

Hypoglycemic effect of triterpenoid-rich extracts from *Euryale ferox* shell on normal and streptozotocin-diabetic mice

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Abstract: The antioxidant effects of the triterpenoid-rich extracts from *Euryale ferox* shell (ES) have been confirmed *in vitro*. This study examined whether the triterpenoid-rich extract from ES eases human hyperglycemia and diabetes caused by metabolic disorders. Normal and streptozotocin (STZ)-induced diabetic mice were used as controls for the four groups that received the triterpenoid-rich extracts of ES suspended in distilled water orally at doses of 200, 300, 400, 500±2 mg/L. Body weight, blood glucose and pancreatic tissue morphology were observed after 4 weeks. The expression of protein tyrosine phosphatase-1B (PTP1B) and insulin receptor substrate (IRS-1) proteins, which are related to the regulation of glucose metabolism *in vivo*, were also investigated. Compared with the model group (LD₅₀ 900±2 mg/L), it was found that the triterpenoid-rich extracts of ES could regulate glucose metabolism (P<0.01) and cause body weight to return to normal levels (P<0.05). Islet morphology recovered well, the expression of the negative regulation protein PTP1B gene was reduced and insulin receptor IRS-1 protein expression was increased. These data prove that the triterpenoid-rich extracts from ES have a therapeutic effect on diabetes by insulin resistance.

Keywords: Euryale; diabetes; insulin resistance.

INTRODUCTION

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both, which is a condition that develops over a period of a few days as the blood glucose levels of a type 1 or type 2 diabetic gradually rise (Tahrani *et al*, 2010). This results in severe micro vascular and macro vascular problems, including neuropathy, nephropathy, retinopathy, cardiovascular and peripheral vascular disease. (Anusha Bhaskar *et al*, 2008). If the serum glucose levels remain over 800 mg/dL for several hours, swelling can occur in the brain that can eventually result in coma (Egawa *et al*, 2001).

Among several targets in diabetes therapy, Protein-tyrosine phosphatase-1B (PTP1B) is a typical non-receptor type from the PTP family in the insulin signaling pathway. PTP1B has been recognized as a therapeutic drug target for type 2 diabetes and over expression of PTP1B in transfected cells inhibits IR and IRS-1 phosphorylation. When knocking down the PTP-1B gene in wild-type mice, we found that insulin sensitivity was greatly increased and that blood glucose tended to be normal. It is worth noting that these mice later received a high-fat diet but have not presented with high blood sugar (Asente-Appiah *et al*, 2004). Over expression of PTP1B blocks the activation of insulin receptor tyrosine and insulin signaling (Row *et al*, 2007). These results

suggested that inhibiting the expression of PTP1B can increase insulin sensitivity and ease hyperglycemia. Numerous studies have searched for an efficient, specific inhibitor of PTP1B to treat type 2 diabetes. In fact, PTP1B causes activated insulin receptor (IR) dephosphorylation, thus blocking the insulin-signaling pathway. Therefore, any IR-related changes in PTP1B expression levels or dynamics may affect insulin signal transduction and lead to insulin resistance. The role of PTP1B for the IR, insulin receptor substrate 1,2 (IRS-1, IRS-2), growth factor receptor binding protein 2 (Grb2), phosphatidylinositol 3-kinase (PI-3K) and other proteins associated with insulin signal transduction is to induce dephosphorylation of the phosphotyrosine residues of these proteins and attenuate insulin signal transduction, resulting in post-receptor insulin resistance (Kim *et al*, 2009).

The use of medicinal plants for the treatment of diabetes mellitus dates back from the Ebers papyrus of about 1550 B.C. A multitude of herbs, spices and other plant materials have been described for the treatment of diabetes throughout the world. The practice of pharmacological has been attracting attention for its advantages, such as effectiveness, low incidence of side effects and also for historical, cultural and economic reasons. The wide diversity of species has led scientists to make great efforts to bioprospect plants that may contribute to the management of diabetes (Qiu *et al*, 2004).

