Effect of a stilbene glycoside-rich extract from Polygoni Multiflori Radix on experimental non-alcoholic fatty liver disease based on principal component and orthogonal partial least squares discriminant analysis

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Abstract. Polygoni Multiflori Radix is a traditional Chinese medicine used clinically to support the functions of the liver and kidneys and to treatment hyperlipidemia. In previous studies, an effective fraction, rich in 2,3,5,4'-tetrahydroxy stilbene-2-O-β-D-glucoside (TSG), was separated from Polygoni Multiflori Radix and demonstrated hypolipidemic activity. The present study aimed to systematically assess the effect of this fraction on non-alcoholic fatty liver disease (NAFLD). A NAFLD model was established by feeding Sprague-Dawley rats a high-fat diet with 10% fructose solution for 18 weeks. Hematoxylin and eosin staining was applied for hepatic histopathological analysis. In addition, enzyme activities, lipid metabolism, inflammatory factors and insulin resistance indices were measured using a fully automatic blood biochemistry analyser and ELISA. Furthermore, cytochrome P450 2E1 (CYP2E1) and peroxisome proliferator-activated receptor α (PPARα) mRNA and protein expression were evaluated using reverse transcription-quantitative polymerase chain reaction and western blot analysis. Principal component analysis and orthogonal partial least squares discriminant analysis were used to analyse the data. The results revealed that the TSG-rich fraction (TSGP) significantly lowered the serum total cholesterol and triglyceride levels, and the liver free fatty acid, CYP2E1 mRNA and malondialdehyde levels, in addition to mitigating hepatic enlargement and alleviating liver steatosis. Furthermore, it upregulated PPARα mRNA expression in the liver tissue. The results indicated that TSGP exhibited a protective effect against NAFLD and the underlying mechanism may involve augmentation of anti-lipid peroxidation capacity via regulation of PPARα and CYP2E1-mediated pathways.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease (1). NAFLD is associated with the current obesity pandemic, and ~20-33% of adults in developed countries suffer from NAFLD (2). The term NAFLD describes a spectrum of liver disease, which may develop from fatty infiltration to steatohepatitis and hepatocellular carcinoma (3) if effective intervention is lacking. Hyperlipidemia-induced fatty infiltration and oxidative injury are considered to be the major factors promoting the occurrence and development of NAFLD (4-7). Although controlling body weight with diet and exercise is effective for NAFLD therapy (8), drug treatment remains an important means of disease management. Agents, including exenatide and statins (9), used for the treatment of diabetes and hyperlipidemia are being tested as potential treatments for NAFLD and non-alcoholic steatohepatitis (10). Considerable attention has been focused on natural products as an alternative means of treating NALFD; a numberof natural products are thought to have functions that ameliorate the symptoms of NAFLD via the restoration of lipid metabolism (11).

Polygoni Multiflori Radix, also known as Heshouwu (HSW), a dried root of *Polygonum multiflorum* Thunb., is a traditional Chinese medicine that has been used for supporting the functions of the liver and kidney, and for regulating hyperlipidemia for several decades (12). HSW is one of the most frequently used crude drugs for the prevention and treatment of hyperlipidemia and NAFLD (13,14), and a previous study revealed that HSW exhibits a pronounced effect on lipid regulation in the treatment

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of early-stage NAFLD (15). Bioactive component analysis has revealed that HSW comprises stilbenes, phenolic acid and flavonoids as potential lipase inhibitors (16), and protocatechuic acid and 2,3,5,4'-tetrahydroxy-stilbene-2-O-β-d-glucoside (TSG), which exhibit antioxidant activity (17). Previous studies have demonstrated that TSG has good hypolipidemic effects, particularly in the reduction of low-density lipoprotein-cholesterol (LDL-C) via the promotion of intracellular cholesterol 7α-hydroxylase (CYP7α) expression (18-20), and is able to reverse NAFLD through gut microbiota and toll-like receptor 4/nuclear factor-κB (NF-κB) pathway modulation (21).

The present authors' research group has focused on the bioactive component analysis, separation, pharmacodynamics and toxicology evaluation of HSW for a number of years, with a particular focus on TSG. In a previous study by the present group, an extract containing >50% TSG was obtained using a macroporous resin. A dose-dependent anti-hyperlipidemic effect was observed for this extract in pharmacodynamic experiments, and a 9-month long-term toxicity test of beagles revealed that a dosage of 1.0 g/kg/day is safe (data not published). Pharmacokinetic studies revealed that TSG was rapidly absorbed and widely distributed throughout the body with great efficiency, followed by rapid elimination and clearance (22), and indicated that the liver was the organ containing the highest amount of TSG (23,24). All the aforementioned factors indicate that TSG is a potential candidate for anti-NAFLD drug development.

Previous studies concerning the anti-NAFLD related effects of TSG have focused on the active component (25) and on a single effect, including lipid regulation and anti-inflammatory functions (26,27), and no comprehensive evaluation of the effect of TSG on anti-NAFLD using multiple indices has been reported. Thus, the present study used an NAFLD model induced by a high-fat diet (HFD) with fructose drinking to systematically assess the effects of the TSG-rich fraction (TSGP) of HSW in the prevention of NAFLD. This was assessed with the aim of elucidating the main efficacy, indices and the potential mechanisms of this composition.

Materials and methods

Reagents. TSGP was prepared through an adaptation of a previous extraction process (28), with several modifications. Briefly, Polygoni Multiflori Radix (purchased from Zhejiang Chinese Medical University, Zhejiang, China) was crushed and extracted with 60% (v/v) ethanol by a refluxing method. Following concentration via evaporation, the fluid ethanolic extract was subjected to open column chromatography (1.5 m x22 cm) with a macroporous resin (NKA II, The Chemical Plant of Nankai University). The column was eluted stepwise with 10, 20 and 50% (v/v) ethanol solution. The 50% eluted fraction was collected, concentrated and dried under vacuum conditions. The content of TSG in this fraction was 54%, which was determined using high-performance liquid chromatography with diode-array detection (Agilent 1100 series; Agilent Technologies, Inc., Santa Clara, CA, USA; Fig. 1). The separation was achieved using an Ultimate XB-C18 column (150x4.6 mm x5 μm; Welch Materials, Inc., Austin, TX, USA) at 25°C with acetonitrile and H_2O_2 (20:80 v/v) as mobile phase at flow rate 1.0 ml/min, and the sample injection volume was

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Primer	Direction	Sequence (5'-3')
PPARα	Forward	GCTTCATCACCCGAGAGTTC
	Reverse	GGGAAATGTCACTGTCATCCA
CYP2E1	Forward	TCTGCTCCTGTCTGCTATTCTG
	Reverse	ACTGCCAAAGCCAACTGTGA
β-actin	Forward	GCTCTCTTCCAGCCTTCCTT
	Reverse	GGTCTTTACGGATGTCAACG

PPARα, peroxisome proliferator-activated receptor α; CYP2E1, cytochrome P450 2E1.

