**Anti-diabetic effect of *Alpinia oxyphylla* extract on 57BL/KsJ db-/db- mice**

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**Abstract.** Diabetes mellitus is characterized by high blood glucose levels. Increased levels of reactive oxygen species (ROS) may disrupt insulin signaling and result in insulin resistance. The *Alpinia oxyphylla* extract (AOE) possesses powerful antioxidant activity and may therefore inhibit the development of insulin resistance. The objective of the present study was to determine the effects of AOE on blood glucose, insulin and lipid levels in a type II diabetic nephropathy animal model (C57BIKs db-/-). All experiments were performed on male C57BL/Ks DB/DB and db-/- mice that were left to acclimatize for 1 week prior to the experimental period. AOE was administered to these mice at different dosages (100, 300 and 500 mg/kg) for 8 weeks. The results demonstrated that AOE did not affect mouse weight, while blood glucose concentrations were found to significantly decrease in a dose-dependent manner (P<0.05). The effect of administering 500 mg/kg AOE (AOE500) to db-/- mice was tested further. Treatment with AOE500 for 8 weeks led to improved glucose tolerance and reduced plasma insulin concentrations (P<0.05), as well as a significant decrease in triglyceride concentrations (P<0.05) and levels of total cholesterol (P<0.05) in db-/- mice. Furthermore, treatment with AOE500 decreased the concentration of malondialdehyde, elevated the concentration of glutathione and increased the activities of the antioxidant enzymes superoxide dismutase and peroxidase (P<0.05) in the livers of db-/- mice. Meanwhile, AOE-treated mice exhibited significantly reduced urine albumin, creatinine and blood urea nitrogen excretion (P<0.05). In parallel, the upregulated expression of phosphatase and tensin homolog (PTEN) in the liver and kidneys of db-/- mice was impaired following AOE500 treatment. The results of the present study suggest that AOE regulates blood glucose and lipid levels and improves renal function by mediating oxidative stress and PTEN expression at the onset of type II diabetes mellitus.

**Introduction**

Diabetes mellitus is a chronic metabolic disease caused by a disorder of insulin secretion that results in the development of insulin resistance in target tissues. This disease that causes complications, such as metabolic disorders and multiple organ damage syndrome, is becoming a major threat to human health, with ~350 million cases in 2014 alone (1-3). Hyperglycemia is a major contributor to oxidative stress and reactive oxygen species (ROS) production (4,5). Increased levels of ROS resulting from hyperglycemia may disrupt the insulin signaling cascade, stimulating the development of insulin resistance (6,7). There has been a growing interest in dietary supplements and/or herbal medicines, which may improve the management of blood glucose due to their perceived safety and efficacy (8,9). A number of plants have been found with potential anti-diabetic capacity, including *Alpinia oxyphylla* (10). This has been used in China for centuries as both a food and medicinal substance (11) and is widely used as a tonic, aphrodisiac and anti-polyuria according to the Chinese Pharmacopoeia (12). A. oxyphylla extracts (AOEs) are rich in polyphenols, polysaccharides, protocatechuic acid and labdane diterpene glycosides (13). These extracts possess potent antioxidant activity, which inhibit nitric oxide production and the biosynthesis of prostaglandin (14). Therefore, they have the potential to be developed as a therapeutic to treat diabetes mellitus.

Phosphatase and tensin homolog (PTEN), is a phosphoinositide phosphatase that negatively regulates the insulin signaling pathway. It has been demonstrated that inhibiting PTEN expression normalizes blood glucose concentrations and improves insulin tolerance in db-/- mice (15). PTEN
overexpression in 3T3-L1 adipocytes inhibited serine-threonine kinase protein kinase B (Akt) activation and glucose uptake. By contrast, reduced PTEN expression enhanced insulin-stimulated Akt and glycogen synthase kinase-3α activity (16). These results suggest that PTEN may be a potential target to treat diabetes.

The primary objective of the present study was to determine the effects of AOE on blood glucose, insulin and lipid levels in a type II diabetic animal model, C57BLKsj db/db-.