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Euryale ferox is a mature seed of the *Euryale* genus of *Nymphaeaceae* which is identified by Nath *et al.* (1985). Recently, a large number of selective small molecules were reported to inhibit the expression of PTP1B (Chen *et al.*, 2002). This study aimed to identify new sources of efficient and safe anti-hyperglycemia drugs. *Euryale ferox* has been used in food and medicine for a long time. Its roots, stems and leaves can also be used as medicine. In this study, the *Euryale ferox* shell was extracted with alcohol and used to reduce the blood sugar of mice with hyperglycemia.

MATERIALS AND METHODS

Triterpenoid-rich extracts from the Euryale shell

Euryale ferox were collected in and around Huainan City in China. A voucher specimen authenticated by the Department of Botany at the Hefei University of Technology, was deposited at the College of Biotechnology and Food Engineering under number 425. The seed was broken and the shell was soaked in 3 volumes of 75% ethanol at 60°C for 16 h to obtain an extract. The extracts were concentrated using a vacuum rotary evaporator at 50°C. The concentrated extracts were then diluted with water, and the solution was extracted 3 times with petroleum ether, ethyl acetate and water-saturated butanol, in that order. The extracts were then pooled and concentrated using a rotary evaporator to yield triterpenoid-rich main fractions and dried to a powder. The powder was then mixed with water to concentrations of 200, 300, 400, 500±2 mg/L.

Animals and experimental protocol

Forty-eight male KM mice were purchased from the animal experiment center of The Anhui University of Medicine. Hyperglycemia was induced in 40 animals at 12 weeks of age by the intraperitoneal injection of STZ (80 mg/kg) buffered in cold sodium citrate (pH 4.4). The experimental protocol was approved by the Animal Experimentation Ethics Committee of the UFPE (Process no. 012974), in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Seven days after STZ injection, hyperglycemia was documented by measuring the glucose content of tail vein blood with a Roche Glucometer (Roche Diagnostics GmbH, Mannheim, Germany). Hyperglycemia was defined as a plasma glucose concentration greater than 16.7 mmol/L. There were eight animals per cage and six groups A (normal group), B(model group), C, D, E and F) that were separated, each with free access to food and water. The water bottles were filled twice each day, and the animals were weighed once each week. Body weights were recorded at the start and the end of the experimental period. Blood was collected by cardiac puncture and livers were rapidly excised, rinsed in ice-cold saline and weighed. Aliquots of liver were snap frozen in liquid

nitrogen and kept at -80°C until analyzed. Each pancreas was fixed in 10% formalin for histology.

Histology and immunohistochemistry

Pancreases from 6 animals of each group were processed, each of which were fixed in 10% paraformaldehyde overnight and processed for paraffin embedding. Sections (4 µm) were mounted on organosilane-coated slides. Hematoxylin/eosin staining was used for histological evaluation under a light microscope (Hu *et al.*, 2005). Images of the pancreatic tissue and islet β-cells were viewed on the monitor of a computer through a microscope connected to a charged coupling device camera. The areas of the islets were contrasted and analyzed.

RT-PCR analysis

Total RNA was isolated from the liver tissues by a UNIQ-10 pillar Trizol total RNA extraction kit (Shanghai Sangon Biological Engineering Technology & Service Corporation Ltd., Shanghai, China). The A260/A280 of total RNA was between 1.8 and 2.0. The primers were designed by Primer Premier 5 according to the PTP1B and the IRS-1 gene sequences in the NCBI gene bank. The primers for mouse PTP1B (Forward 5'-CCTA CCTG GCTG TCAT CG-3', Reverse 5'-CCAC CATC CGTC TCCT AAC-3'; 365 bp) and IRS-1 (Forward 5'-GTTG AGTT GGGC AGAA TAGG -3', Reverse 5'-TCCA AAGG GCAC CGTA TT-3'; 512 bp) were synthesized by the Shanghai Sangon Biological Engineering Technology & Service Corporation Ltd.

