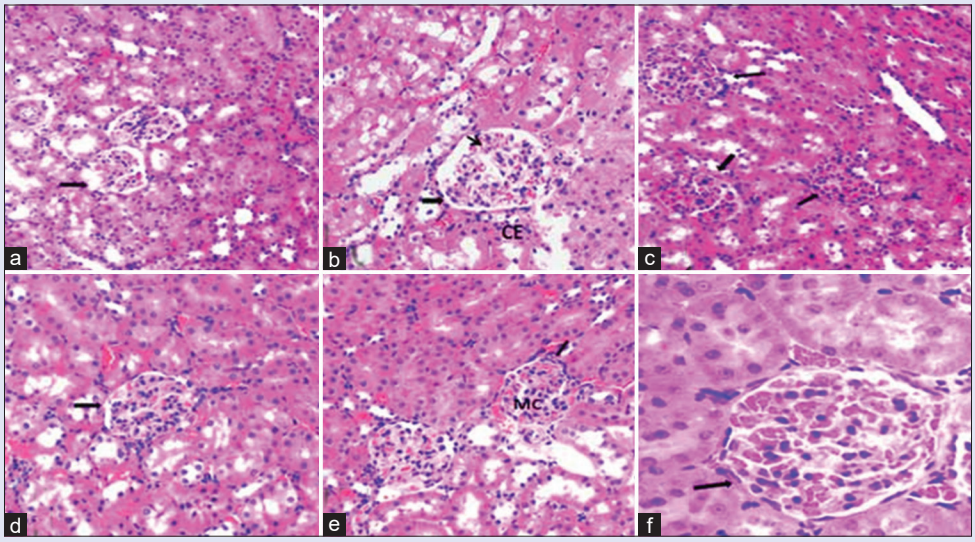

Figure 7: Effect of purified anthocyanin of mulberry marc on histopathological of the kidney. Histological observation (a-e, ×200, f, ×400), (a) NC group, (b) type 2 diabetes group, (c) met group, (d) 50 mg/kg group, (e) 100 mg/kg group, (f) 200 mg/kg group, (↑): Glomerular; (↑): Base membrane; CE: Epithelial cell; MC: Mesangial cell

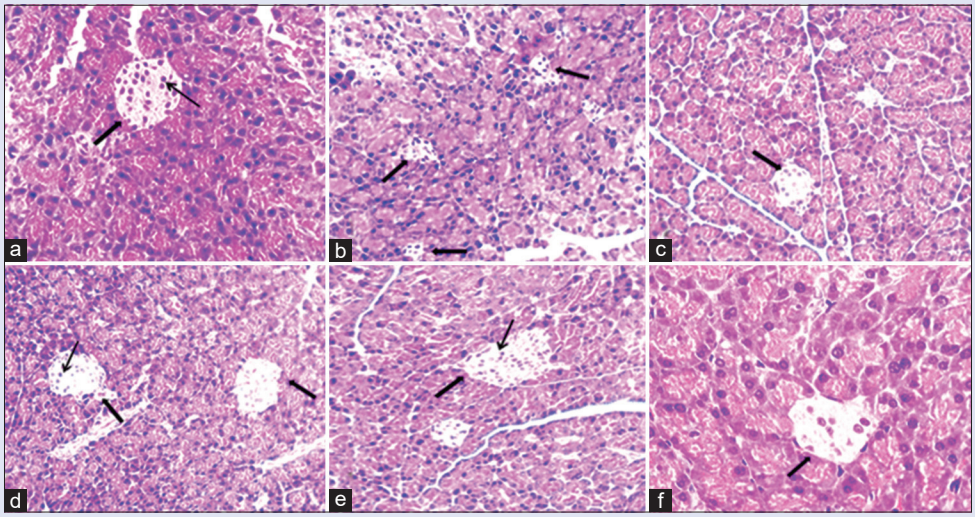

Figure 8: Effect of purified anthocyanin of mulberry marc on histopathological of the pancreas. Histological observation (a-f, ×200), a NC group; b, type 2 diabetes group; c, Met group; d, 50 mg/kg group; e, 100 mg/kg group; f, 200 mg/kg group; (↑): Islet cell; (↑): Islet

compound that can pass cellular membrane via GLUT2 transporters[25] which make DNA alkylation and lead to β-cell apoptosis and eventually develop diabetes.[26] Furthermore, GLUT2 transporters are not only expressed in the pancreas but also in liver and kidney, and this is the reason why STZ can injure these organs.[27] The fat-enriched diet has an important influence on insulin resistance.[28] The T2D was induced by high-fat diet combined with STZ which can well simulate the development of human diabetes and changes that gradually occur in metabolism of humans.[29] We found that the body weight was obviously ($P < 0.05$) decreased, and FBG ($P < 0.01$) was significantly increased after induced by high-fat diet and STZ. The average weight of induced rats in each group was at least lighter than the NC group 9.44 g and FBG was higher 8.74 mmol/mL. It indicated that the T2D was occurred.

