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Studies on the extraction of pumpkin components and their biological effects on blood glucose of diabetic mice

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ABSTRACT

Pumpkin crude extract (PCE) was extracted from pumpkin powder with water and 95% ethanol at 60°C. Several components were isolated and further purified by solvents purification and dialysis as well as column chromatography. The anti-diabetic activities of the hypoglycemic components extracted from pumpkin were identified using diabetic model mice which were induced by alloxan intraperitoneal injection. The diabetic mice were treated with pumpkin extracts by intraperitoneal injection at dosages of 100, 200 and 400 mg/kg body weight. Blood samples for glucose assays were taken from the diabetic mice before injection and 4, 7 and 11 hours after injection. The results showed that the blood glucose levels in the diabetic mice were significantly reduced by PCE-C from 15.32 ± 4.38 mM to 5.77 ± 1.46 mM ($p < 0.001$) in 7 hours. PCE-C was further purified by deproteinization, dialysis and Sephadex G-100 column chromatography and a fraction as PCE-F was collected. Seven hours after PCE-F injection at a dosage of 200 mg/kg body weight, blood glucose levels dropped significantly from 15.90 ± 3.21 mM to 7.19 ± 2.54 mM ($p < 0.01$). PCE-F was identified by gas chromatography as a polysaccharide consisting of heterogeneous monosaccharides, such as glucose, galactose, arabinose and rhamnose.

1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by a high level of glucose in the blood due to the non-secretion of insulin or insulin insensitivity. Diabetes mellitus is considered a common, growing, serious, costly, and potentially preventable public health problem. The number of people with diabetes is estimated to increase from 117 million in 2000 to 366 million in 2030. Individuals, families and nations will continue to bear the economic burden of healthcare due to the prevalence of diabetes [1]. Current therapeutic options include lifestyle adjustments (exercise and diet), oral hypoglycemic agents (factors that enhance the activity of the β-cells) [2,3], inhibitors of α-glucosidase at the level of the small intestine [4,5], factors that enhance peripheral insulin sensitivity [6,7], and insulin treatment [8,9]. However, the drugs used to treat this disorder are expensive, have side effects or contraindications. Dietary intervention, particularly the use of traditional food and medicine derived from natural sources, is a mainstay in the management of diabetes mellitus [10]. Natural plant-derived drugs provide several potential options for the control of diabetes [11,12]. These plant-derived components may be less toxic and have fewer side effects than synthetic agents [13].

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Pumpkin (*Cucurbita moschata* Duch, a member of the Cucurbitaceae family) consists of succulent coarse vine and numerous seeds. It is a popular traditional healthy food with hypoglycemic and anti-diabetic activities. It has been reported that *Cucurbita* species exhibit diverse pharmacological activities [1], including antioxidant activities, antimicrobial and hypoglycemic effects [14–21]. Li et al reported that protein-bound polysaccharides extracted from pumpkin significantly increased levels of serum insulin, reduced blood glucose levels and improved glucose tolerance [22,23]. Zhu et al found that pumpkin polysaccharides can decrease the blood glucose level of diabetic rats, enhance the activity of superoxide dismutase, reduce the production of malondialdehyde and nitrogen monoxide, and improve the ultra-structural features of islet cells [24].

In this study, we isolated and purified the most effective hypoglycemic components from pumpkin, investigated their anti-diabetic activities using alloxan-induced diabetic mice, and analyzed the main monosaccharide composition of the polysaccharides in the pumpkin extracts.

2. Methods

2.1. Plant materials and chemicals

Fresh and uniformly shaped pumpkins were selected from a commercial pumpkin farm in Wuxi (Jiangsu Province, China). The fresh pumpkin was peeled, the seeds were removed, and the pumpkin sliced into pieces measuring 0.5 × 2.0 × 5.0 cm. The slices were sun dried and ground into powder.

Alloxan was purchased from Sigma Chemical Co. (St Louis, MO, USA). A one-touch glucometer was purchased from Roche Diagnostics GmbH (Mannheim, Germany) for the analysis of blood glucose (BG). All other chemicals were of analytical grade.