Total RNA was reverse transcribed into cDNA using the AMV First Strand cDNA Synthesis Kit (Shanghai Sangon Biological Engineering Technology & Service Corporation Ltd., China) and amplified by PCR. Each 25µl of RT reaction contained 11.4µl of sterile de-ionized H₂O, 2.5µl of 1 × PCR Buffer, 2.5µl of 0.2 mmol/L dNTP mixture, 1.25µl of each 0.5 µmol/L PTP1B primer or 1.25 µl of each 0.5µmol/L IRS-1 primer, 0.1µl of 0.5 µg/25µl Taq DNA polymerase, 1.5µl of 1.5mmol/L MgCl₂, and 2 µl of 10 pg⁻¹ µg cDNA. The amplification profile was as follows: 1 cycle of 95°C for 2 min; 30 cycles of 95°C, 45 s; the PTP1B primer at 57°C or the IRS-1 primer at 55°C for 1 and 30 s; 72°C for 45 s; and a final elongation step of 72°C for 10 min.

Each PCR product was mixed with 2µl of loading dye (25% bromophenol blue, 25% glycerol) and electrophoresed on a 1.5% agarose gel containing ethidium bromide (0.5µg/ml) for 1 h at 100 V.A low DNA mass ladder was used as a molecular weight marker. The DNA bands were visualized and densitometric analysis was performed using a UV transilluminator.

STATISTICAL ANALYSIS

The statistical software Origin 6.1 (Origin Lab Corporation, USA) was used by the first author of this

study. All experimental data were expressed as the mean $\pm$ SD. The differences between the test and control groups were evaluated using Student's t-test.

RESULTS

The effect of the ES extracts on serum and body weight levels

After 4 weeks of the ES diet, the body weights and blood glucose values of each group of mice were evaluated at the beginning and the end of this experiment. The experimental data were analyzed, and the results are as follows:

After four weeks of feeding, the mice that did not receive STZ grew well, and their fasting blood glucose values were normal from the beginning to the end of the experiment. The other groups injected with STZ exhibited destruction of the islet β cells, and their blood glucose values were elevated. After four weeks of feeding with the triterpenoid-rich extracts from ES, the blood glucose levels were reduced. The higher concentrations of the ES extract showed enhanced reduction of blood glucose levels. As indicated by statistical analysis, the D, E and F groups are significantly different with regard to the initial blood glucose data ($P < 0.05$). Obviously, the ES extracts can regulate blood sugar metabolism and lower the blood sugar of hyperglycemic mice (fig. 1).

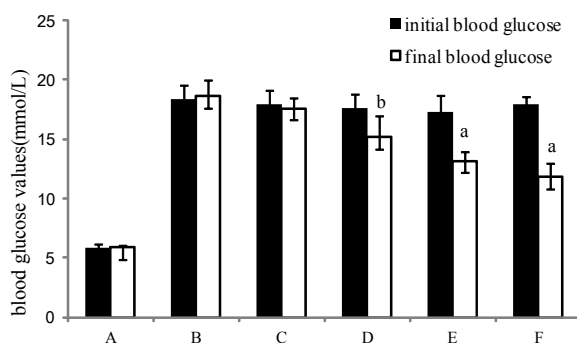


Fig. 1: Comparison of blood glucose values of experimental mice in each group. The A group is a normal group, the B group is a model group, the C group received 200 mg/L ES extract, the D group received 300 mg/L ES extract, the E group received 400 mg/L ES extract and the F group received 500 mg/L ES extract.

Comparing the experimental groups with the normal group and model group with regard to body weight, mice under normal conditions grew larger, while mice in the model group showed a decrease in body weight. After four weeks of feeding with the ES extracts, mice suffering from hyperglycemia showed increased body weight, with groups E and F showing significant increases when compared with the model group ($P < 0.05$). Thus, the

triterpenoid-rich extracts from ES can ease the weight loss caused by hyperglycemia (fig. 2).