5 μl. The HFD consisted of standard fodder 76.5%, lard 12%, cholesterol 1%, yolk powder 5%, whole milk powder 5% and cholate 0.5%, and was formulated by the Animal Supply Centre of Zhejiang Academy of Medical Science (Hangzhou, China).

Animals and treatments. A total of 38 male, 8-week-old Sprague-Dawley rats, weighing between 180 and 200 g, were purchased from the animal supply centre of Zhejiang Academy of Medical Science [certificate no.: SCXK (Zhe)2014-0001, Hangzhou, China]. The animals were housed at 25±1°C with humidity of 55±5%, and exposed to a 12-h light/dark cycle for 1-week acclimatization prior to the experiment. All rats were fed rodent laboratory chow with tap water *ad libitum* and were fasted but had free access to water for 12 h prior to the experiment. All procedures were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals and with the Animal Management Rules of the Health Ministry of PR China (document no. 55, 2001). The study was approved by the Ethics Committee of Zhejiang Chinese Medical University.

Animals were divided into the normal (n=10), model control (n=10), positive control (polyene phosphatidylcholine, PPC; n=10) and TSGP (n=8) groups according to their blood lipid levels, which were measured prior to the experiment. Animals were provided with free access to water and those in the normal group were fed a control diet (CD), while those in the model, PPC and TSGP group were fed the HFD with 10% fructose solution for 18 weeks. Distilled water was provided to the rats in the normal and model control groups, while the positive control and TSGP groups were administered 136.8 mg/kg PPC preparation (Essentiale; Sanofi-Aventis Beijing Pharmaceutical Co. Ltd., Beijing, China) and 160 mg/kg TSGP, respectively. All the water or test substances were orally administrated once daily. Throughout the study, animals were weighed once weekly, and the levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), LDL-C, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured at 4, 6, 10, 14 and 18 weeks. At the end of the experiment all animals were anesthetized and sacrificed. Blood was collected from the abdominal aorta and centrifuged at 1,500 x g for 15 min at 4°C to separate the serum, and liver tissue was harvested for histopathology,
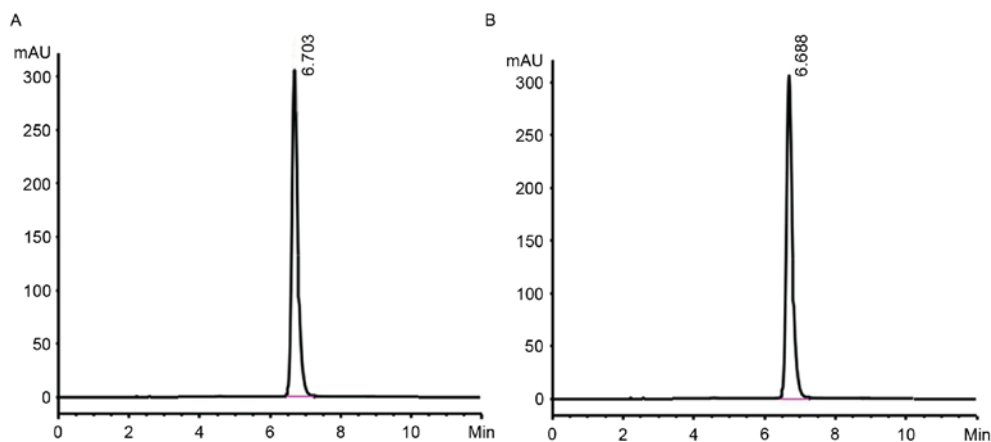
Figure 1. High-performance liquid chromatography of TSGP detected at 320 nm. (A) TSG standard and (B) TSGP that had been separated from Polygoni Multiflori Radix using macroporous resin. TSG, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside; TSGP, TSG-rich fraction.

reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

Biochemical assays and enzyme-linked immune sorbent assay (ELISA). ALT, AST, TC, TG, HDL-C, LDL-C, serum creatinine, blood urea nitrogen, uric acid and glucose levels were measured usinga fully automatic blood biochemistry analyzer (Toshiba TBA-40FR; Toshiba Medical Systems Corporation, Otawara, Japan). Serum apolipoprotein A-I (apoA1), apolipoprotein B (apoB), cholic acid (CA), cholesterol ester, CYP7α1, lecithin-cholesterol acyltransferase, insulin, adiponectin, leptin, nitric oxide (NO, 20150115), heme oxygenase-1,tumor necrosis factor α, interleukin-6, endothelins, thromboxane, 6-keto-prostaglandinF1α (6-Keto-PGF1α), liver free fatty acid (FFA), lower-density lipoprotein receptor (LDL-R), superoxide dismutase (SOD, 20150101), malondialdehyde (MDA, 20150112), glutathione (GSH), catalase (CAT, 20150115, purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and β-hydroxy-β-methylglutaryl-coenzyme A (HMG-CoA), acyl coenzyme A-cholesterol acyltransferase (ACAT), transforming growth factor β1(TGF-β1) and NF-κB (20150101, obtained from Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) levels were analyzed using ELISA kits according to the manufacturer's instructions.

Liver histopathological examination. The left lobe of the liver was fixed in 10% neutral formalin for 48 h at 25-27°C, dehydrated in a 70-100% gradient of ethyl alcohol, dealcoholized in xylene, embedded in paraffin and sectioned (5-μm thickness). Tissue slides were deparaffinized in xylene, rehydrated in a reverse-gradient series of ethyl alcohol and stained with hematoxylin for 3 min and eosin for 1 min (H&E; Merck KGaA, Darmstadt, Germany). Pathological changes were observed under a light microscope with an advanced 3.2 image analysis system (Motic China Group Co., Ltd., Xiamen, China).

RT-qPCR. The total RNA in liver tissue was extracted using TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized by reverse transcription (RT) using random hexamer primers (Verso cDNA kit; Thermo Fisher Scientific, Inc.). The RT system consisted of 1 μl M-mlv, 4 μl

5X RT Buffer, 1 μl Rnase A inhibitor, 1 μl OligdT, 1 μl dNTP, and added Rnase-free water up to 20 μl. The reaction conditions were 42°C for 45 min and 70°C for 10 min. qPCR was performed using mRNA against the housekeeping gene 18s as an internal control. qPCR was performed by the TaqMan method with RQ1 Rnase-Free Dnase (cat. no. M6101, Promega Corp, Madison, WI, USA), and fluorescence biotin quantitation kit (cat. no. PM10003, Hangzhou Biosci Biotech Co., Ltd., Hangzhou, China) coupled with a Step One Plus Real-Time PCR System (Agilent Stratagene Mx3005P; Agilent Technologies, Inc., Santa Clara, CA, USA). The qPCR system consisted of 10 μl 2X qPCR mix, 0.4 μl forward primer, 0.4 μl reverse primer, 0.4 μl cDNA, 8.8 μl nuclease-free water (total volume 20 μl). Thermocycler conditions were as follows: 94°C for 1 min, 95°C for 10 sec, 58°C for 10 sec, 72°C for 10 sec (40 cycles). A melting curve was also constructed to ensure that only a single product was amplified. The sequences of the primers used are provided in Table I. Furthermore, the relative mRNA expression was calculated following normalization of values to that of β-actin, and the relative amounts of the RNAs were calculated using the comparative Cq method (29).