2.2. Animals

Young adult (6–7 weeks, 20–25 g, male and female) Kunming mice were purchased from Wuxi Atom Medicine Research Institute (Jiangsu, China). The mice were housed in cages on a 12-hour light–dark cycle with accessibility to food and tap water. Temperature and humidity in the cages were held at 25 ± 2°C and 55–60%, respectively.

2.3. Extraction of hypoglycemic components from pumpkin powder

Pumpkin powder was mixed with distilled water at a ratio of 1:3 (w/w) at 60°C for 2 hours and then centrifuged at 3000 rpm for 20 minutes. The supernatant was collected as a water-soluble extract while the sediment was mixed with 95% alcohol at a ratio of 1:3 (w/w) for 48 hours at 4°C followed by centrifugation at 3000 rpm for 10 minutes. The sediment was discarded while the supernatant, the alcohol-soluble extract, was mixed with the previously collected water-soluble extract. The mixture of the water-soluble and alcohol-soluble extracts was named "pumpkin crude extract" (PCE). The PCE was further extracted 3–5 times with ether at 20°C. The ether phase was collected and rotary evaporated and the collected residue was named PCE-A. The aqueous phase was further extracted 3–5 times with n-butyl alcohol at 20°C. The n-butyl alcohol phase was collected and rotary evaporated and the collected residue was named PCE-B. The aqueous phase was mixed with 95% alcohol at a ratio of 1:2 (w/w) at 4°C and the collected precipitate was named PCE-C while the alcohol extract was evaporated and the collected residue was named PCE-D.

2.4. Purification of effective components

To remove proteins and some low molecular weight compounds from PCE-C, Sevag reagent [25] and dialysis were employed successively. The crude polysaccharide solution was rotary evaporated under vacuum at a temperature below 50°C before being dried in a vacuum oven for 1 week. The dried crude polysaccharide powder was named PCE-E; it was light yellow and easy to dissolve in water.

PCE-E was purified over a Sephadex G-100 (Amersham Pharmacia, Sweden) column (108 cm × 1.5 cm). Fifty mM of sodium phosphate buffer was used as the mobile phase at a flow rate of 10 mL/h. Fractions were collected and the polysaccharide content was assayed by the phenol-sulfuric acid method [26] using glucose as the standard. The fractions containing polysaccharide were dialyzed, concentrated, precipitated with alcohol, and lyophilized to obtain PCE-F and PCE-G.

2.5. Quantification of polysaccharides in PCE-F

The content of carbohydrates in the hypoglycemic components from pumpkin was measured by the phenol-sulfuric acid method. The polysaccharide content was calculated using the following formula:

$$\text{Polysaccharide content}(\%) = (\text{measured amount of carbohydrates}/\text{weight of sample}) \times 100\%$$

The protein content of the hypoglycemic components was determined by the Bradford method using bovine serum albumin as the standard [27].

2.6. Determination of monosaccharide composition of PCE-F by gas chromatography

The main monosaccharide composition of the hypoglycemic components from pumpkin was determined by gas chromatography. Ten milligrams of sample was hydrolyzed in 10 mL of 2 M trifluoroacetic acid at 105°C for 2 hours and monosaccharides therein reacted with trimethylsilylating reagent. The trimethylsilylated derivatives were loaded onto an HP-5 capillary column and monitored by a flame ionization detector. The following program was adopted for gas chromatography analysis: injection temperature, 230°C; detector temperature, 230°C; column temperature was programmed to increase from 130°C to 180°C at 5°C/min, hold at 180°C for 5 minutes, increase to 220°C at 5°C/min and finally hold at 220°C for 3 minutes. Nitrogen was used as the carrier gas and

maintained at a flow rate of 1.0 mL/min. Rhamnose, arabinose, galactose, glucose, mannose, xylose, and fructose were used as standards. Inositol was used as the internal standard.