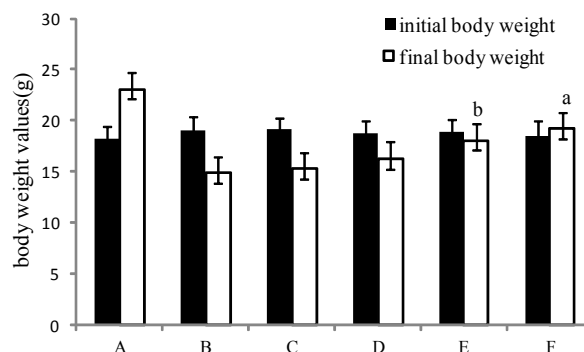


Fig. 2: Comparison of body weights among experimental mice. The A group is a normal group, the B group is a model group, the C group received 200mg/L ES extract, the D group received 300mg/L ES extract, the E group received 400 mg/L ES extract and the F group received 500 mg/L ES extract.

Effect on islets observed through hematoxylin/eosin staining

We can see in photo A that the pancreatic cells are full of cytoplasm, show islet morphology integrity and no signs of fibrosis, and have islet cells that are more tightly packed. In photo B, the boundary between the exocrine tissue and the islet perimeter is fuzzy, the size of the islet is becoming smaller and irregular, the cytoplasm of the islet cells is decreased, the pyknotic cell nuclei are darkly stained and there is tissue fibrosis. In photo C, the islet cells are also shrunken and darkly stained. In photo D, the islets do not continue to shrink; additionally, the cell structures become clear, the cytoplasm begins to increase, the tissue is generally lighter in color and fibrosis is reduced. In photo E, the pancreatic cell morphology becomes clearer and complete and there is an increase of cytoplasm in the islet cells and a significant decrease in fibrosis-like cells. In photo F, the islet morphology is full, the cells are closely arranged, there is an abundance of cytoplasm, the staining is shallow, the cell structures maintain their integrity and fibrosis has disappeared (fig. 3).

Expression of PTP1B and IRS-1 protein in livers of treated hyperglycemic mice

To confirm that the treatment of the hyperglycemic mice with ES extracts resulted in PTP1B and IRS-1 protein expression in the livers, the total RNA was extracted from the livers and reverse transcribed into cDNA; Then, the PCR products were amplified using the corresponding specific primers. The PTP1B PCR products were 365 bp, while the IRS-1 PCR products were 512 bp. The PCR products in each group were compared by agarose gel electrophoresis. The DNA marker ladder indicates 100 bp, 150 bp, 200 bp, 300 bp, 400 bp and 500 bp.