Western blot analysis. 100 mg liver tissues were ground with liquid nitrogen and the total protein was extracted with a total protein extraction kit (KGP250, Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). In brief, the ground liver tissue was added with 0.5 ml lysis buffer containing 10 mM Trise HCl (pH 7.5), 10 μl 0.25 M sucrose and protease inhibitors, followed lysis for 10 min on ice and centrifugation at 20,392 x g for 5 min at 4°C. The total proteins were quantified by the Bradford method with a protein quantitation kit (cat. no. MR04001, Hangzhou Biosci Biotech Co., Ltd.). Protein (60 μg/lane) was separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore; Billerica, MA, USA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween-20 for 2 h at room temperature. Following overnight incubation at 4°C with primary antibodies PPRA-α (cat. no. SC-9000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; dilution ratio, 1:500), CYP2E1 (cat. no. SC-133491, Santa Cruz Biotechnology, Inc.; dilution ratio, 1:500) and β-actin (cat. no. 4970, Cell Signaling
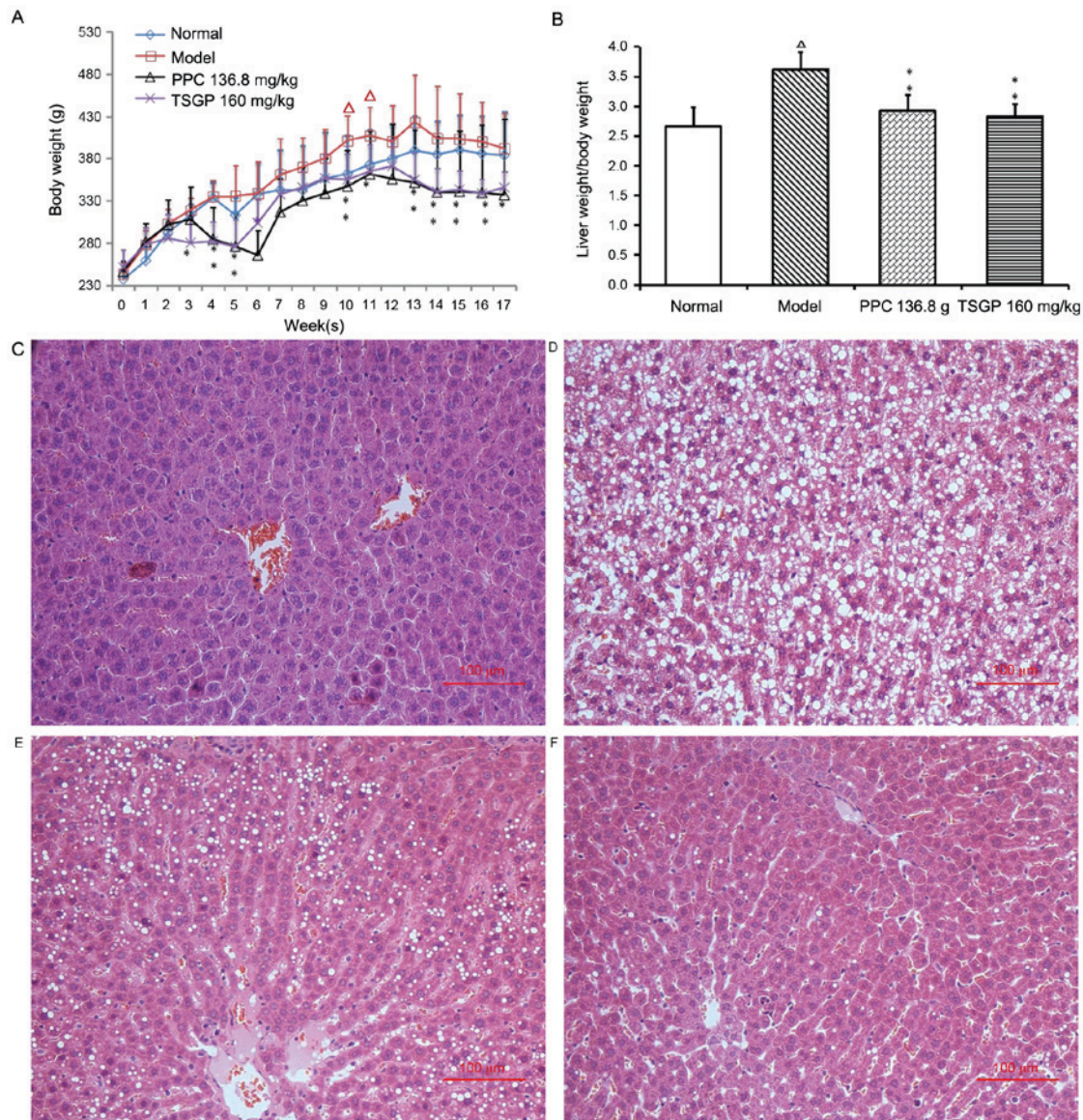
Figure 2. Effects of TSGP on body weight, liver weight/body weight ratio and liver histopathology. (A) Body weight and (B) liver weight/body weight ratio. Data are presented as the mean ± standard error of the mean. (C-F) Representative images of liver histopathology (hematoxylin and eosin staining, magnification, x200) for the (C) normal, (D) model, (E) PPC 136.8 mg/kg and (F) TSGP 160 mg/kg groups. $^{\Delta}$P<0.05 vs. the normal control group; *P<0.05 and **P<0.01 vs. the model group. TSGP, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside rich fraction; PPC, polyene phosphatidylcholine.