2.7. Preliminary screening for the components with hypoglycemic effects

The mice were segregated into the following groups: normal control group, hyperglycemic model group, and the group treated with various components from pumpkin (PCE group). The PCE group was further divided into three dosage subgroups: 10 mice in each subgroup were injected with PCE at dosages of 100, 200 and 400 mg/kg BW. Mice were fasted overnight and diabetes was induced by a rapid intravenous injection of alloxan (200 mg/kg BW) freshly dissolved in saline. The mice were then given 5% glucose in drinking water overnight to prevent hypoglycemia. Alloxan-diabetic mice were allowed free access to food and water until further experiment. After 72 hours, mice with blood glucose levels greater than 11.3 mM were selected as the alloxan-induced diabetic mice [28]. The mice in the normal control group and hyperglycemic model group were intraperitoneally injected with the same volume of normal saline instead of PCE. During the experimental period, the mice had free access to food and water. BG was measured by drawing blood from the tail veins of the mice at 4, 7 and 11 hours after intraperitoneal injection.

2.8. Experimental design

The mice were randomly separated into 20 groups with 10 animals in each group. Groups 1–8 were normal mice. Mice in Group 1, the control group, were injected with saline (0.86% NaCl). The mice in Groups 2, 3 and 4 were injected with 100, 200 and 400 mg/kg BW of PCE, respectively. The mice in Groups 5, 6, 7 and 8 were injected with 200 mg/kg BW of PCE-A, -B, -C and -D, respectively. The other groups comprised mice with alloxan-induced diabetes (Groups 9–20). The mice in Group 9, the hyperglycemic model group, were injected with 0.86% NaCl only. The mice in Group 10, the positive control, were injected with the Xiaoke pill, a Chinese medicine widely used in the clinical treatment of diabetes in China, at 750 mg/kg BW in 0.86% NaCl. The mice in Groups 11, 12 and 13 were injected with PCE at dosages of 100, 200 and 400 mg/kg BW, respectively. The mice in Groups 14, 15, 16, 17, 18, 19 and 20 were injected with 200 mg/kg BW of PCE-A, -B, -C, -D, -E, -F and -G, respectively. Blood samples were taken from the tail vein of the mice before and 4, 7 and 11 hours after intraperitoneal injection, and BG was measured using the one-touch glucometer.

2.9. Statistical analysis

Data are presented as $\bar{x} \pm$ SD from multiple experiments. Statistical analyses were performed using Statview (version 1.30; Abacus Concepts, Inc., Berkeley, CA, USA). Variations within groups of animals were determined by analysis of variance and t test. A probability value of $p < 0.05$ was considered significant.

3. Results

3.1. Preliminary screening of the components with hypoglycemic effects

Before intraperitoneal injection and 4, 7 and 11 hours after intraperitoneal injection of PCE, the BG concentrations in mM in the diabetic mice decreased to different levels (Table 1). Compared to the BG concentrations of the mice in the hyperglycemic model group, the BG concentrations in both the normal and diabetic groups injected with 200 and 400 mg/kg BW of PCE decreased significantly, especially 7 hours after intraperitoneal injection. At 7 and 11 hours after injection with 200 mg/kg BW of PCE, the BG concentrations in the diabetic mice were significantly lower ($p < 0.05$ at both times) than those in the hyperglycemic model group. At the same dosage of 200 mg/kg BW, the BG concentrations at 7 and 11 hours after treatment in the normal group were significantly lower ($p < 0.01$ and <0.05, respectively) than those in the control group. The BG levels of the diabetic mice in the 200 mg/kg BW PCE group were 55.25% lower than those in the hyperglycemic model group, while the BG levels in the positive

Table 1 – Effects of PCE on BG concentrations (mM) in normal mice and alloxan-induced diabetic mice.