Materials and methods

Animals and treatment. All experiments were performed on 4-week old male C57BL/Ks DB/DB (normal mice, 12.6±1.8 g) and db/db-mice (type II diabetic animal model, 17.8±1.9 g). A total of 60 mice were purchased from the Model Animal Research Center of Nanjing University (MARC; Nanjing, China). Mice were left to acclimate for 1 week prior to the experimental period. The room that animals were housed in was maintained under a constant 12-h light-dark cycle with a temperature of 23±3˚C and relative humidity of 70±10% throughout the experimental period. All mice were housed in group cages with two animals per cage and were given free access to standard pellets and water. Animal experiments were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and all protocols and studies were approved by the Ethics Committee of Hainan Medical College for Animal Care and Use (Hainan, China). For the care and use of animals utilized in this research, animals were monitored twice a week and none of animals succumbed or exhibited severe illness that would require early euthanasia during the whole experiment. Early sacrifice would have been performed if one of the following criteria were met: Loss of >20% body weight, a wound that cannot be improved following medication or the development of neurological symptoms in animals stopping them from being able to feed themselves. For anesthesia and sacrifice, mice were treated with 2-3% isoflurane inhalation and 3% CO₂ inhalation, respectively.

Preparation of the plant extract. The ripe fruit of A. oxyphylla was purchased from a market specializing in herbs (Herb Market, Haikou, China) in February 2014. The plant was authenticated by Dr Qiang Liu of the Department of Pharmacognosy, Hainan Medical college (Haikou, China). A. oxyphylla was extracted with 640 ml water for 16 h at 90°C. This process was conducted twice. The plant extract was then lyophilized and stored at room temperature until use in experiments I and II. The dry yield was 8% (w/w).

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The ability of the prepared extracts to scavenge the DPPH radicals was determined by the method described by Wang et al (17). Briefly, 10 µl AOE of the aforementioned different concentrations was added to 290 µl methanol solution of DPPH (0.1 mM). The solution was mixed well and then left at room temperature for 30 min in the dark. The absorbance of the resulting solution was read at 519 nm using an Eon™ Microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA). Ascorbic acid was employed as a reference and the radical scavenging activity was calculated as a percentage: of DPPH discoloration using the following equation.

\[
\text{DPPH radical scavenging (％) = } \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%
\]

where \(A_{\text{sample}}\) is the absorbance of the solution when the extract/reference has been added at a particular level and \(A_{\text{control}}\) is the absorbance of the DPPH solution without the extract added. All analyses were run in triplicate. The IC₅₀ values calculated denote the concentration of a sample required to decrease the absorbance at 519 nm by 50%.

Experiment I. A total of 30 mice that purchased from MARC were divided into five groups with six animals in each group. DB/DB group mice and db/db-H₂O group (db/db-mice) were administered placebo (saline) only, while groups db/db-AOE100, db/db-AOE300, and db/db-AOE500 were administered 100, 300 and 500 mg/kg AOE, respectively, via the intragastric route, once a day for 8 weeks (~0.2 ml in volume). The effect of AOE on db/db-mice was determined by changes in body weight assessed by weighing once a week, while non-fasting blood glucose concentrations measurements were analyzed from tail blood taken once a week.

Experiment II. Another group of 30 4-week-old male mice also purchased from MARC, which included 10 DB/DB (12.1±1.6 g) and 20 db/db-mice (17.3±1.8 g). These mice were divided into 3 groups, with 10 animals in each group and were housed in the same conditions as described previously. DB/DB mice group and db/db-H₂O group were administered placebo (saline) only, whereas the db/db-AOE500 group was administered 500 mg/kg AOE via the intragastric route once a day for 8 weeks (~0.2 ml in volume). At the end of the 8-week period, following fasting overnight, each animal was weighed. Individual mice were placed in metabolic cages to obtain 24 h urine collections. Then, the animals were sacrificed using 3% isoflurane inhalation and 3% CO₂ inhalation, and blood and tissue samples were collected for analysis. Blood samples were collected from the hepatic portal vein into a tube for EDTA anticoagulation and centrifuged (1,000 x g for 15 min at 4°C) to separate the plasma. The plasma was then frozen at -70°C for biochemical analysis. The liver and kidneys were excised, weighed and homogenized in a 3:1 w/v of 0.25 M sucrose, 10 mM HEPES, 1 mM EDTA (pH 7.5) buffer. Samples were homogenized for 30 sec at 6.45 m/s in an Omni Bead Ruptor (OMNI International IM, Kennesaw, GA, USA). Protein concentrations in each sample were determined using a Bradford protein assay kit (Tiangen Biotech Co., Ltd., Beijing, China).