Technology, Inc., Danvers, MA, USA; dilution ratio, 1:1,000), the membranes were incubated with HRP-conjugated rabbit anti-mouse IgG (Cell Signaling Technology, Inc.) for 1 h at room temperature. Immunodetection was performed with Amersham enhanced chemiluminescence detection reagent (GE Healthcare, Chalfont St. Giles, UK), with β-actin used as an internal control. The expression levels were quantified by ImageJ 1.46r image analysis software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The obtained data were imported into SIMCA-P 11.5 (Umetrics AB, Umea, Sweden) for principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). PCA was used to visualize whether the groups could be differentiated on the basis of pharmacodynamic indices. PCA was used to differentiate the characteristic indices and was conducted using MATLAB

7.10 (The Math Works, Inc., Natick, MA, USA). Data were auto-scaled prior to performing PCA. Variable importance for projection (VIP) values produced during OPLS-DA were applied to identify potential effective indices, and variables with VIP values >1 were considered to be significant. All values were expressed as the mean ± standard deviation. One-way analysis of selected variance with least-significant difference post hoc analysis multiple comparisons was applied to compare the differences amongst groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of TSGP on body weight (BW), liver weight/BW ratio and liver histology. The effect of TSGP on the BW of the rats fed with a HFD for 18 weeks was investigated, and a significant reduction in the mean BW was observed in the TSGP group
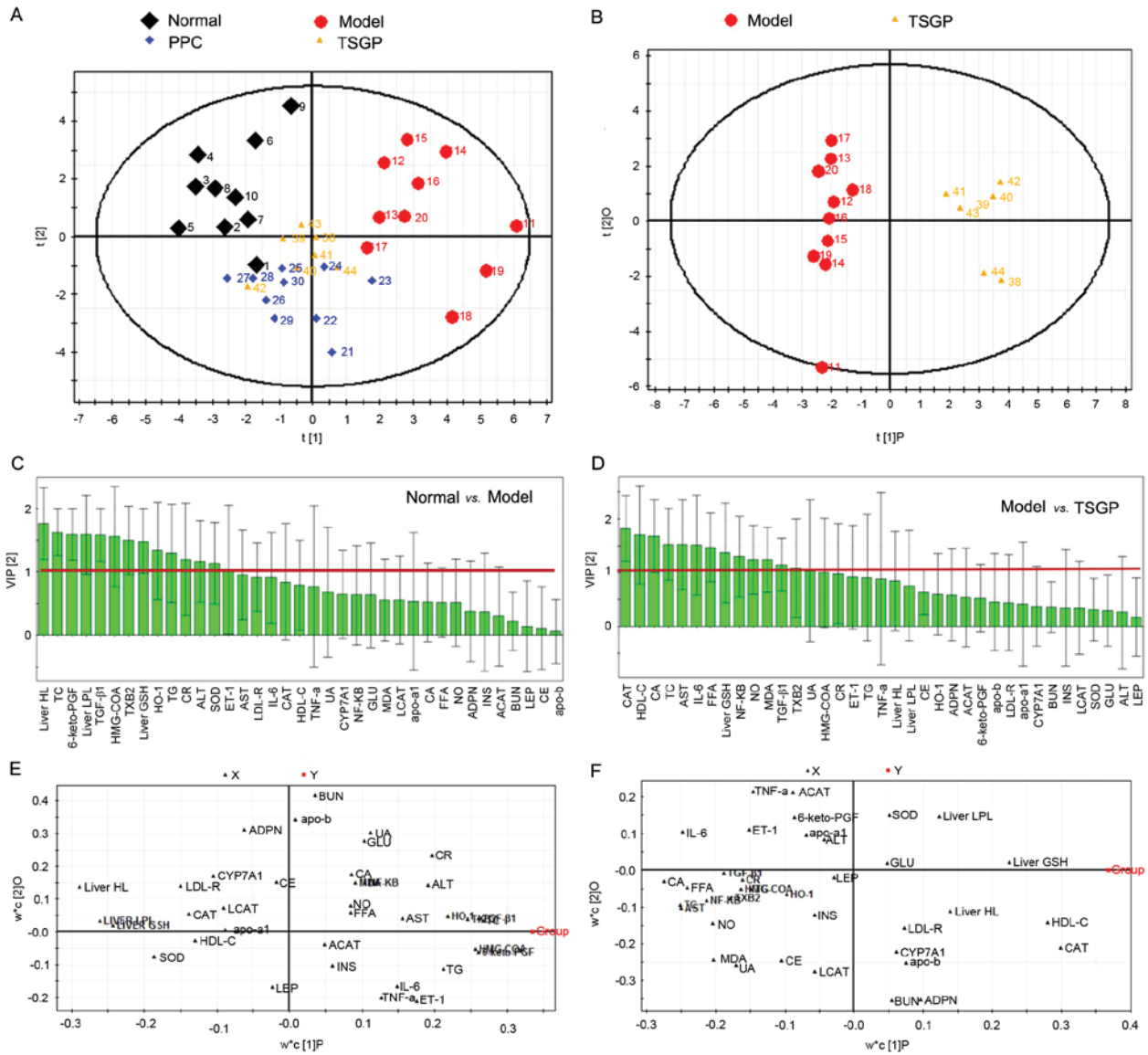
Figure 3. Overall efficacy of TSGP in non-alcoholic fatty liver disease models induced by a high-fat diet evaluated via PCA and OPLS-DA. (A) PCA score plot for the four groups; (B) OPLS-DA score plot for the model vs. TSGP groups; (C and D) variable importance for projection plots of the OPLS-DA results; (E and F) loading plots for OPLS-DA showing (E) normal vs. model and (F) model vs. TSGP.TSGP, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside rich fraction; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; PPC, polyene phosphatidylcholine.

compared with the HFD group after 3 weeks of feeding. The final mean BW of rats in the TSGP group was significantly lower than that of the model group (345.6±18.9 vs. 391.7±42.1 g; P<0.05; Fig. 2A). The liver weight/BW ratio was significantly increased in the model group compared with the normal control group (P<0.01), and the TSGP group presented a significantly lower liver weight/BW ratio compared with the model group (P<0.01; Fig. 2B).

Representative images of liver histology are depicted in Fig. 2C-F. The animals in the normal group presented normal liver histology, hepatocytes were observed with a common radial array encircling the central veins and no hepatocyte lipid degeneration was observed (Fig. 2C). In the model group, the lobular structures of hepatocytes were disrupted, and inflammatory cell infiltration and evident lipid droplets were visible in the hepatic plates (Fig. 2D). These histopathological variations revealed that the NAFLD rat model was established successfully. Compared

with the model group, PPC and TSGP markedly reduced the hepatic steatosis and vacuolar degeneration and effectively alleviated the degree of NAFLD lesions (Fig. 2E and F).