Group	PCE dosage (mg/kg BW)	Time after intraperitoneal injection (h)			
		0	4	7	11
Control group	0	5.07 ± 0.45	5.05 ± 0.32	5.05 ± 0.49	5.02 ± 0.35
Model group	0	12.85 ± 2.92	13.32 ± 2.87	13.15 ± 2.59	13.18 ± 3.04
Positive control group	750	12.36 ± 2.72	10.18 ± 2.76	9.02 ± 2.54[a]	9.88 ± 2.97
Normal group (PCE)	100	4.70 ± 0.42	4.54 ± 0.78	4.12 ± 0.59	4.09 ± 0.46
	200	5.50 ± 0.85	4.94 ± 0.97	3.92 ± 0.88[c]	3.96 ± 0.79[b]
	400	4.82 ± 0.69	4.53 ± 0.87	4.08 ± 0.59[b]	4.11 ± 0.54
DM group (PCE)	100	12.46 ± 2.86	12.02 ± 3.11	11.27 ± 2.77	11.32 ± 2.57
	200	13.01 ± 3.67	10.42 ± 3.15	8.47 ± 2.14[a]	8.64 ± 2.52[a]
	400	13.12 ± 3.45	11.24 ± 3.02	9.25 ± 2.73[a]	9.97 ± 2.86

a Compared with the hyperglycemic model group, $n = 10$, mean ± SE, $p < 0.05$.
b compared with the control group, $n = 10$, mean ± SE, $p < 0.05$.
c compared with the control group, $n = 10$, mean ± SE, $p < 0.01$.

control group were 45.78% lower than those in the hyperglycemic model group at 7 hours after intraperitoneal injection. The results indicated that PCE has a dose-dependent BG-lowering activity in diabetic mice.

3.2. BG-lowering effects of PCEs in diabetic mice

The BG concentrations of the diabetic mice decreased to different extents before and 7 hours and 11 hours after intraperitoneal injection of 200 mg/kg BW of PCE-A, PCE-B, PCE-C and PCE-D (Table 2). These results indicated that PCE-A and PCE-B had significant effects on reducing the BG levels of normal mice from 5.77 ± 1.02 mM to 3.88 ± 0.48 mM ($p < 0.01$) and from 5.88 ± 0.72 mM to 3.98 ± 0.78 mM ($p < 0.01$), respectively. However, there was no significant difference in BG levels before and after PCE-A and PCE-B treatment in the alloxan-diabetic mice group. The BG levels in the normal and alloxan-diabetic mice treated with PCE-D showed no significant difference ($p > 0.05$), indicating that PCE-D does not have any BG-lowering effect on the tested mice.

PCE-C showed a BG-lowering effect on normal mice. The BG level decreased significantly ($p < 0.05$) from 5.39 ± 0.75 mM before PCE-C treatment to 4.40 ± 0.84 mM 7 hours after PCE-C injection. However, no significant decrease was observed 11 hours after PCE-C treatment. For alloxan-diabetic mice, PCE-C treatment resulted in a significant decrease ($p < 0.001$) in BG level from 15.32 ± 4.38 mM before PCE-C injection to 5.77 ± 1.46 mM 7 hours after PCE-C treatment. The hypoglycemic effect of PCE-C may not be due to the stimulation of insulin secretion but by other pathways.

3.3. Purification of PCE-C by column chromatography

PCE-C was separated from PCE by partition with different organic solvents with a yield of 13.6%, and its crude protein content was 2.37%. PCE-E was obtained after protein removal by the Sevag reagent and the protein content decreased to 0.31%. PCE-F and PCE-G were two fractions of PCE-E separated by column chromatography using Sephadex G-100 as the stationary phase. The yield of PCE-F was 57.48% while the yield of PCE-G was 34.18% (Fig. 1). These two fractions were both light yellow powders and easy to dissolve in water but not in organic solvents such as acetone, ether, chloroform or butanol. The reactions of the two PCE subfractions with the phenol-sulfuric acid reagent were positive while the ninhydrin tests were negative, which indicated that these two fractions did not contain any amino acids or proteins. Furthermore, the reactions of the two PCE subfractions with the iodine-potassium iodide reagent were negative, indicating that they were non-starch polysaccharides.

