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Original Article

Trapa natans pericarp extract ameliorates hyperglycemia and hyperlipidemia in type 2 diabetic mice

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ABSTRACT

The pericarp of *Trapa natans* L., an annual aquatic floating herb belonging to Lythraceae family, is used as a folk medicine in China. In this study, extracts of *Trapa natans* pericarp were tested both *in vitro* and *in vivo* through a high-fat diet with a single medium dosage streptozotocin injection induced type 2 diabetic mice. Different solvent extracts of *Trapa natans* pericarp showed α-amylase and α-glucosidase inhibitory activity. After four weeks administration, the ethyl acetate extract of *Trapa natans* pericarp (50 and 100 mg/kg b.w.) reduced fasting blood glucose level, ameliorated oral glucose tolerance and insulin resistance, improved serum lipids alterations in type 2 diabetic mice as well. Additionally, ethyl acetate extract significantly elevated the insulin receptor substrate 1 and Akt serine/threonine kinase phosphorylation compared to diabetic group. HPLC-MS and HPLC-DAD analysis showed that the ethyl acetate extract was rich in hydrolysable tannins. Results support the notion that *Trapa natans* pericarp extract has a potential hypoglycemic activity.

Introduction

Trapa natans L., is an annual aquatic floating herb belonging to Lythraceae family (Institute of Botany, the Chinese Academy of Sciences, 2004). The fruits of *T. natans* and some related *Trapa* species, commonly known as water caltrop or LingJiao in Chinese, were widely cultivated as food in China and Southeast Asia for a long history. The pericarp of water caltrop has been used as a folk medicine in China to cure diarrhea, dysentery, gastric ulcer, haemorrhoids and furunculosis. Previous studies showed that pericarp of water caltrops contains hydrolysable tannins, which have indicated several biological activities like antioxidant, liver-protective and anti-diabetic activity (Wang et al., 2011; Kang, 2011; Yasuda et al., 2014; Huang et al., 2016).

Diabetes mellitus (DM) is a worldwide metabolic disease. DM may be categorized into two main types. Patients of Type 1 DM have little or no endogenous insulin secretory capacity. Type 2 DM always develops from a combination of insulin resistance and an inadequate compensatory insulin secretory response (American Diabetes Association, 2009). The anti-diabetic properties of extracts from natural herbs have been widely demonstrated (Ahmad, 2013). For the serious side effectives of the present anti-diabetic medicines, it became a tendency to find effective and safe novel agents from natural products (Li et al., 2004).

The fruit of water caltrop has been used as an adjuvant function food to cure DM in south China, while the pharmacological actions and the specific active compound still remains unclear. In this study, a high-fat diet with single medium dosage streptozotocin (STZ)-induced Type 2 DM (T2DM) model was established in mice to investigate the potential anti-diabetic action of the extract of pericarp of *T. natans*—a common cultivated specie of water caltrop in south China. To indicate the anti-diabetic mechanism, the carbohydrate-hydrolyzing enzymes inhibitory effects and insulin resistant related proteins expression were also tested. HPLC-MS and HPLC-DAD methods were developed to investigate the structure and content of active compounds in the extract.

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Materials and methods

Plant material

Trapa natans L., Lythraceae, pericarp was collected in Zaozhuang, Shandong Province, China (35.115109 °N,116.760235 °E), in September 2014 and identified by Prof. Weilin Li at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing (China). A voucher specimen of *T. natans* (NO. 140910) has been deposited in Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing (China).

Chemicals

α-Amylase (Taka-Diastase, from *Aspergillus oryzae*), α-glucosidase (from *Saccharomyces cerevisiae*), 4-nitropheny-α-D-glucopyranose, streptozotocin and Folin-Ciocalteu's phenol reagent of analytical grade were obtained from Sigma–Aldrich (St. Louis, MO, USA). Acarbose was purchased from Bayer HealthCare Co. Ltd. (Beijing, China). Metformin hydrochloride was purchased from Zhonglian pharmaceutical Co. Ltd. (Shenzhen, China). 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose was isolated from *T. natans* by semi-preparative HPLC and identified by HPLC-MS and NMR, the purity is more than 95%. Methanol (HPLC grade) was obtained from Tedia Co. Inc. (Fairfield, OH, USA). Other reagents of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Extraction