In vivo pharmacodynamic analysis. PCA and OPLS-DA, which are unsupervised and supervised pattern recognition methods for the multivariate statistics of mass data, were performed to explore the differences of indices among the groups. The PCA score plot (Fig. 3A) visibly demonstrated the distribution for the four groups. The clear separation between the normal control and model groups implied that the NAFLD model was established successfully. The OPLS-DA score plot (Fig. 3B) confirmed that there was an evident difference between the model and TSGP groups. In addition, the small overlap of the normal and TSGP groups indicated amelioration of the condition of rats treated with TSGP, indicating that TSGP has an inhibitory effect on NAFLD development. VIPs of the OPLS-DA results are

presented in Fig. 3C and D. CAT, HDL-C, CA, TC, FFA, liver GSH, NF-κB, NO and MDA, which had VIP values for the OPLS-DA of >1, were selected as significant indicators demonstrating a clear difference between the model and TSGP groups. OPLC-DA loading plots (Fig. 3E and F) also revealed that these indices were far from the origin. The differential abundance of indices presented in a heat map (Fig. 4) confirmed that TC, HDL-C, CAT, SOD and MDA are the main pharmacodynamic indicators of the anti-NAFLD effects of TSGP. Therefore, the present study focused on the antioxidation properties of TSGP in experimental NAFLD.

Effects of TSGP on serum TG, TC, HDL-C and ALT levels. Serum TC and TG levels were significantly elevated in the model group compared with the normal group from week 2 to 18 ($P<0.05$) and TSGP significantly lowered the levels of TC on weeks 14 and 18 ($P<0.01$) and of TG ($P<0.05$) from week 10 to 18 (Fig. 5A and B). The serum HDL-C levels in animals fed with a HFD were slightly lower compared with those in normal rats, and no differences were observed between the TSGP and model groups, with the exception of at the end of week 14 and week 18 ($P<0.05$ and $P<0.01$, respectively; Fig. 5C). Furthermore, the ALT levels in model rats revealed a tendency to increase compared with those in normal control rats, with a significant increase in week 14 ($P<0.05$), but no significant difference was observed between the TSGP and model groups (Fig. 5D).

Effects of TSGP on hepatic MDA, FFA, CAT, SOD and GSH levels. MDA and FFA were significantly elevated in model rats compared with the normal group at the end of the experiment ($P<0.05$), and TSGP significantly lowered the levels of MDA and FFA ($P<0.01$; Fig. 6A and B). By contrast, the levels of SOD, CAT and GSH in the liver of model rats were lower than those in normal rats fed with a CD, although the reduction was only significant for CAT ($P<0.05$; Fig. 6C-E). Finally, TSGP revealed a significant elevation of CAT levels compared with the model group ($P<0.01$; Fig. 6D), but no significant differences in the SOD and GSH levels between the TSGP and model groups were observed.

RT-qPCR and western blot analysis. mRNA and protein expression of cytochrome P450 2E1 (CYP2E1) and peroxisome proliferator-activated receptor α (PPARα) were tested. Compared with the control rats fed a CD, the mRNA levels of CYP2E1 in model rats fed with a HFD were upregulated and those of PPARα were downregulated, although neither change was significant, and TSGP reversed the changes in expression of CYP2E1 and PPARα mRNA that were observed in the model rats ($P<0.05$ and $P<0.01$, respectively; Fig. 7A). Additionally, TSGP reduced CYP2E1 protein expression compared with that in the model group, although the reduction was not significant, and no significant difference in PPARα protein expression was observed between the TSGP and model rats (Fig. 7B).

Discussion

The present study investigated the effects of TSGP on an established experimental NAFLD model induced by a high-fat high-cholesterol diet with fructose drinking. A single dataset analysis may be limited and insufficient to provide a holistic
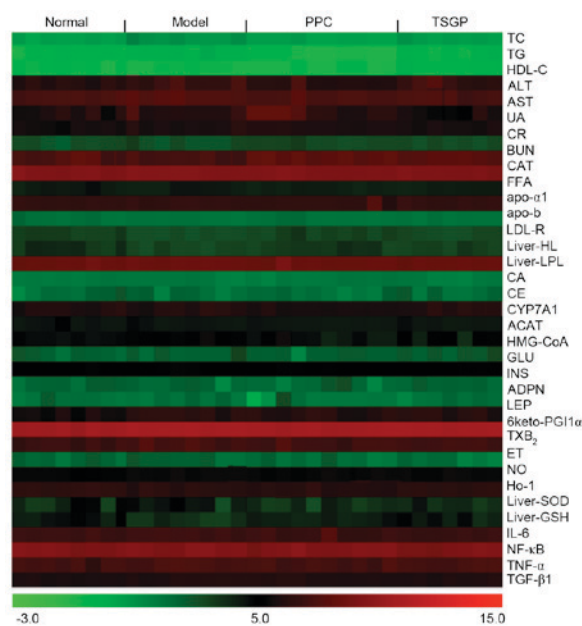

Figure 4. Heat map demonstrating differential abundance of indices in each group. Each column represents a sample, and each row represents an index. The mean signal intensity is coloured black; red indicates above-mean intensity, green denotes below-mean intensity and the degree of colour saturation reflects the magnitude of intensity relative to the mean. PPC, polyene phosphatidylcholine; TSGP, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside rich fraction.

picture of the phenomenon being studied; therefore, the mode recognition methods PCA and OPLS-DA were applied to analyze the mass of data obtained in the present study. PCA is a powerful and versatile method capable of providing an overview of complex multivariate data and is used to reveal an association between variables and relations between sample patterns (30). OPLS-DA is an improved partial least squares method and is a powerful tool for distinguishing the classes of observations and providing a meaningful interpretation of the differences observed (31).

