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A polysaccharide from *Acanthopanax senticosus* improves the antioxidant status in alloxan-induced diabetic mice

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ABSTRACT

The effects of *Acanthopanax senticosus* polysaccharide (ASP) on antioxidant status had been investigated both in vitro and in alloxan-induced diabetic mice. In vitro antioxidant assays, ASP had a potent scavenging ability to superoxide radical and hydroxyl radicals. In vivo experiment, male Wistar rats were made diabetic by injection of alloxan and ASP (50, 100 and 200 mg/kg) was administered to diabetic mice for two months. ASP treatment could significantly and dose-dependently reduce the levels of lipid peroxidation markers, namely thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LP), and elevate enzymic antioxidants, such as superoxide dismutase (SOD) and catalase (CAT) activities in the plasma, liver, kidney and heart of diabetic rats. Moreover, increased level of serum insulin and decreased level of blood glucose (FBG) were observed in the plasma of diabetic control rats. The results demonstrated that ASP oral administration had an efficacious amelioration effect on the antioxidant status in alloxan-induced diabetic mice.

1. Introduction

Diabetes mellitus (DM) is the most common endocrine disorder, which is mainly characterized by hyperglycemia, and sometimes accompanied by long-term complications affecting the eyes, kidneys, nerves and blood vessels. Although the underlying mechanism of diabetic complications is unknown, much attention has been focused on the role of oxidative stress. Oxidative stress is believed to be a potential causes bringing about the development of complications in diabetes mellitus. Oxidative stress may result from overproduction of precursors to oxygen free radicals and/or decreased efficiency of antioxidant system. Currently available synthetic antidiabetic agents produce serious side effects, such as hypoglycaemic coma and hepatorenal disturbances (Suba, Murugesan, Arunachalam, Mandal, & Sahu, 2004). Moreover, they are not safe for use during pregnancy (Rahman & Zaman, 1989; Shanmugam, Mallikarjuna, Nishanth, Kuo, & Reddy, 2011). Therefore, the search for safer and more effective hypoglycaemic agents to treat diabetes mellitus and its complications has focused on the usage of plant extracts and their constituents. Much interest has grown in the role and usage of natural antioxidants as a means to prevent oxidative damage in diabetes with high oxidative stress (Kamalakkannan & Stanely Mainzen Prince, 2006).

Acanthopanax senticosus have been used as a traditional Chinese medicine for invigorating the liver and kidney, replenishing the vital essence, strengthening bone, stimulating appetite and improving memory for a long history time (Zhang, Huang, Ye, & Qin, 2011). In our early report, we successfully purified one polysaccharide (ASP) from *A. senticosus* and evaluated its antidiabetic effect in alloxan-induced diabetic mice. The results showed that ASP not only could significantly decrease blood-fat and fasting blood levels, but also increase the body weight and serum insulin level of diabetic rats. Consequently for the purpose of better understanding the potential mechanism, in this study we aim to explore whether ASP has ameliorating effects of on antioxidant status in alloxan-induced diabetic mice.

2. Experimental

2.1. Materials and chemicals

A. senticosus were purchased from local drug market in Xian city of China. Alloxan and D-glucuronic acid, deoxyribose, trichloride

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ferric ($FeCl_3$), ethylene diamine tetra-acetic acid (EDTA), ferrozine, nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), dihydromicotineamidadenine dinucleotide (NADH), Tris–HCl buffer, H_2O_2, ascorbate acid, and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glucose Analyzer and strips were purchased from Roche Diagnostic Co. (USA). Reagents for serum insulin were purchased from Adlitteram Diagnostic Laboratories Co. (USA). All of other reagents were analytical grade from Peking Chemical Co. (Peking, China).

2.2. Preparation of polysaccharides

The dried *A. senticosus* was extracted with 95% ethanol (3 × 10 vol) at 75 °C for 5 h under reflux to remove lipid. And then the residue was then extracted with distilled water (4 × 10 vol) at 24 °C for 3 h. After centrifugation (1700 × *g* for 15 min, at 20 °C), the supernatant was concentrated to one-tenth of the volume, and precipitated with 95% ethanol (5 vol) at 4 °C overnight. The precipitate collected by centrifugation, was suspended in distilled water, followed protein removing by proteinase digestion and the Sevag method (Staub, 1965). Then the solution was exhaustively dialyzed against water for 2 days before being collected and freeze dried. At the end we get the crude polysaccharide (CASP).