The air-dried *T. natans* pericarp (10 kg) was ground to crude powder. The sample was extracted twice with 50 l of 80% ethanol by soaking for 14 days at room temperature. Then the resultant ethanol extract was concentrated *in vacuum* at 50 °C. The concentrated ethanol extract was suspended with water and successively partitioned by petroleum ether, ethyl acetate and *n*-butanol. Each extract was concentrated in vacuum at 50 °C to yield petroleum ether extract (TQP, 30.1 g), ethyl acetate extract (TQE, 238.9 g), *n*-butanol extract (TQB, 30.4 g).

Total phenolic content analysis

Total phenolic content measured by Folin–Ciocalteu phenol reagent was performed according to the published method (Lu et al., 2015).

HPLC-MS and HPLC-DAD analysis

TQE was dissolved in methanol (1 mg/ml) and filtered through a 0.45 μm PVD filter for HPLC-MS and HPLC-DAD analysis. HPLC-MS analysis was generated on an Agilent 6530 accurate-mass quadrupole time-of-flight system (Agilent, USA) with an ESI source operating in the negative ionization mode. Analysis was made using a MassHunter Qualitative Analysis software (B.05.00). Separation was performed with an Agilent ZORBAX SB-C_{18} column (4.6 × 100 mm, 1.8 μm, Waldbronn, Germany). A mixture of methanol (A) and 0.1% formic acid (B) was used as the mobile phase under gradient conditions (0–40 min, 10–50%A; 40–50 min, 50–100% A; 50–60 min, 100%). The HPLC-DAD analysis was carried out on a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, USA) equipped with a diode array-UV detector and a Inertsustain C_{18} column (4.6 × 250 mm, 5 μm, GL sciences, Japan) using the similar mobile phase to HPLC-MS. The UV detection was monitored at 280 nm.

α-Amylase and α-glucosidase inhibition test

The α-amylase and α-glucosidase inhibitory assay were determined by the method with small modification (Flores et al., 2013; Kazeem et al., 2013). In brief, samples were diluted with dimethylsulfoxide. Acarbose was used as a reference compound. 10 μl sample solution was added to 50 μl α-amylase (1 U/ml in 0.1 M sodium phosphate buffer (pH 6.9 with 6.7 mM sodium chloride).The mixture in each case was incubated at 37 °C for 10 min. Then, 50 μl of 1% starch solution in 0.1 M sodium phosphate buffer was added to each tube. The reaction mixture was incubated at 25 °C for 10 min and stopped with 10 ml of dinitrosalicylic acid reagent. The mixture was incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding 790 μl of distilled water, and absorbance was measured at 520 nm. A complete reaction mixture without sample was used as control.

The α-glucosidase inhibitory assay was performed in 96-cell microplates. Acarbose was used as a reference compound. Samples were diluted with dimethylsulfoxide. Sample (2 μl) was added to 4 μl of α-glucosidase (0.5 U/ml in 0.1 M sodium phosphate buffer, pH 6.9) and 154 μl of 0.1 M sodium phosphate buffer (pH 6.9). The mixture was incubated at 37 °C for 10 min. Then, 40 μl of 1.5 mM 4-nitropheny-α-D-glucopyranose (dissolved in 100 mM sodium phosphate buffer, pH 6.9) was added and incubated for 20 min. Absorbance was read at 405 nm. A complete reaction mixture without sample was used as control.

Animals and treatment

Male ICR mice (6-week-old, 18–22 g) were provided from Comparative Medicine Center of Yangzhou University, China. All mice were kept in an animal house controlled at a constant temperature of 22 ± 3 °C, and relative humidity of 55 ± 5% with 12 h dark-light cycle. All the experimental procedures and animal treatments were followed according to the Guide for the Care and Use of Laboratory Animals, which was approved by the Animal Ethics Committee of China Pharmaceutical University (certificate number: SYXK2016-0011).