PCA and OPLS-DA have been widely used in chemometrics and omics data analysis, and in the present study, they were used to evaluate the overall efficacy of TSGP in NAFLD models and to identify the main iconic pharmacodynamics indicators. As the results demonstrate, a clear separation among the normal control and model rats, and the model and TSG-treated rats was observed in PCA and OPLS-DA analysis, indicating successful construction of the NAFLD model and an efficient protective effect of TSGP against NAFLD. VIP and loading plots for OPLS-DA enabled the detection of characteristic indices, including serum TC and liver GSH levels amongst rats in the normal, NAFLD and TSGP-treated groups. The results of further analysis revealed that TSGP significantly inhibited the elevation of serum TC and TG in the later stages of NAFLD induction. TSGP treatment also mitigated hepatic enlargement and alleviated liver steatosis. It also exhibited the effects of a HFD on the levels of hepatic MDA, FFA, CAT, and a significant reduction of CYP2E1 mRNA expression and elevation in PPARα mRNA expression were observed. These data indicate that TSGP in the context of the model of the present study has a good effect in NAFLD prevention.
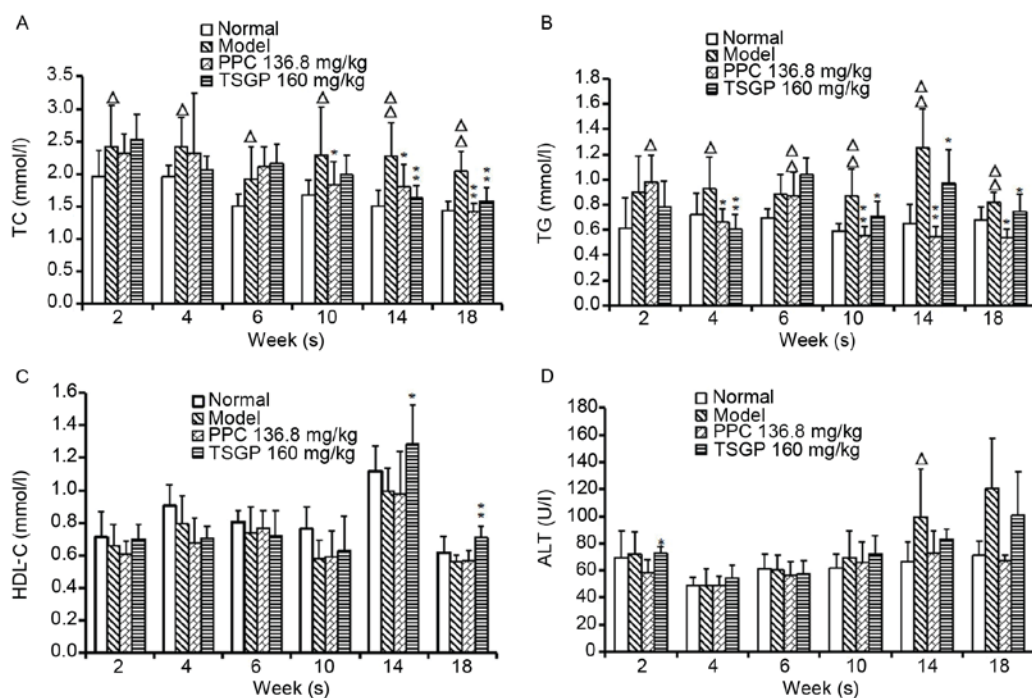
Figure 5. Levels of serum lipids and ALT measured at different time points throughout the study period. Serum (A) TC, (B) TG, (C) HDL-C and (D) ALT levels. $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.01 vs. the normal control group; *P<0.05 and **P<0.01 vs. the model group. ALT, alanine aminotransferase; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; PPC, polyene phosphatidylcholine; TSGP, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside rich fraction.
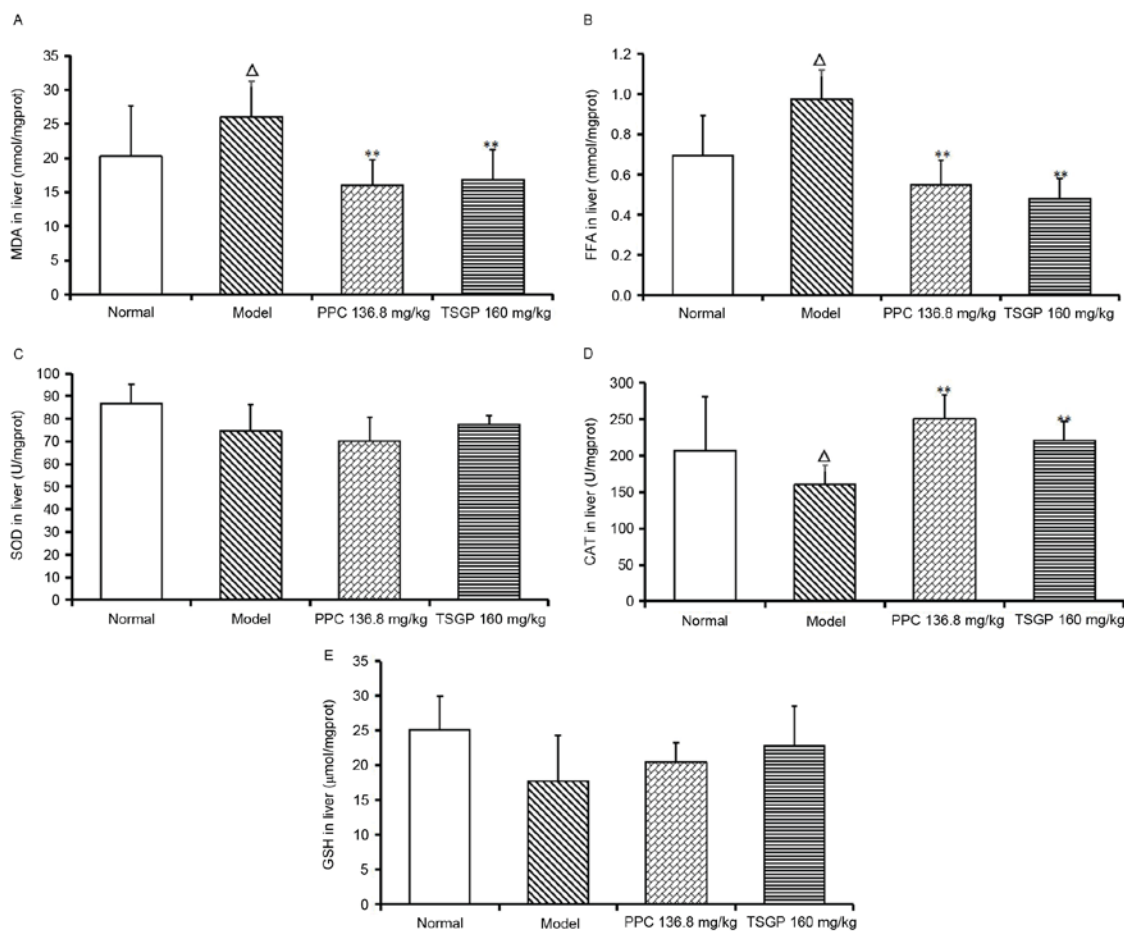