CASP was dissolved in distilled water and centrifuged, and then the supernatant was loaded onto a column of DEAE-cellulose column (2.6 cm × 40 cm) equilibrated with 0.9% sodium chloride. After loading with sample, the column was eluted at a flow rate of 1 ml/min with a gradient of NaCl aqueous solution (0 and 1 M). Fractions were collected by the automated fraction collector, and monitored with the phenol–sulfuric acid method at 490 nm absorbance for polysaccharides, and protein absorption at 280 nm was measured for each fraction. The eluted solution was separated into two fractions (CASP and CASPA), and then CASP was further purified by gel-permeation chromatography on a Sephadex G-100 column (2.6 cm × 100 cm), eluted with 0.15 M NaCl with a flow rate of 1 ml/min. Fractions (test tube nos. 36–39) were collected and lyophilized to get a white purified polysaccharide ASP (Fu et al., 2011).

2.3. Assay for antioxidant activity in vitro

2.3.1. Hydroxyl radical scavenging activity assay

Evaluation of the scavenging ability of ASP on hydroxyl radicals was performed according to the method previously described by Halliwell, Gutteridge, and Aruoma (1987), with a minor modification (Halliwell et al., 1987). Reaction mixtures in a final volume of 1.0 ml contained deoxyribose (60 mM), phosphate buffer (pH 7.4, 20 mM), ferric trichloride (100 μM), EDTA (100 μM), H_2O_2 (1 mM), ascorbic acid (100 μM) and different concentrations of ASP (0, 0.1, 0.2, 0.4, 0.8, or 1.6 mg/ml). Solutions of ferric trichloride and ascorbic acid were made immediately before use. The reaction solution was incubated for 1 h at 37 °C, and then 1 ml of 1% TBA and 1 ml of 20% (v/v) HCl were added to the mixture. The mixture was boiled for 15 min and cooled on ice. Deionized water and ascorbic acid served as blank and positive control, respectively. The absorbance of the resulting mixture was measured at 532 nm. The scavenging activity of hydroxyl radical (%) was calculated according to the following equation:

$$\text{Scavenging effect } (\%) = \frac{(A_{532(\text{blank})} - A_{532(\text{sample})})}{A_{532(\text{blank})}} \times 100$$

where $A_{532(\text{blank})}$ was the absorbance of the control (deionized water, instead of sample) and $A_{532(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

2.3.2. Superoxide radical scavenging activity assay

The superoxide radical scavenging activity of ASP was evaluated by the method of photoreduction of NBT detailed by Hakkim, Arivazhagan, and Boopathy (2008) with some modifications (Hakkim et al., 2008). Briefly superoxide anions were generated in a nonenzymatic PMS-NADH system by the oxidation of NADH and assayed by reduction of NBT. Reaction mixtures in a final volume of 3 ml contained the following reagents at final concentration: 16 mM Tris–HCl buffer (pH 8.0), containing 78 mM reduced nicotinamide adenine dinucleotide (NADH), 50 μM nitroblue tetrazolium (NBT), 10 μM phenazin methosulfate (PMS), and various concentrations of samples. The coloration reaction of superoxide radicals with NBT was determined at 560 nm. The deionized water was used as the blank control and ascorbic acid was used as positive control. Each value was expressed by the mean of triplicate measurements with standard deviation. The capability of scavenging the superoxide radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = \frac{(A_{560(\text{blank})} - A_{560(\text{sample})})}{A_{560(\text{blank})}} \times 100$$

where $A_{562(\text{blank})}$ was the absorbance of the control (deionized water, instead of sample) and $A_{562(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

2.4. Assay for antioxidant activity in vivo

2.4.1. Animals

Male Wistar rats (180–200 g each) were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, and were maintained in a 12 h light:12 h dark cycle, temperature (25 ± 1 °C) and relative humidity (30–70%).