The T2DM mouse model was established by the method (Ibrahim and Islam, 2014) with some modification. After one week acclimatization, animals were randomly divided into two groups. Mice were administered either a normal chow diet (10% of calories from fat) or a high-fat diet (HFD, 60% of calories from fat) which were provided by Shanghai Laboratory Animal Co. Ltd. In the fourth week, the HFD-fed mice were intraperitoneally injected once with a low dose of STZ (100 mg/kg b.w./*i.p.*, dissolved in citrate buffer (pH 4.4). After six weeks, mice with fasting serum glucose level >11 mmol/l, were classified as T2DM. The diabetic mice were randomly divided into four groups: the test groups were treated with low dose 50 mg/kg b.w. (TQEL) and high dose 100 mg/kg b.w. (TQEH) of TQE per day by intragastric administration. The reference group was treated with 200 mg/kg b.w. of metformin (MET) per day by intragastric administration. Diabetic mice control (DBC) and normal control groups (NC) were fed with 0.5% CMC-Na solution. During the experiment, blood glucose was determined weekly. After ten weeks of treatment, the food for animals was removed for 12 h but drinking water was free to tap. Animals were sacrificed under chloral hydrate anesthesia, the blood samples were collected from orbital vein and tissues samples were collected by dissection.

Oral glucose tolerance test (OGTT)

The oral glucose tolerance test was carried out after overnight fasting at 9th week, blood glucose was determined using a glucometer via the tail vein. And then they were orally loaded with a glucose solution (2 g/kg b.w.). Blood glucose concentrations were determined at 30, 60, 120 and 180 min. The area under the concentration versus (AUC) glucose 0–180 min was calculated.

Table 1
α-Amylase and α-glucosidase inhibitory assay.

Sample	Enzyme inhibition	
	α-amylase (IC_{50}, mg/ml)	α-glucosidase (IC_{50}, g/ml)
TQP	0.994 ± 0.132^{a}	0.104 ± 0.011^{b}
TQE	0.026 ± 0.008^{c}	0.0025 ± 0.00011^{b}
TQB	0.256 ± 0.098^{b}	0.055 ± 0.014^{b}
Acarbose	0.103 ± 0.014^{b}	560.3 ± 29.7^{a}

TQP, petroleum ether extract of *Trapa natans*; TQE, ethyl acetate extract of *T. natans*; TQB, n-butanol extract of *T. natans*.
Data were expressed as mean ± SD; [a]Columns with the same letter are not statistically different at $p < 0.05$.

Measurement of biochemical parameters in plasma

Blood samples were centrifuged at 2500×*g* for 15 min at 4 °C to obtain serum, which was then stored at −80 °C for further biochemical analysis. The levels of serum insulin concentration, triacylglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) were detected by commercial kits. Insulin sensitivity was assessed by computing homeostatic model assessment (HOMA).

Western blot analysis

Liver tissues were homogenized and lysed using a commercial protein extraction kit. The tissue lysate samples were centrifuged at 11,270×*g* at 4 °C for 5 min. The supernatants were collected and the protein concentrations were measured by BCA protein assay. Denatured proteins (25 μg) were applied to 10% SDS–PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were incubated overnight at 4 °C with primary antibodies (anti-phospho-IRS-1, anti-IRS-1, anti-Akt, anti-phospho-Akt, Cell Signaling Technology, Beverly, MA, USA) in 5% milk. They were then incubated with anti-mouse IgG, HRP-linked antibody or anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, Beverly, MA, USA) at room temperature for 1.5 h. Membrane-bound antibodies were detected by an enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Luminescent signal was captured with a Fuji medical X-ray film (Fujifilm, Tokyo, Japan).

Statistical analysis

All data were presented as the mean ± standard error of the mean (SEM). Statistical significance were evaluated using one-way ANOVA analysis followed by a Tukey's-HSD post-hoc test (GraphPad Software, Inc., San Diego, CA, USA). A value of $p < 0.05$ was considered as statistically significant.

Results and discussion

α-Amylase and α-glucosidase inhibitory assay

Postprandial hyperglycaemia may lead to β-cell dysfunction (Baron, 1998). What is more, the increased concentration of blood glucose will induce many disorders including retinopathy, nephropathy, neuropathy, and angiopathy. Decreasing postprandial hyperglycaemia plays a key role in the treatment of T2DM and pre-diabetic states. Inhibiting carbohydrate-hydrolyzing enzymes in the digestive tract, such as α-amylase and α-glucosidase contributes to reducing the absorption of glucose, thereby alleviating postprandial hyperglycaemia (Lebovitz, 2001).