Glucose tolerance test. Following 6 weeks AOE administration, the blood glucose concentration of mice in experiment II was measured by the oral glucose tolerance test (OGTT). Blood
samples were collected via the tail vein after an overnight fast and measured for fasting blood glucose concentration. The animals were then administered glucose (1.0 g/kg) solution orally. After 2 h, blood samples were again collected via the tail vein and measured to determine 2 h postprandial blood glucose concentration.

**Measurement of plasma concentration levels of glucose, triglycerides and total cholesterol.** Commercial kits for glucose (F006), triglycerides (F001-1) and cholesterol (F002-1) were all purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and used to measure all parameters, according to the manufacturer's instructions. Glucose concentration was assayed by the glucose oxidase method (18) and total cholesterol and triglyceride levels in plasma were tested using the enzymatic colorimetric method (19,20).

**Measurement of hepatic concentrations of reduced glutathione (GSH), malondialdehyde (MDA), peroxidase (POD) and superoxide dismutase (SOD).** These parameters were measured using Commercial kits for GSH (A006-2), MDA (A003-1), POD (A084-3) and SOD (A001-1) were all purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and used to measure all parameters, according to the manufacturer's instructions. GSH concentration was assayed using a chromogenic assay. Briefly, 0.5 ml cold EDTA tissue homogenate (0.02 mol/l) was added to 0.2 mol/l Tris buffer (pH 8.2) and 0.1 ml 5,5’-dithiobis-(2-nitrobenzoic acid). Samples were centrifuged at 1,350 x g for 10 min at room temperature. The absorbance of the clear supernatant was measured at 412 nm using a Eon™ Microplate spectrophotometer (Biotek Instruments, Inc.). MDA concentration was assayed using a chromogenic assay. This assay measures free and protein-bound MDA without undue interference from the other lipid peroxidation products (21). The standard curves for the 0–20 µmol/l range were prepared for each assay using the chromogen supplied in the kits. POD was measured by monitoring oxidation of 16 mM guaiacol in 50 mM potassium phosphate buffer (pH 6.5), following addition of 10 µl 10% H₂O₂ in a 3 ml volume. POD activity was measured as the absorbance increase at 470 nm. SOD activity was measured using the Beauchamp and Fridovich method (22). The total reaction mixture consisting of phosphate buffer (0.5 M, pH 7.4), post-mitochondrial supernatant, xanthine (1 mM) and NBT (57 µM) was incubated for 15 min at room temperature and the reaction was initiated by addition of xanthine oxidase (50 µU). The reaction rate was measured by recording the change in the absorbance at 550 nm.

**Measurement of urine concentration of creatinine and BUN.** These parameters were measured using Commercial kits for creatinine (C011-2) and BUN (C013-2) were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and used to measure concentrations of creatinine and BUN, according to the manufacturer's instructions. The creatinine concentration was determined by the sarcosine oxidase method (23) and BUN concentration was determined using the Urease method (24).

Urine albumin assay. Urine albumin was measured using an Albumin Mouse ELISA kit (ab108792), which was purchased from Abcam (Cambridge, UK). Absorbance was read using an automated microplate ELISA reader (Biotek Instruments, Inc.) and concentrations were calculated by the standard curve run on each assay plate. All samples were measured in duplicate.