They were fed with standard laboratory diet and given tap water ab libitum. The experimental protocol was approved by Institutional Animal Ethical committee. Animal described as fasted were deprived of food for 16 h but allowed free access to water.

2.4.2. Induction of diabetes in experimental animals

After 2 weeks of acclimatization, male rats were rendered diabetic by intraperitoneal injection with a freshly prepared solution of alloxan monohydrate in saline (300 mM NaCl) at a dose of 150 mg/kg of body weight (BW). Since alloxan injection to mice could result in a fatal hypoglycemia due to massive pancreatic insulin release, diabetic rats were orally remedied with 20% glucose solution (5–10 ml) after 6 h. The rats were then fed with 5% glucose water solution for the next 24 h on to prevent severe hypoglycemia. When the condition of diabetes was stabilized, animals were fasted for 8 h, then blood sample was taken from their tail veins and the plasma glucose concentration was measured by glucose assay kit (BIOSINO Inc., Beijing, PR China). Rats exhibiting glycosuria and hyperglycemia (blood glucose concentration >250 mg dl^{-1}) were considered diabetic and only uniformly diabetic rats were included in the study (Ananthan et al., 2004).

2.4.3. Experimental design and oral administration

This experimental study was conducted as follows:

Group I: Normal rats (NC)

Group II: Alloxan-diabetic rats (DC)

Group III: Diabetic + ASP (50 mg/kg BW/D) (DL)

Group IV: Diabetic + ASP (100 mg/kg BW/D) (DM)

Group V: Diabetic + ASP (200 mg/kg BW/D) (DH)

24 diabetic rats were randomly divided into four groups consisting of six rats each. Six healthy rats were served as normal control. The polysaccharides were administered using vehicle solution

(0.9% NaCl). The animal in NC and DC groups serving as normal control and diabetic control were orally received 0.9% NaCl only.

Groups III–V (DL, DM and DH) were given orally with ASP at the dose of 50 mg/kg, 100 mg/kg and 50 mg/kg BW/D in the same vehicle, respectively. After two months of treatment, the animals were deprived of food overnight prior to being sacrificed by decapitation. The trunk blood collected and serum was separated by centrifugation for 5 min and was kept at −20 °C. The liver, kidney and heart were removed promptly and got rid of fat; all these samples were stored at −80 °C until required. Plasma was separated for the estimation of FBG and FINS level.

2.4.4. Determination the level of blood glucose and insulin in mice

The blood glucose levels were measured by using a glucose analyzer (Bellamkonda et al., 2011). The fasting serum insulin (FINS) levels were determined using an insulin radioimmunoassay kit (Beijing North Institute of Biological Technology, Beijing) (Buccolo & David, 1973).

2.4.5. Measurement of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LP) levels

The concentrations of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LP) were estimated by the methods of Daňová et al. (2005) and Shang, Qin, Cheng, and Miao (2006), respectively.

2.4.6. Superoxide dismutase (SOD) activity assay

SOD activity was estimated by the method of Beauchamp and Fridovich (1971). Tissue homogenates in potassium phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), L-methionine (13 mM), riboflavin (2 μM) and nitroblue tetrazolium (75 μM) were mixed completely together, and then the generated blue color in the reaction was measured at 560 nm. The potency of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as units per milligrams of protein (Beauchamp & Fridovich, 1971).

2.4.7. Catalase activity (CAT) activity assay

CAT activity was evaluated according to the method of Aebi (1984). Enzymatic reaction was initiated by adding an aliquot of 20 μl of the homogenized tissue and the substrate (H_2O_2) to a concentration of 0.5 M in phosphate buffer (100 mM, pH 7.4). The variances in absorbance were measured at 240 nm. CAT activity was calculated in terms of nanomoles H_2O_2 consumed per minute per milligram of protein.

2.5. Statistical analysis

All the data were expressed as mean ± standard deviation (SD) of three replicates and were analyzed statistically by one-way analysis of variance (ANOVA) using SPSS version 10.0 software (SPSS Inc, Chicago, USA). The significance of the difference between the means of test and control studies was established by Student's *t*-test. The results were considered to be significant if $P < 0.05$.