Enzymatic inhibitory assay indicated that three extracts had inhibitory activity against α-amylase and α-glucosidase *in vitro*. TQE showed strongest activity among all the extracts (Table 1).

Effects of TQE on fasting blood glucose (FBG) and OGTT

From 6th week to 10th week, the NC mice maintained a stable glucose level while all the diabetic mice groups had a steady growth in glucose level (Fig. 1A). TQEL and TQEH significantly ($p < 0.01$) decreased the FBG level in 8th week compared with DBC group. TQEH significantly ($p < 0.05$) reduced the FBG level in 9th week and 10th week.

OGTT is a crucial parameter which has commonly been used to evaluate apparent insulin release and insulin resistance for diabetes screening (Stumvoll et al., 2000). In OGTT assay, the blood glucose levels and AUC of DBC group were significantly ($p < 0.0001$) higher than the NC group, suggesting a significant impaired glucose tolerance. TQEH significantly ($p < 0.01$) reduced the glucose level from 30 to 180 min compared with DBC group, similar activity was also observed in TQEL or MET group during the whole OGTT period. The total AUC of TQEL and TQEH were also significantly ($p < 0.01$, $p < 0.0001$) lower compared to the DBC group (Fig. 1B, C).

Fasting or postprandial hyperglycemia is implicated in the development of type 2 diabetes and macro- and microvascular complications associated with diabetes (Baron, 1998). It is vital to maintain the glucose homeostasis to prevent the effects of hyperglycemia and its associated complications. In this study, TQE indicated remarkably anti-hyperglycemic activity. Furthermore, TQE ameliorated the impaired glucose tolerance in diabetic mice.

Effects of TQE on serum insulin and HOMA-IR

A single low-dose injection of STZ may lead to a mild impaired to the pancreas which was used to mimic pancreas failure in the pathogenesis of T2DM. The serum insulin concentration and HOMA-IR value of the DBC group were significantly ($p < 0.0001$) increased compared with NC group. TQEL and TQEH significantly decreased the serum insulin concentration ($p < 0.01$, $p < 0.001$) and HOMA-IR scores ($p < 0.001$, $p < 0.0001$) compared with the DBC group (Fig. 1D, E).

TQE induced changes pathways involved in insulin resistance

In insulin signaling pathway, insulin induces insulin receptor substrate 1/2. Meanwhile, it stimulates the PI3K pathway which leads to Akt phosphorylation and glycogen synthase kinase-3 inhibition, and Akt2 might stimulate other transcription factors leading to inhibition of gluconeogenesis and glycogen accumulation in liver (Jian et al, 2018). As shown in Fig. 4, the phosphorylation of IRs-1 and Akt were significantly decreased in the liver of DBC group ($p < 0.001$).TQEH treatment significantly increased IRs-1 phosphorylation ($p < 0.01$). In addition, TQEL hand TQEH increased the Akt phosphorylation in a dose-related manner ($p < 0.05$, $p < 0.01$) (Fig. 2A, B).

In T2DM, the insulin receptor becomes less sensitive to insulin resulting in hyperinsulinemia and disturbances in insulin release (Shanik et al., 2008). In our study, high fat diet with a single low-dose injection of STZ induced an insulin resistance in mice. Both TQEL and TQEH significantly decreased the insulin level and HOMA-IR scores. Meanwhile, Subdued IRs-1 and Akt phosphorylation in the liver were also detected in DBC mice. These molecular events were significantly repaired by TQE administration. These results suggested that TQE could upregulate IRs-1and Akt phosphorylation which may lead to the ameliorated insulin resistance in T2DM mice.

Effects of TQE on serum lipid profiles

High-fat diet treatment also induced significant ($p < 0.0001$, $p < 0.001$, $p < 0.0001$, $p < 0.05$) boosted TC, TG, LDL-c levels and decreased HDL-c value in DBC group compared with the NC group.