Western blot analysis. Western blot analysis was performed on tissue extracts from the liver and kidney. Antibodies against PTEN (ab32199) and β-actin (ab129348) were purchased from Abcam. Homogenate (30 µg) was separated by 10% SDS-PAGE and transferred to polyvinyldene difluoride membranes. Membranes were blocked with Thermo Scientific SuperBlock (TBS) Blocking Buffer (cat. no. 37355; Thermo Fisher Scientific, Inc., Waltham, MA, USA) overnight at room temperature. Membranes were then incubated with either anti-PTEN (1:1,000; ab32199, Abcam) or anti-β-actin (1:5,000; ab129348, Abcam) antibodies for 2 h. Membranes were subsequently incubated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:4,000; ab150088, Abcam) for 1.5 h. All membranes were visualized using the Amersham ECL Prime Western Blotting Detection Reagent enhanced chemiluminescence (RPN2232, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and exposure to ECL Hyperfilm (GE Healthcare Bio-Sciences). All western blot analyses were performed at least three times.

**Statistical analysis.** Results are presented as the mean ± standard deviation. Data were analyzed by the Statistical Product and Service Solutions (SPSS) program ver. 16 (SPSS, Inc., Chicago, IL, USA). Comparisons between two groups were analyzed by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Free radical scavenging activity of AOE. The DPPH radical is widely used for the assessment of radical scavenging (25). The soluble free radical DPPH is known to be a good hydrogen acceptor that yields DPPH-H as a by-product (26). The antioxidant activities of AOE and vitamin C (a positive control) (15) were measured based on the scavenging activities for a stable DPPH radical as presented in Fig. 1A. On increasing the doses of AOE from 500 mg/l to 20 g/l and that of vitamin C from 0.5 to 30 mg/l, the values of the DPPH scavenging activity were found to be 1.72% (500 mg/l), 5.63% (1,000 mg/l), 12.74% (2,000 mg/l), 27.63% (4,000 mg/l), 44.61% (6,000 mg/l), 64.34% (8,000 mg/l), 72.35% (10,000 mg/l) and 73.75% (20,000 mg/l) for AOE and 1.37% (0.5 mg/l), 8.23% (1 mg/l), 17.94% (2 mg/l), 40.62% (4 mg/l), 69.53% (8 mg/l), 80.32% (15 mg/l) and 90.71% (30 mg/l) for vitamin C. The half maximal effective concentration (EC₅₀) values of AOE for the scavenging of DPPH radicals were 6,543.2 mg/l (AOE) and 5.2 mg/ml (vitamin C). These results suggest that 10 g/l AOE exhibits the optimum antioxidant capacity to scavenge DPPH free radicals.

Effects of AOE on body weight and blood glucose. Changes in body weight in DB/DB and db/db- mice, and following AOE administration over 8 weeks, did not differ significantly among the groups.
any of the groups (Fig. 1B). Plasma glucose levels decreased significantly [12% (P<0.05) in AOE100, 18% (P<0.05) in AOE300 and 28% (P<0.05) in AOE500] compared with the db/db-H2O group in a dose-dependent manner (Fig. 1C). The results demonstrated that the highest dose of AOE, 500 mg/kg was most effective in decreasing blood glucose levels.

**Effect of AOE on OGTT.** OGTTs were performed to determine the effect of a single oral dose of AOE on glucose tolerance in db/db mice (Fig. 2A and B). Glucose challenge dramatically increased the blood glucose concentration in db/db-H2O group mice, whereas AOE500 mice exhibited significantly suppressed blood glucose concentration 30, 60 and 90 min following the glucose load (Fig. 2A). When the area under the curve was compared between the groups, that of the DB/DB mice was only 39% of db/db-H2O group (P<0.05), whereas the AOE500 group showed 19.4% reduction in blood glucose levels compared with db/db-H2O group (P<0.05; Fig. 2B).

**Effects of AOE on the plasma insulin concentration.** The effect of AOE on plasma lipid concentration was studied following AOE administration for 8 weeks in db/db mice to reveal the mechanism of the *A.oxyphylla* effect. The plasma insulin concentration in the db/db-H2O group was found to be significantly higher than in DB/DB mice and db/db-AOE500 group (both P<0.05; Fig. 2C).