Many clinical researches showed that abnormal blood glucose metabolism is often accompanied by abnormal blood lipid metabolism.[30] TG and TC are the main parameters of lipid metabolism in diabetic patients; the rate of synthesis of TC is altered by the influence of insulin, that is to say, insulin can promote the conversion of sugars to TC.[31] In general, patients with T2D have insulin resistance which will cause the increase of TC. In general, the TG was esterification by free fatty acids in peripheral circulation that released from fat tissues[32] or by acetyl-CoA which is the glucose oxidation product.[33] Under normal physiological conditions, esterification of free fatty acids dominates the TG generation, followed by glycolipid conversion. Moreover, in T2D patients, the TC produced by glycolipid conversion will increase. The increase of TG and TC is a risk factor for complications of cardiovascular and cerebrovascular diseases in diabetes.[34,35] Therefore, improving lipid metabolism can normalize glucose metabolism. Our study showed that TC and TG levels were significantly ($P < 0.05$) decreased and the reduction range is 1.71–2.25 and 0.46–0.55 mmol/mL in the MMA treatment groups when compared to the T2D group; it indicated that MMA can improve the T2D rats' lipid metabolism.

In patients with T2D, the body cannot fully utilize glucose because of dysglycemia which accelerates the decomposition of fat and protein to supplement energy and calories. As a result, the body's carbohydrates, fats, and proteins are hugely consumed, coupled with the loss of water, leading to weight loss.[36] Moreover, in hyperglycemia, free radicals can come from many pathways; the free radicals are closely related to the activation of angiotensin II.[37] Studies have shown that the content of angiotensin II is increased in T2D.[38] Angiotensin II is not only a vasoactive substance but also a growth factor that can induce hypertrophy, proliferation of renal cells, and protein production to cause an increase of kidney weight.[39] In recent decades, a large number of studies have proved that T2D is an autoimmune disease, which is related to multi-organ inflammation, and the spleen, as one of the main organs of immune response, may gain weight due to splenomegaly caused by inflammation.[40] In addition, clinical and pathophysiological studies have shown that the liver and pancreas of T2D patients would accumulate excessive fat, which will lead to an increase in their liver weight.[41] Our results showed that body and organ weight was significantly ($P < 0.05$) reversed after 4-week MMA treatment, the range of body weight increased was 10.76–14.01 g, and the range of the liver, kidney, and spleen decreased was 1.66–1.73, 0.11–0.23, and 0.24–0.41 g in the MMA treatment groups, respectively, which may indicate that diabetes was improved after MMA treatment.

Metabolic disorders of lipids and glucose can cause the increase of oxidative stress level and damage the antioxidant defense system in T2D patients,[42] and the oxidative stress is defined as excessive of ROS, such as superoxide, hydrogen peroxide, and hydroxyl radical ions.[43] Excessive ROS will destroy the basic structure of cells, induces mitochondrial division, and results in actions on both the insulin receptor pathway and stress proteins.[44] In addition, the increased ROS will regulate the expression of Bcl-2 and induce the changes of mitochondrial membrane potential, leading to the release of cytochrome C, and then activate the caspase cascade reaction, leading to apoptosis. Therefore, insufficient insulin secretion is appeared because quantity and quality of β-cells are reduced.[45] Furthermore, the accumulation of oxidation products will directly damage the sensitive macromolecules in the islet tissue and reduce the sensitivity of insulin.[46] MDA is a biomarker of intensified lipid peroxidation and also indirect evidence of high free radical production in diabetes.[47] SOD plays a pivotal role in the balance of oxidation and antioxidation, scavenging free radical and protecting against cell damage.[48] As a strong antioxidant, we found that the SOD was significantly ($P < 0.05$) increased (26.88–43.32 U/mL) while MAD significantly ($P < 0.05$) decreased (1.83–4.46 mmol/mL) in the MMA treatment groups compared to the NC group. It suggested that MMA increased antioxidant capacity of T2D rats.

Persistent hyperglycemia can cause damage to various tissues, especially the kidney, pancreas, and liver, which can aggravate the disease and cause a series of complications. The liver plays an important role in maintaining blood glucose homeostasis, balancing the uptake, storage, and production of glucose via glycogenesis, glycogenolysis, and gluconeogenesis.[49] Therefore, liver injury will lead to excessive glucose output, increased the expression of gluconeogenesis, and reduced glycogen synthesis, resulting in hepatic insulin resistance.[50] Insulin is secreted by irregular cells scattered in the pancreas, and the destruction of the pancreas directly affects the secretion of insulin and aggravates diabetes. It was found in the pathological observations that the pathological changes of liver, kidney, and pancreas of diabetic rats treated with MMA were obviously improved, which suggested that the MMA can improve diabetes and its complications.

CONCLUSION

The study concluded that the content of anthocyanin in MM was very high, and there was a good hypoglycemic effect of MMA. Moreover, it possessed hypoglycemic and amelioration diabetes properties by improving glucose and lipid metabolism and decreasing oxidative stress and thereby reduced insulin resistance and β-cell apoptosis. MMA is expected to become an inexpensive natural antioxidant and hypoglycemic agent as a raw material for the development of hypoglycemic functional foods and health-care products, so as to realize waste utilization, save resources, and protect the environment.

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Conflicts of interest

There are no conflicts of interest.

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