Figure 6. Levels of MDA, FFA, CAT, SOD and GSH in liver tissue at the end of the experiment. (A) MDA, (B) FFA, (C) SOD, (D) CAT and (E) GSH levels in the liver. $^{\Delta}$P<0.05 vs. the normal control group; **P<0.01 vs. the model group. MDA, malondialdehyde; FFA, free fatty acid; CAT, catalase; SOD, superoxide dismutase; GSH, glutathione; PPC, polyene phosphatidylcholine; TSGP, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside rich fraction.
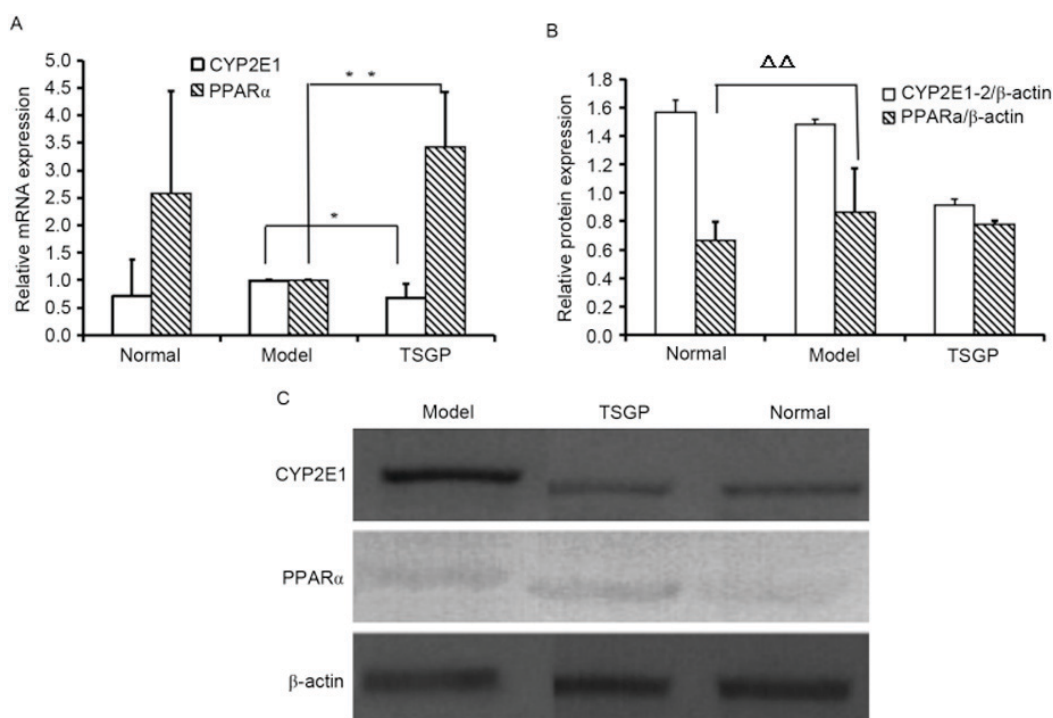
Figure 7. mRNA and protein expression of selected genes in liver tissue. (A) mRNA expression of CYP2E1 and PPARα (shown as 2-DDCq). (B) Quantified protein expression of CYP2E1 and PPARα and (C) representative western blots. $^{ΔΔ}P<0.01$ vs. the normal group; $^{*}P<0.05$ and $^{**}P<0.01$ vs. the model group. CYP2E1, cytochrome P450 2E1; PPARα, peroxisome proliferator-activated receptor α; TSGP, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside rich fraction.

A previous study revealed that lipid metabolism and oxidative stress are critical in the development of HFD-triggered NAFLD (32). During metabolic processing, FFAs released into the liver stimulate the expression of the key factor controlling cholesterol synthesis (3-hydroxy-3-methylglutaryl-coenzyme A reductase) as well as the critical fatty acid synthesis factors, such as sterol regulatory element binding protein 1c, which promote the synthesis of liver cholesterol and TG (33,34). The inhibition of PPARα reduces the metabolism of fatty acids and leads to the development of NAFLD (35), while an excess of hepatic FFA upregulates CYP2E1 expression and increases the production of reactive oxygen species, subsequently inducing oxidative stress (36), followed by intracellular superoxide species production and hepatic injury. Elevated MDA and reduced SOD, CAT and GSH-peroxidase (GSH-Px) are the common biomarkers of oxidative stress *in vivo* (37-39). High serum MDA levels are considered a key feature in liver injury (40). SOD, CAT and GSH-Px are three enzymes that act against oxidative stress by catalyzing the dismutation, decomposition and reduction of superoxide anions, H_2O_2 and hydroperoxides into non-toxic products (41,42) to eliminate intracellular superoxide species and thus prevent hepatic injury.

The current results revealed that hepatic FFA and MDA levels, and the expression of CYP2E1 mRNA were elevated significantly, while CAT, SOD and GSH-Px activities and the expression of PPARα mRNA were decreased (although only the reduction in CAT activity was statistically significant) in response to HFD intake, indicating that oxidative damage existed in the liver. A previous report has revealed that TSG exhibited antioxidant activity on ROS (17), and the experiments in the present study revealed that administration of TSGP significantly decreased liver FFA and MDA levels, inhibited CYP2E1 mRNA

expression, promoted the activity of CAT and the expression of PPARα mRNA in the liver of rats with NAFLD. These changes reduced oxidative stress in NAFLD model rats and improved the symptoms. Therefore, it is suggested that the administration of TSGP effectively protects against HFD-induced hepatic lipid peroxidation via hepatic antioxidant enzyme regulation.

Overall, the present study demonstrated that PCA and OPLS-DA were useful in the systematic assessment of the protective effect of TSGP against experimental NAFLD using a multi-index analysis. Blood lipid regulation and hepatic lipid blocking may be the main mechanisms underlying the effects of TSGP in NAFLD prevention. Therefore, the present study provides a basis for the application of TSGP in the treatment of NAFLD and for novel anti-NAFLD drug development.

References

1. Tiniakos DG, Vos MB and Brunt EM: Nonalcoholic fatty liver disease: Pathology and pathogenesis. Annu Rev Pathol 5: 145-171, 2010.
2. Preiss D and Sattar N: Non-alcoholic fatty liver disease: An overview of prevalence, diagnosis, pathogenesis and treatment considerations. Clin Sci (Lond) 115: 141-150, 2008.