3. Results and discussion

3.1. Preparation of polysaccharide and its chemical properties

As our previous study, one homogeneous polysaccharide, named ASP, was purified from this plant with a molecular weight of 59 kDa. ASP containing 95.5% carbohydrate content was composed of glucose, galactose and arabinose in a molar ratio of 2.1:1.9:1, as determined by gas chromatography (GC) (Fu et al., 2011).

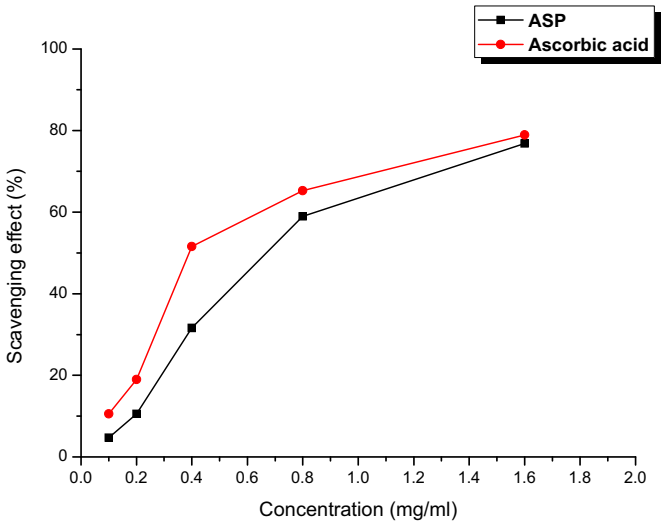

Fig. 1. Scavenging effects of ASP on hydroxyl radicals. Results were presented as mean ± SD ($n = 3$).

3.2. Antioxidant activity of polysaccharides in vitro

3.2.1. Hydroxyl radical scavenging activity

The scavenging effects of different concentrations of samples (0.1–1.6 mg/ml) on hydroxyl radicals were shown in Fig. 1. ASP presented potent hydroxyl radical-scavenging activity in a concentration-dependent manner. Especially beyond the concentration of 0.8 mg/ml, scavenging effect of ASP on hydroxyl radical was nearly close to that of ascorbic acid.

3.2.2. Superoxide radical scavenging activity

From Fig. 2, we can see that the scavenging ability of ASP on superoxide radicals correlated positively well with increasing concentrations (0.1–1.6 mg/ml). After concentration over 0.4 mg/ml, there is a prompt enhancing effect to scavenge the peroxide radicals by ASP, which were similar to that of ascorbic acid at the concentration of 1.6 mg/ml.

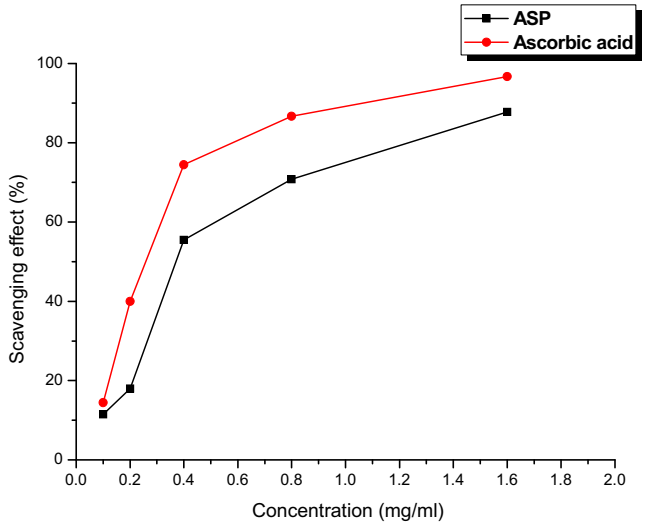

Fig. 2. Scavenging effects of ASP on superoxide radicals. Results were presented as mean ± SD ($n = 3$).

Table 1
The effect of ASP on FBG and FINS levels of normal and alloxan-induced diabetic rats.