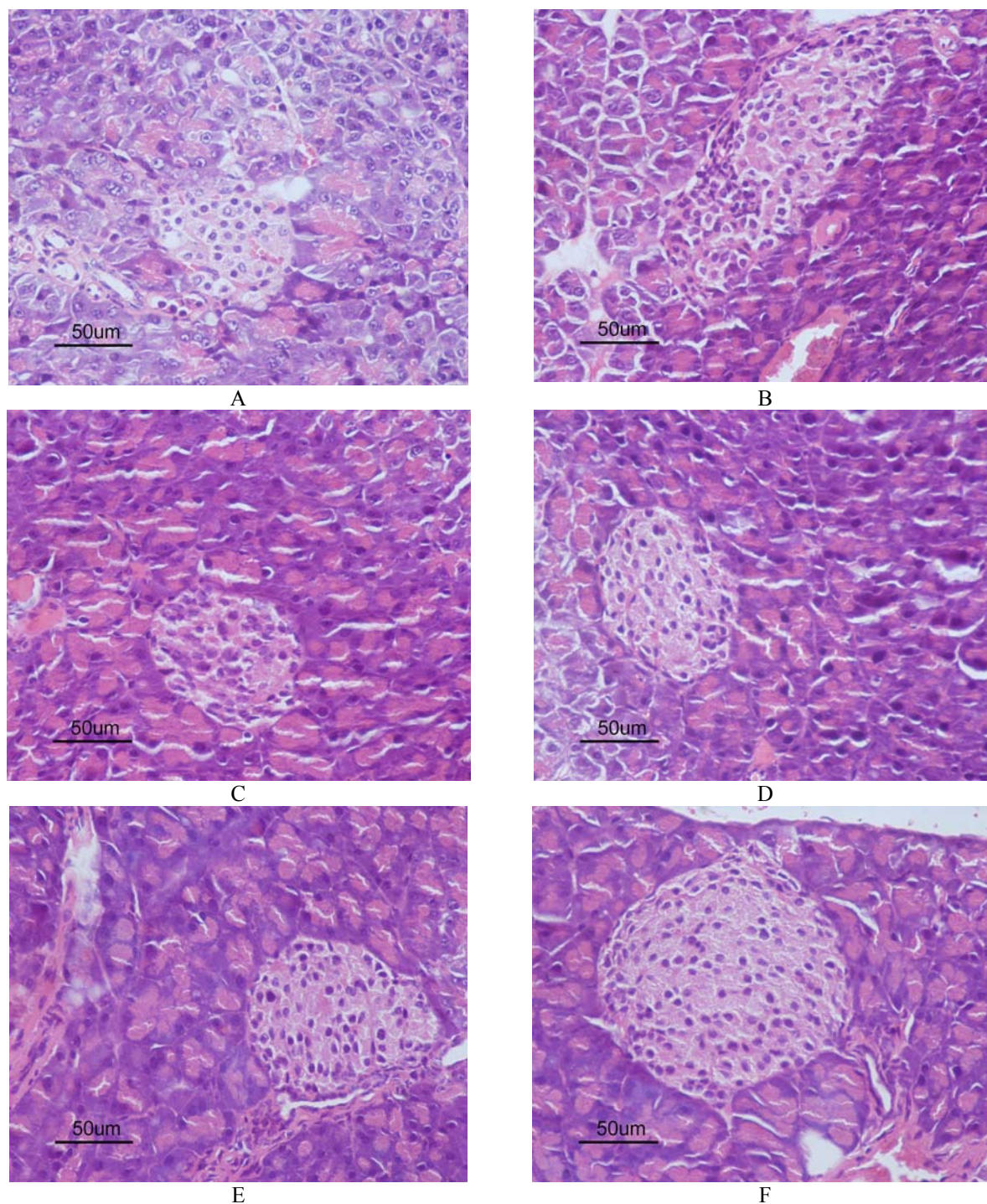


Fig. 3: Photos of pancreas histology in mice with HE staining. Photo A is the normal group, photo B is the model group, photo C received 200 mg/L ES extract, photo D received 300 mg/L ES extract, photo E received 400 mg/L ES extract and photo F received 500 mg/L ES extract.

It can be observed that, compared to model group B, groups C, D, E and F showed reduced expression of the PTP1B protein, with Group F showing significant reduction. This result indicated that feeding the mice ES extracts can inhibit the expression of PTP1B. The IRS-1 protein expression was performed. Group A showed a

large amount of protein expression, while group B showed little. Groups C, D, E and F expressed increasing amounts of protein, all of which were greater than that of Group B. This finding demonstrates that triterpenoid-rich extracts from ES may increase the amount of IRS-1 protein and that IRS-1 protein can improve the insulin

signal transductions needed to decrease blood sugar concentration (fig. 4).