**Effects of AOE on dyslipidemia.** The effect of AOE500 on plasma lipid concentration revealed significant differences in most of the lipid profiles between the *A. oxyphylla*-treated and db/db-H2O group mice (Fig. 3A and B). The plasma
Effects of AOE on renal function. To assess the effect of AOE on renal function, urine albumin, creatinine and BUN were measured. The results indicate that urine albumin (P<0.05), creatinine (P<0.05) and BUN (P<0.05) were all significantly increased in db-/db-H2O group mice compared with DB/DB mice (Fig. 5). Urine albumin excretion was 0.62±0.17 mg/24 h in AOE500 mice, significantly lower than that of the db-/db-H2O group mice (1.27±0.31 mg/24 h, P<0.05; Fig. 5A), although still higher than that of DB/DB group (0.32±0.04 mg/24 h, P<0.05). The concentrations of creatinine and BUN were also significantly reduced following AOE500 treatment (P<0.05), while the BUN-to-Creatinine ratio was decreased. These results indicate that AOE500 treatment improved renal function (Fig. 5B and C).

Effects of AOE on the PTEN expression. In order to detect whether AOE500 impairs diabetic nephropathy development via downregulation of PTEN, PTEN protein expression was examined by western blot analysis. The present study showed that PTEN protein expression was enhanced both in the liver and renal tissue of db-/db-H2O group mice. However, PTEN protein expression was significantly decreased (P<0.05) following AOE500 treatment in the db-/db- mice (Fig. 6).

Discussion

*A. oxyphylla* is rich in eudesmane sesquiterpenes, diterpenes, flavonoids and diarylheptanoids, the components found to possess potent antioxidant properties (27). It has been reported that the extract of *A. oxyphylla* fruit exhibits concentration-dependent antioxidant capacity (17). AOE serves a neuroprotective role by attenuating oxidative stress, including increasing the activity of glutathione peroxidase, decreasing levels of MDA and decreasing the neuronal damage and apoptosis that occur in the frontal cortex and hippocampus in mice (28,29). The active phenolic components yakuchinone A and yakuchinone B that exist in *A. oxyphylla* fruit exhibit anti-inflammation and antioxidant capacity *in vitro* (30). In addition, yakuchinone A exhibit anti-adipocyte differentiation capacity (31). The diarylheptanoids isolated from the fruits of *A. oxyphylla* have potent antioxidant activities in the DPPH assay (32) and eudesmane sesquiterpenes may inhibit nitric oxide production in lipopolysaccharide-induced and interferon-gamma-induced murine macrophages (14,33). Furthermore, protocatechuic acid from *A. oxyphylla* may protect against hydrogen peroxide-induced oxidative pheochromocytoma cell death (34). In the present study, the ability of the prepared extract of *A. oxyphylla* to scavenge DPPH radicals was determined. The tested AOE showed a promising effect on DPPH scavenging in a concentration-dependent manner. Compared with the extracts, ascorbic acid showed higher radical scavenging ability with 37.3 mg/ml IC50. Although ethanol extracts are reported to have the highest flavonoids and diarylheptanoids, the components found in the fruits of *A. oxyphylla* fruit exhibit antioxidant activity and antioxidant enzyme activity. The effects of AOE500 on concentrations of lipid peroxides and activity of antioxidant enzymes in the liver are shown in Fig. 4. The concentration of hepatic thiobarbituric acid reactive substance (TBARS) in the db-/db-H2O group was significantly increased compared with that of DB/DB mice (P<0.05). There was a significant decrease in the MDA concentration of AOE500 treated db-/db- mice (P<0.05; Fig. 4A). As presented in Fig. 4B, the GSH content was significantly decreased in db-/db-H2O group mice (P<0.05) and partially recovered following AOE500 treatment (P<0.05). Activities of superoxide dismutase (SOD) and peroxidase (POD) in the liver of the db-/db-H2O group were inhibited compared with those of DB/DB group (both P<0.05). Meanwhile, the activities of SOD and POD in the liver of the AOE500 group were partly restored compared with db-/db-H2O group (both P<0.05; Fig. 4C and D).
impairing glucose-stimulated insulin secretion and increasing β-cell apoptosis (36). Under normal circumstances, ROS maintains an optimal oxidative balance for appropriate biological cell function by antioxidants, including GSH, vitamin C and vitamin E, as well as antioxidant enzymes, such as SOD, POD and catalase. However, in type II diabetes mellitus (T2DM), there are insufficient endogenous antioxidant defenses to balance the increased ROS production. In the present study, db/db- mice showed decreased GSH concentration, inhibited SOD and POD activities and enhanced MDA levels when compared with the DB/DB mice. In accordance with this finding, Ihara et al (36) reported that the concentration of protein carbonyls and lipid hydroperoxides increases in the kidneys of db/db- mice, and that oxidative stress serves an important role in the progression of early diabetic nephropathy. The present study found that AOE-treated db/db- mice treated with AOE exhibited partially restored antioxidative capacity. Following AOE treatment, the primary non-enzyme antioxidant GSH concentration was significantly higher and antioxidase SOD and POD activity were also increased, whereas lipid peroxidation MDA concentration was decreased in db/db- mice. AOE anti-oxidative capacity may underlie the beneficial effects of AOE in diabetes.