Parameters and treatments	NC	DC	DL	DM	DH
FBG (mmol/l)	4.41 ± 0.42**	14.11 ± 1.59	9.67 ± 1.36**	8.23 ± 1.11**	6.38 ± 0.93**
FINS (μIU/ml)	27.34 ± 1.95**	12.27 ± 1.56	20.81 ± 1.91**	23.87 ± 2.10**	25.38 ± 1.87**

All values represent the mean ± standard deviation ($n = 6$ for each group). Significant differences with DC group were designated as $^{**}P < 0.01$.

Table 2
The effect of ASP on TBARS and LP levels of normal and alloxan-induced diabetic rats.

Parameters	NC	DC	DL	DM	DH
Plasma					
TBARS	0.24 ± 0.04**	0.47 ± 0.05	0.36 ± 0.07*	0.28 ± 0.06**	0.25 ± 0.03**
LP	11.45 ± 0.54**	36.45 ± 0.75	20.65 ± 0.91**	19.62 ± 0.71**	13.41 ± 0.84**
Liver					
TBARS	0.87 ± 0.12**	3.59 ± 0.21	3.08 ± 0.23*	1.98 ± 0.31**	1.05 ± 0.13**
LP	68.98 ± 2.34**	165.4 ± 4.24	100.56 ± 3.14**	89.54 ± 2.44**	70.51 ± 2.11**
Kidney					
TBARS	0.78 ± 0.25**	1.79 ± 0.31	1.04 ± 0.17**	0.89 ± 0.14**	0.80 ± 0.16**
LP	54.20 ± 2.34**	140.93 ± 2.34	100.13 ± 2.34**	68.04 ± 2.34**	59.10 ± 2.34**
Heart					
TBARS	0.65 ± 0.11**	1.43 ± 0.13	1.04 ± 0.09**	0.87 ± 0.10**	0.71 ± 0.08**
LP	34.02 ± 1.46**	100.04 ± 3.21	78.32 ± 2.73**	54.98 ± 1.56**	40.15 ± 1.34**

All values represent the mean ± standard deviation ($n = 6$ for each group). The unit of TBARS is μmol MDA equivalents/ml serum (in plasma) or μmol MDA equivalents/mg protein (in tissue); the unit of LP is $\times 10^{-5}$ mM (in plasma) or mM/100 g tissue (in tissue). Significant differences with DC group were designated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

Table 3
The effect of ASP on SOD and CAT activity of normal and alloxan-induced diabetic rats.

Parameters	NC	DC	DL	DM	DH
Plasma					
SOD	5.42 ± 0.33**	2.53 ± 0.27	3.39 ± 0.35*	4.23 ± 0.64**	5.09 ± 0.45**
CAT	45.89 ± 2.34**	19.93 ± 1.04	25.42 ± 1.11**	34.98 ± 1.67**	40.12 ± 1.72**
Liver					
SOD	10.24 ± 0.89**	4.32 ± 0.76	5.24 ± 0.67*	7.89 ± 0.58**	9.83 ± 0.66**
CAT	47.62 ± 1.69**	19.48 ± 1.25	25.35 ± 1.21*	39.20 ± 1.34**	46.42 ± 1.91**
Kidney					
SOD	21.43 ± 1.01**	8.49 ± 0.39	14.34 ± 0.64**	17.98 ± 0.42**	20.51 ± 0.89**
CAT	39.43 ± 1.09**	18.43 ± 0.93	23.42 ± 1.01*	31.4 ± 1.34**	37.42 ± 1.26**
Heart					
SOD	14.34 ± 0.43**	3.32 ± 0.23	7.45 ± 0.35**	9.20 ± 0.44**	12.24 ± 0.52**
CAT	31.54 ± 1.32**	16.32 ± 0.78	21.54 ± 0.72**	25.43 ± 0.87**	27.50 ± 1.02**

All values represent the mean ± standard deviation ($n = 6$ for each group). The unit of SOD and CAT is U/ml serum (in plasma); the unit of SOD and CAT is U/mg protein (in tissue). Significant differences with DC group were designated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.3. The effect of polysaccharides on fasting blood glucose and insulin level

Table 1 shows the level of blood glucose and plasma insulin in normal and experimental groups after two months' treatment. The level of blood glucose was significantly increased, whereas the level of plasma insulin was significantly decreased in diabetic control rats as compared with that of normal rats ($^{**}P < 0.01$). ASP oral administration at dose of 50 mg, 100 mg and 200 mg/kg BW/D to diabetic rats dose-dependently reversed all these changes to near-normal levels.