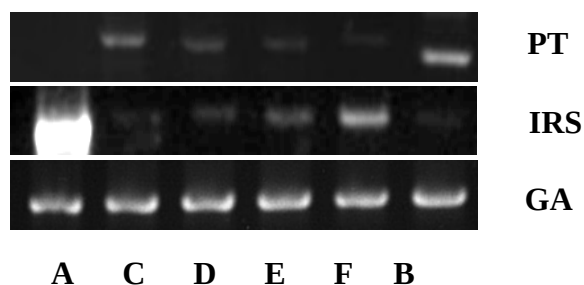


Fig. 4: The treatment of the hyperglycemic mice with ES extract resulted in PTP1B and IRS-1 protein expression in the livers.

DISCUSSION

We know that blood sugar elevation is associated with the amount of insulin and that the lack of insulin may lead to type 2 diabetes. In recent years, the incidence and mortality rates of type 2 diabetes have been increasing. Many experts are concerned about the causes of insulin insufficiency. One of the many reasons for this insufficiency is insulin resistance caused by a glucose metabolism disorder in a target tissue, which undermines the secretion of insulin by the pancreas (Brandy Panunti *et al*, 2004). The protein tyrosine phosphatase-1B (PTP1B) is the main negative regulator of the insulin signaling pathway and has received significant attention in recent years (Salar Bakhtiyari *et al*, 2010). It is associated with the protein tyrosine kinase (PRK), which controls many cellular functions via tyrosine phosphorylation levels. These functions include regulating cell growth, differentiation, metabolism, cell cycle, cell communication, cell migration, gene transcription, ion channel activity, immune response and other processes (Zhang, 2002). Activation of PTP1B can lead to the abnormal phosphorylation of tyrosine residues, and the disharmony of these signals in any part of the network may cause a variety of human diseases, including cancer and diabetes. The incidence of type 2 diabetes has gradually increased around the world (Kennedy *et al*, 2000, Gum *et al*, 2003).

This study proves that PTP1B has an important role in human endocrine diseases, especially in type 2 diabetes. Gum used PTP1B antisense oligonucleotide as a therapy in diabetic mice and found that this therapy decreased the amount and activity of PTP1B, while phosphorylation of the insulin receptor and substrate increased (Zinker *et al*, 2002). Zinker *et al*. used the PTP1B antisense oligonucleotide on the db / db mice model and found that after treatment, the 2 h postprandial fasting blood glucose level of these mice was decreased, the glycated hemoglobin (HbA1c) was higher and close to the normal levels, and the amounts of PTP1B and its mRNA in liver

and fat tissues were decreased (Malamas, 2000). Numerous studies have shown that PTP1B is a potential target for the treatment of diabetes and obesity and has attracted more research into finding an efficient and specific inhibitor of PTP1B to treat type 2 diabetes. Recently, articles have reported a large number of selective small molecule inhibitors of PTP1B (Bhavna Sharma *et al*, 2008), but the triterpenoid-rich extracts from ES have not been described. In this paper, it was shown that the ES extracts can inhibit the expression of PTP1B, indicating that the ES extracts can be used to decrease blood glucose levels in hyperglycemic patients with type 2 diabetes.

IRS-1 is a substrate capable of directly activating the insulin receptor tyrosine kinase. IRS-1 binds several proteins through its SH2 region and can accelerate glucose uptake, transport and substrate phosphorylation (Bradley *et al*, 2002). As a regulator, PTP1B can reduce the phosphorylation levels of IRS-1 (Janice M *et al*, 2004). The above data show that ES extracts improve the expression of IRS-1, implying that the triterpenoid-rich extracts from ES can inhibit the expression of PTP1B and accelerate IRS-1 phosphorylation and glucose transport. Treatment with ES extracts also increases the level of IRS-1 expression (fig. 4), improves insulin substrate concentration and accelerates the reduction in blood glucose levels in hyperglycemia and diabetes.

From histology, we know that after treatment with the ES extracts, the morphology of the pancreas in the experimental groups recovered well. This finding indicated that the damaged pancreatic tissues can be repaired by the triterpenoid-rich extracts from ES.

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