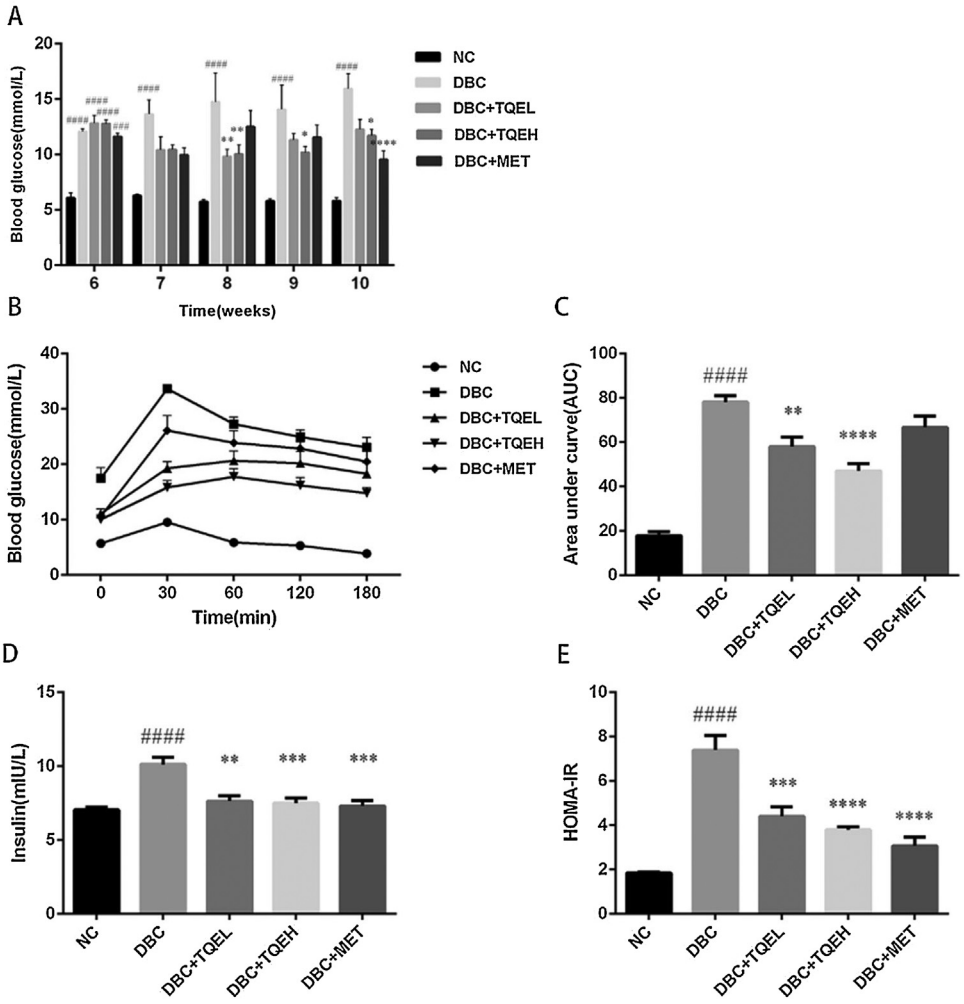

Fig. 1. The effects of TQE on hyperglycemia and insulin resistance in T2DM diabetic mice. A. Fasting blood glucose in 6–10 weeks. B. Oral glucose test (OGTT). C. Area under the curve of OGTT. D. Fasted serum insulin level in 10th week. E. HOMA-IR values, which were calculated as glucose (mmol/l) × insulin (mIU/l)/22.5. NC:, normal control; DBC: diabetic control; TQEL: low dosage (50 mg/kg b.w.) of ethyl acetate extract of *Trapa natans*; TQEH: high dosage (100 mg/kg b.w.) of ethyl acetate extract of *T. natans*; MET: Metformin hydrochloride (200 mg/kg b.w.). Each value represents the mean ± *SEM* (n = 8); $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs. NC, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. DBC).