Previous studies have demonstrated that insulin resistance, the inability of cells to efficiently respond to stimulation by insulin, precedes the onset of T2DM by many years (37,38). In the current study, db/db- mice exhibited higher insulin concentrations and reduced OGTT compared with DB/DB mice. These results are in accordance with those from previous studies, highlighting that the decreased insulin sensitivity in db/db- mice results in poor glucose regulation. Following long-term AOE treatment, insulin concentration was significantly decreased and notably, db/db-AOE500 mice exhibited better OGTT than that of db/db-H2O mice. These results suggest that AOE may mediate db/db- mouse insulin sensitivity to ameliorate the symptoms of diabetes.

At a later stage of T2DM, certain patients develop a progressive increase in the urinary albumin excretion rate, creatinine and BUN, which has been identified as diabetic nephropathy (39,40). The development of renal failure in diabetic nephropathy is due to oxidative stress or inefficient antioxidant systems (41). Oxidative stress may disrupt renal sodium regulation and lead to hypertension (42). The present study demonstrated that AOE decreased urine albumin excretion and reduced the increase of plasma creatinine and BUN that occurred in db/db-diabetic mice, indicating that AOE may protect the kidney glomeruli against diabetic nephropathy. PTEN overexpression may act as an originator or promoter of diabetic nephropathy. Oxidative stress is one of the activators that regulates the increase of nuclear PTEN expression and additionally inhibits PTEN nuclear export (43). Long-term oxidative stress may induce diabetes by upregulating PTEN expression. Furthermore, the partial knockdown of PTEN

Figure 5. Effect of AOE on renal function. AOE not only significantly decreased the level of (A) urine albumin excretion but also inhibited the decreased levels of (B) urea Creatinine and (C) BUN in comparison with db/db-H2O group mice. Data represent the mean ± standard deviation (n=10). *P<0.05. BUN, blood urea nitrogen; AOE, Alpinia oxyphylla extract; AOE500, 500 mg/kg AOE; db/db- mice, type II diabetic animal model; DB/DB, C57BL/Ks DB/DB mice.

Figure 6. Effect of AOE on PTEN expression. Chronic administration of AOE attenuates high glucose-enhanced PTEN expression in the liver and kidney of db/db- mice (A). The gray level ratio of PTEN to β-actin in in the (B) liver and (C) kidney of db/db- mice. *P<0.05. AOE, Alpinia oxyphylla extract; AOE500, 500 mg/kg AOE; PTEN, Phosphatase and tensin homolog; db/db-mice, type II diabetic animal model; DB/DB, C57BL/Ks DB/DB mice.
ameliorates ROS-induced insulin resistance (44). The suppression of PTEN expression produces a marked improvement in blood glucose concentration and insulin sensitivity in diabetic mice (15). Inhibitors of PTEN may therefore serve as target proteins in future drug screens. Interestingly, AOE significantly impaired the PTEN protein level in parallel to the reduced glucose level, and attenuated oxidative stress. Further studies should be conducted to characterize the anti-diabetic component of AOE.

In conclusion, the present study demonstrated that AOE exhibited significant amelioration in hyperglycemia and hyperlipidemia by reducing blood glucose concentration and oxidative stress, increasing plasma insulin levels, improving renal function and impairing PTEN expression in the type II diabetic C57BL/KsJ db/db- mice. Therefore, A. oxyphylla may be developed as a novel medicine or functional dietary food supplement to act against diabetes.

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