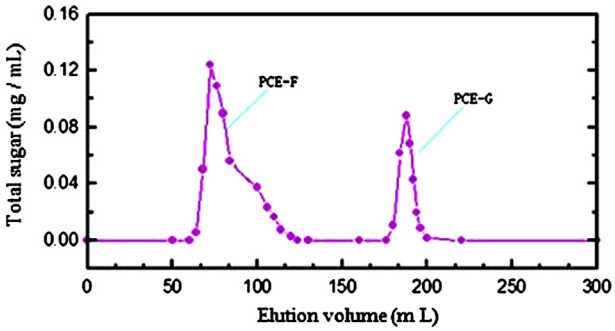

Fig. 1 – Chromatogram of PCE-E using a Sephadex G-100 gel column. Fractions were analyzed by the phenol-sulfuric acid reaction to estimate the sugar content (490 nm).

3.4. BG-lowering effects of PCE-E, PCE-F and PCE-G

Before and 7 hours and 11 hours after intraperitoneal injection of 200 mg/kg BW of PCE-E, PCE-F and PCE-G, the BG concentrations of the diabetic mice were measured (Table 3). The results indicated that PCE-E exerted a significant effect on diabetic mice. The BG in diabetic mice decreased from 16.01 ± 3.51 mM (before PCE-E treatment) to 7.64 ± 2.32 mM ($p < 0.001$) 11 hours after PCE-E treatment, which indicates that PCE-E had a BG-lowering effect on diabetic mice.

There was no significant difference in the BG levels of diabetic mice before PCE-G treatment and 11 hours after PCE-G treatment, indicating that PCE-G did not have any BG-lowering effect. BG levels decreased significantly from 15.90 ± 3.21 mM (before PCE-F treatment) to 7.19 ± 2.54 mM 7 hours after PCE-F treatment. The BG level was maintained at 8.23 ± 2.26 mM 11 hours after PCE-F treatment, indicating that PCE-F not only had a BG-lowering effect but also a BG-maintaining effect on diabetic mice.

Table 2 – Effects of PCE-A, PCE-B, PCE-C and PCE-D on BG levels (mM) in normal and alloxan-induced diabetic mice.

Group		Time after PCE treatment (h)		
		0	7	11
Normal	PCE-A	5.77 ± 1.02	4.30 ± 0.77[a]	3.88 ± 0.48[b]
	PCE-B	5.88 ± 0.72	3.98 ± 0.78[b]	4.29 ± 0.79[a]
	PCE-C	5.39 ± 0.75	4.40 ± 0.84 [a]	4.61 ± 0.92
	PCE-D	5.76 ± 0.66	5.70 ± 0.72	5.24 ± 0.81
Diabetic	PCE-A	15.40 ± 4.76	14.62 ± 3.04	15.63 ± 3.32
	PCE-B	16.03 ± 4.16	13.12 ± 2.87	13.33 ± 3.68
	PCE-C	15.32 ± 4.38	5.77 ± 1.46[c]	5.80 ± 0.94[c]
	PCE-D	13.50 ± 3.87	12.76 ± 2.81	13.04 ± 2.97

a $p < 0.05$, compared to 0 h PCE treatment, $n = 10$, mean ± SE.
b $p < 0.01$, compared to 0 h PCE treatment, $n = 10$, mean ± SE.
c $p < 0.001$, compared to 0 h PCE treatment, $n = 10$, mean ± SE.

Table 3 – Effects of PCE-E, PCE-F and PCE-G on BG levels (mM) in alloxan-induced diabetic mice.

Group	Time after PCE treatment (h)		
	0	7	11
PCE-E	16.15 ± 3.43	8.04 ± 2.46[b]	7.91 ± 2.38[b]
PCE-F	15.90 ± 3.21	7.19 ± 2.54[a]	8.23 ± 2.26[a]
PCE-G	17.65 ± 3.12	14.55 ± 3.24	13.67 ± 2.86

a $p < 0.01$, compared to 0 h PCE treatment, $n = 10$, mean ± SE.
b $p < 0.001$, compared to 0 h PCE treatment, $n = 10$, mean ± SE.