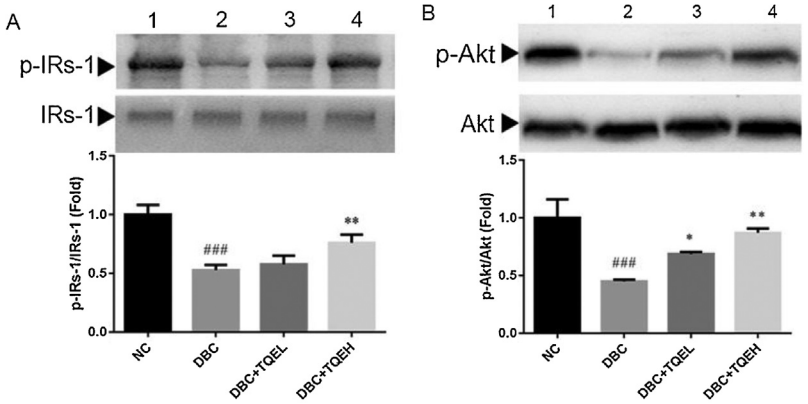

Fig. 2. Effect of TQE on the IRs-1(A) and Akt (B) expression in liver issue. 1. NC; 2. DBC; 3. DBC + TQEL; 4. DBC + TQEH. All the results were presented as mean ± SE (n = 3). $^{\#\#\#}p < 0.001$ vs. NC; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. DBC.

After four weeks administration, TQEH and TQEL significantly ($p < 0.05$) reduced the TG level and the LDL-c level, respectively. Neither TQEH nor TQEL made significant ($p > 0.05$) decrease in TC level. Both TQEH and TQEL group showed significant ($p < 0.01$, $p < 0.01$) increase in HDL-c level compared with DBC group (Fig. 3A–D).

Boosted TC, TG, LDL-c levels, reduced HDL-c are the key abnormalities that constitute diabetic dyslipidaemia. The development of T2DM often associated with dyslipidaemia, which increased the risk of complications-coronary heart disease, diabetic nephropathy, non-alcoholic fatty liver disease. (Best and O'Neal, 2000; Bardini

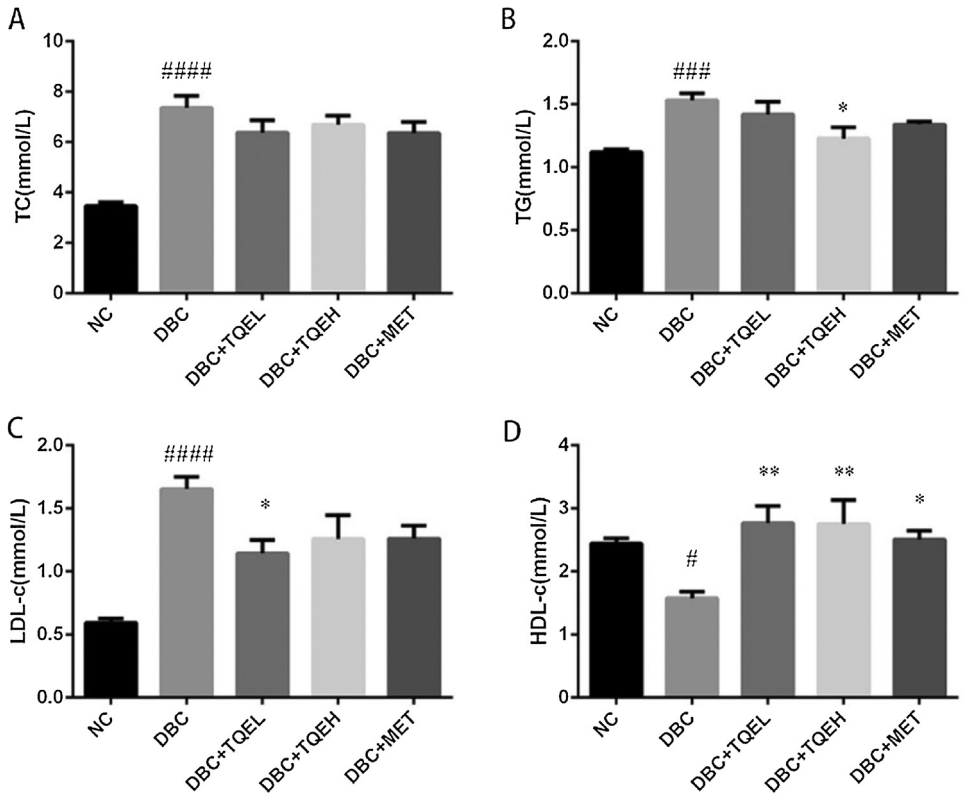

Fig. 3. The effect of TQE on the serum lipid profile, TC (A), TG (B), LDL-c (C), HDL-c (D) in T2DM diabetic mice. Each value represents the mean ± SEM (n = 8); $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs. NC, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. DBC).