3.4. Antioxidant activity of polysaccharides in vivo

3.4.1. TBARS and LP levels

As shown in Table 2, the levels of TBARS and LP, as markers of lipid peroxidation, were evaluated in plasma, liver, kidney and heart of normal and experimental rats. The levels of TBARS and LP were significantly increased in diabetic control rats. Administration of ASP to diabetic rats significantly decreased the levels of TBARS and LP. The results indicated that treatment of diabetic rats with ASP could significantly decrease lipid peroxidation.

3.4.2. SOD and CAT activity

The effect of ASP on the activities of antioxidant enzyme SOD and CAT in plasma, liver, kidney and heart of normal and experimental rats were measured (Table 3). In diabetic group, a significant decrease of SOD activity in plasma (−53%), liver (−58%), kidney (−60%) and heart (−77%) was observed as compared to normal control group ($^{*}P < 0.05$ and $^{**}P < 0.01$). At the same time the activities of CAT in plasma, liver, kidney and heart were attenuated by −57%, −59%, −53% and −48%, respectively. Treatment of diabetic rats with ASP improved significantly the antioxidant enzyme activities of the above organ compared to those of diabetic group ($^{*}P < 0.05$ and $^{**}P < 0.01$) in a dose-dependent manner.

4. Conclusion

Diabetes is a complex metabolic disorder with a characteristic modulation of glucose metabolism. In diabetic patients, chronic hyperglycemia weakens the efficiency of antioxidative defense systems (Baynes, 1991), resulting in an increased generation of ROS (Rajasekaran, Ravi, Sivagnanam, & Subramanian, 2006). Many previous studies have focused on diabetes mellitus and its complications, in which pathogenic oxygen free radicals play a great role.

Alloxan is one of the chemical agents used for the induction of type II diabetes mellitus in animal, which produces a massive oxygen radicals in the body. Accordingly ROS lead to reduction in insulin release by the destruction of β-cells of the islets of langerhans, thereby inducing hyperglycaemia (Grover, Vats, & Rathi, 2000). As a strategy to counteract the negative effect of oxidative stress, antioxidant-based therapy is promising to minimize the complications associated with oxidative stress in diabetes mellitus. It is interesting that nondigestible carbohydrates such as fructans have been shown to exert systemic effects (Cummings, MacFarlane, & Englyst, 2001; Roberfroid, Bornet, Bouley, & Cummings, 1995). This work is the first investigation dealing with the protective effects of a polysaccharide from *A. senticosus* on diabetes and their complications in liver, kidney and heart functions. As we know, the body weight of the Wistar rats was more than 10 times than that of the Kun-ming mice. Therefore based on our previous preliminary investigation of potential therapy for type II diabetes by ASP, we replaced the Kun-ming mice with the Wistar rats for better understanding the hypoglycemic capacity and the mechanism by which ASP take effect.

In summary, in vitro antioxidant assays, ASP had a potent scavenging ability to superoxide radical and hydroxyl radicals. Besides, ASP oral administration to diabetic rats induced by alloxan could not only decrease FGB, TBARS and LP levels, but also increase the expression level of FINS, SOD and CAT in plasma, liver, kidney and heart of experimental rats. The results demonstrated that ASP had potential ameliorating effects on antioxidant status in alloxan-induced diabetic mice, through alleviating oxidative stress and free radicals as well as enhancing enzymatic defenses. Therefore based on the present study, it can be concluded that *A. senticosus* polysaccharide can be considered as a potent antioxidant for the therapy of diabetes mellitus and its complications. Further chemical and pharmacological investigation is needed to carry out to evaluate the anti-diabetic mechanism of ASP.

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