3.5. *The monosaccharide composition of PCE-F*

Purified PCE-F was hydrolyzed by trifluoroacetic acid into monosaccharides, which were further trimethylsilylated for gas chromatography analysis. The results are shown in Fig. 2. Four monosaccharides—glucose, galactose, arabinose and rhamnose—were identified with a molar ratio of 2.0:1.0:1.5:2.5 (with galactose as the reference).

4. Discussion

In the present study, the anti-diabetic potential of pumpkin polysaccharides to alloxan-induced diabetic mice was investigated. Alloxan is a known compound that induces insulin deficiency in most animal models with some remarkable similarities to human insulin-dependent diabetes mellitus [29]. The diabetogenic agent is a hydrophilic and chemically unstable pyrimidine derivative that is toxic to pancreatic β-cells [30]. This compound, due to its similarity in three-dimensional structure to glucose molecules, undergoes uptake by pancreatic β-cells via low-affinity GLUT2 glucose transporters in the plasma membrane [31,32]. There is a possibility for the survival of a few β-cells and this has been shown by several research groups who observed anti-hyperglycemic activity with oral hypoglycemic agents in alloxan-induced diabetic rats [33,34].

The fundamental mechanism underlying hyperglycemia in diabetes mellitus involves overproduction of glucose by excessive hepatic glycogenolysis and gluconeogenesis, and decreased utilization of glucose by tissues. The pathogenesis of diabetes always involves disturbances in carbohydrate, fat and protein metabolism. These complex multifactorial metabolic changes often lead to functional impairment of many organs, most importantly that of the cardiovascular system, in both types of diabetes [35]. Our results indicated that PCE significantly reduced BG concentrations in diabetic mice ($p < 0.05$). Compared to the normal and diabetic groups, the BG-lowering effect of PCE at a dosage of 200 mg/kg BW was stronger than that of 100 or 400 mg/kg BW. In normal mice, PCE-C treatment caused their BG level to drop significantly 7 hours after treatment ($p < 0.05$), but no significant difference was observed 11 hours after treatment. In alloxan-diabetic mice, PCE-C treatment significantly decreased BG level 7 hours after treatment. Based on the results described above, we propose that the major function of PCE-C is not to stimulate β-cells in the islets of Langerhans to increase insulin secretion, but rather restore the islets of Langerhans, repairing impaired islets or act as an insulin sensitizer to enhance insulin action by improving the insulin sensitivity of target tissues such as the liver, muscle and adipose tissue. Pumpkin polysaccharides may also play an important role in the recovery of liver function and glucose utilization, which is similar to the activity of polysaccharides found in Chinese medicine, such as *Hedysarum polybotrys* polysaccharides and *Opuntia dillenii* polysaccharides [36,37].

Based on this study's results, further chemical and pharmacological investigations should be carried out to evaluate the mechanisms of the anti-diabetic activity of pumpkin polysaccharides.

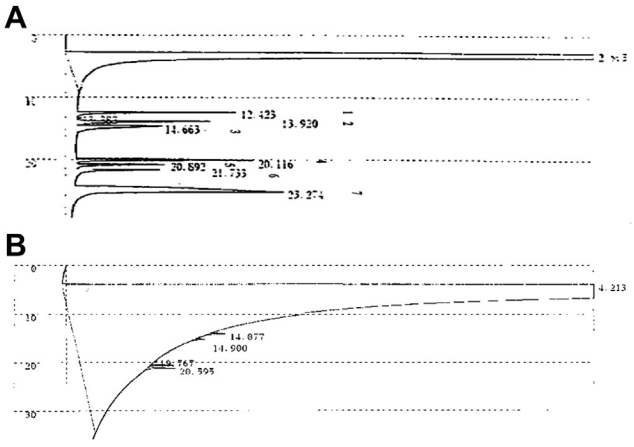

Fig. 2 – Gas chromatogram of the monosaccharide composition of PCE-F. (A) The sugar standards. (B) The tested PCE-F. 1. Rhamnose 2. Arabinose 3. Xylose 4. Mannose 5. Glucose 6. Galactose 7. Inositol.

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