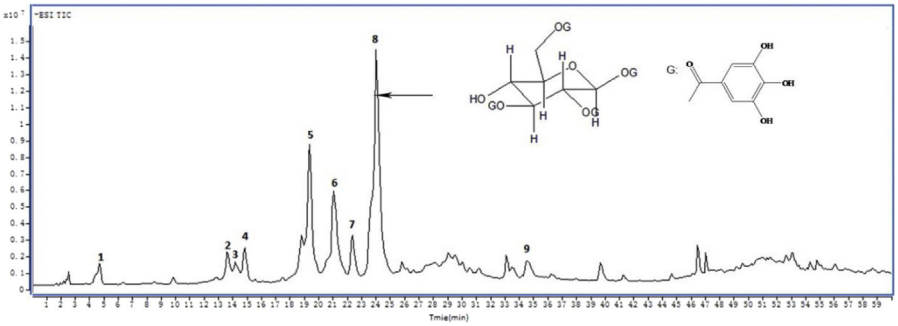

Fig. 4. Chromatograms of TQE by HPLC-MS analysis. gallic acid (1), 1,2,3-tri-*O*-galloyl-β-D-glucopyranose (2), 1,6-di-*O*-galloyl-β-D-glucopyranose (3), 1,3-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxy-diphenoyl-β-D-glucopyranose (4), 1,2,6-tri-*O*-galloyl-β-D-glucopyranose (5), eugeniin (6), ethyl gallate (7), 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (8), ellagic acid (9).

et al., 2011; Komiya et al., 2016). Hence, it should be given a high priority to control the disordered lipid metabolism in the clinical care of diabetes patients. In our study, TQE ameliorated the lipid metabolism disorder by reducing the TG, LDL-c and increasing HDL-c level, suggesting that TQE may be helpful to reduce the risk of diabetic complications.

Total phenolic content

The total phenolic content of TQP, TQE, TQB were 207 ± 11 mg/g, 960 ± 56 mg/g, 552 ± 18 mg/g (as gallic acid equivalent), respectively. Among them, TQE has the highest total phenolic content.

HPLC-MS and HPLC-DAD analysis

In a further study, nine phenolic compounds were assigned from TQE through a HPLC-MS analysis (Fig. 4) (Niemetz and Gross, 2005; Yasuda et al., 2014; Huang et al., 2016). Among of these compounds, compounds 2–6,7,8 were hydrolysable tannins. TQE contains 398 ± 17 mg/g 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (compound 8).

Results showed that TQE was rich in phenolic compounds, and most of which were hydrolysable tannins. It was reported that some hydrolysable tannins (including compounds 2,5,6,7,8 assigned in TQE) isolated from *T. natans* could enhanced glucose uptake activity in C2C12 myotubes *in vitro* (Huang et al., 2016). Compounds 6 and 8, which were isolated from *T. japonica*, displayed both α-amylase and α-glucosidase inhibitory activities and they could reduce blood glucose in normal mice (Yasuda et al., 2014). These reports suggested that the hydrolysable tannins may be the potential pharmacological active compounds responsible for the hypoglycemic effects.

Conclusions

The fruit of *T. natans* was used as an anti-diabetes preparation in China. This study showed that ethyl acetate fraction of pericarp

of the fruit has anti-T2DM activity as well. This pharmaceutical activity is possibly mediated through interfering with gastrointestinal glucose absorption and stimulation of insulin sensitivity. What is more, the fraction was able to ameliorate the diabetes-associated lipid disorder. The componential analysis showed that the fraction was abundant in phenolic compounds, especially in hydrolysable tannins. The further work should be focus on the molecular mechanism as well as the active phenolic compounds for the pharmaceutical activities of the extract.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

Formal analysis, HL; Investigation, HLTYJ, and XQD; Methodology, YYZ and JC: Project administration, H.L. , X.L. , J.W.C. and W.L.L.; Writing – original draft, HL; Writing – review and editing, XL and JWC.

Conflicts of interest

The authors declare no conflicts of interest.

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