3. Kopec KL and Burns D: Nonalcoholic fatty liver disease: A review of the spectrum of disease, diagnosis, and therapy. Nutr Clin Pract 26: 565-576, 2011.
4. Chalasani N, Deeg MA and Crabb DW: Systemic levels of lipid peroxidation and its metabolic and dietary correlates in patients with nonalcoholic steatohepatitis. Am J Gastroenterol 99: 1497-1502, 2004.
5. Day CP and James OF: Steatohepatitis: A tale of two 'hits'? Gastroenterology 114: 842-845, 1998.
6. Phung N, Pera N, Farrell G, Leclercq I, Hou JY and George J: Pro-oxidant-mediated hepatic fibrosis and effects of antioxidant intervention in murine dietary steatohepatitis. Int J Mol Med 24: 171-180, 2009.
7. Seki S, Kitada T, Yamada T, Sakaguchi H, Nakatani K and Wakasa K: In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. J Hepatol 37: 56-62, 2002.
8. Zivkovic AM, German JB and Sanyal AJ: Comparative review of diets for the metabolic syndrome: Implications for nonalcoholic fatty liver disease. Am J Clin Nutr 86: 285-300, 2007.
9. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M and Sanyal AJ; American Gastroenterological Association; American Association for the Study of Liver Diseases; American College of Gastroenterology: The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology 142: 1592-1609, 2012.
10. Nakajima K: Multidisciplinary pharmacotherapeutic options for nonalcoholic Fatty liver disease. Int J Hepatol 2012: 950693, 2012.
11. Xu JY, Zhang L, Li ZP and Ji G: Natural products on nonalcoholic fatty liver disease. Curr Drug Targets 16: 1347-1355, 2015.
12. Pharmacopoeia CoC: Pharmacopoeia of the People's Republic of China. China Medical Science Press Beijin, 2015.
13. Chen CQ and Zhang TJ (eds): Regulation of Blood Lipid Research and Application of Modern Traditional Chinese Medicine. People's Medical Publishing House, Beijing, 2007 (In Chinese).
14. Jiang LD, He YS, Chen X, Tao O, Li GY and Zhang YL: Study on anti-hyperlipidemia mechanism of high frequency herb pairs by molecular docking method. Zhongguo Zhong Yao Za Zhi 40: 2413-2419, 2015 (In Chinese).
15. Li N, Chen Z, Mao X, Yu J and Zhao R: Effects of lipid regulation using raw and processed radix polygoni multiflori in rats fed a high-fat diet. Evid Based Complement Alternat Med 2012: 329171, 2012.
16. Chang YX, Ge AH, Jiang Y, Teye Azietaku J, Li J and Gao XM: A Bioactivity-based method for screening, identification of lipase inhibitors, and clarifying the effects of processing time on lipase inhibitory activity of Polygonum multiflorum. Evid Based Complement Alternat Med 2016: 5965067, 2016.
17. Chen HF, Chen YH, Liu CH, Wang L, Chen X, Yu BY and Qi J: Integrated chemometric fingerprints of antioxidant activities and HPLC-DAD-CL for assessing the quality of the processed roots of Polygonum multiflorum Thunb. (Heshouwu). Chin Med 11: 18, 2016.
18. Wang M, Zhao R, Wang W, Mao X and Yu J: Lipid regulation effects of Polygoni Multiflori Radix, its processed products and its major substances on steatosis human liver cell line L02. J Ethnopharmacol 139: 287-293, 2012.
19. Liu QL, Xiao JH, Ma R, Ban Y and Wang JL: Effect of 2,3,5,4'-tetrahydroxystilbene-2-O-beta-D-glucoside on lipoprotein oxidation and proliferation of coronary arterial smooth cells. J Asian Nat Prod Res 9: 689-697, 2007.
20. Wang W, He Y, Lin P, Li Y, Sun R, Gu W, Yu J and Zhao R: In vitro effects of active components of Polygonum Multiflorum Radix on enzymes involved in the lipid metabolism. J Ethnopharmacol 153: 763-770, 2014.
21. Lin P, Lu J, Wang Y, Gu W, Yu J and Zhao R: Naturally occurring stilbenoid TSG reverses non-alcoholic fatty liver diseases via gut-liver axis. PLoS One 10: e0140346, 2015.
22. Lv G, Gu H, Chen S, Lou Z and Shan L: Pharmacokinetic profile of 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside in mice after oral administration of Polygonum multiflorum extract. Drug Dev Ind Pharm 38: 248-255, 2012.
23. Lou ZH, Lu GY, Chen SH and Gu H: Determination of 2,3,5,4'-tetrahydroxy-stilbene-2-O-β-D-glucoside in Shouwugan preparation and its tissue distribution in mouse. Zhongguo Xian Dai Ying Yong Yao Xue 28: 89-91, 2011 (In Chinese).
24. Lv G, Lou Z, Chen S, Gu H and Shan L: Pharmacokinetics and tissue distribution of 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside from traditional Chinese medicine Polygonum multiflorum following oral administration to rats. J Ethnopharmacol 137: 449-456, 2011.
25. Dong LH, Guo PP, Yan WY, Yang H and Wang CY: Comparative study on hypolipidemic effect of cis-2,3,5,4'-tetrahydroxy stilbene-2-O-β-D-glucoside and trans-2,3,5,4'-tetrahydroxy stilbene-2-O-β-D-glucoside. Shenyang Yao Ke Da Xue Xue Bao 31: 989-993, 2014 (In Chinese).
26. Wang CY, Zhang LT, Yuan ZF, Jin YB and Zhang Z: Blood lipid regulation of ethyl acetate extracting fration and stilbene glycoside from tuber of Polygonum multiflorum. Chin Traditional Herbal Drugs 39: 78-83, 2008 (In Chinese).
27. Zhang W, Wang CH, Shen Y, Li F and Wang YQ: Treatment of 2,3,4',5-tetrahydroxystilbene-2-O-β-d-glucoside on atherosclerosis in rats. Zhongguo Yao Ke Da Xue Xue Bao 38: 261-264, 2007 (In Chinese).
28. Lv L, Gu X, Tang J and Ho CT: Antioxidant activity of stilbene glycoside from Polygonum multiflorum Thunb in vivo. Food Chemistry 104: 1678-1681, 2007.
29. Schmittgen TD and Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101-1108, 2008.
30. Bro R and Smilde AK: Principal component analysis. Anal Methods 6: 2812-2831, 2014.
31. Boccard J and Rutledge DN: A consensus orthogonal partial least squares discriminant analysis (OPLS-DA) strategy for multiblock Omics data fusion. Anal Chim Acta 769: 30-39, 2013.
32. Nobili V, Donati B, Panera N, Vongsakulyanon A, Alisi A, Dallapiccola B and Valenti L: A 4-polymorphism risk score predicts steatohepatitis in children with nonalcoholic fatty liver disease. J Pediatr Gastroenterol Nutr 58: 632-636, 2014.
33. Liu J, Han L, Zhu L and Yu Y: Free fatty acids, not triglycerides, are associated with non-alcoholic liver injury progression in high fat diet induced obese rats. Lipids Health Dis 15: 27, 2016.
34. Papackova Z and Cahova M: Fatty acid signaling: The new function of intracellular lipases. Int J Mol Sci 16: 3831-3855, 2015.
35. Leamy AK, Egnatchik RA and Young JD: Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease. Prog Lipid Res 52: 165-174, 2013.
36. Serviddio G, Bellanti F and Vendemiale G: Free radical biology for medicine: Learning from nonalcoholic fatty liver disease. Free Radic Biol Med 65: 952-968, 2013.
37. Qiang M, Xu Y, Lu Y, He Y, Han C, Liu Y and He R: Autofluorescence of MDA-modified proteins as an in vitro and in vivo probe in oxidative stress analysis. Protein Cell 5: 484-487, 2014.
38. Sayed AA: Ferulsinaic acid modulates SOD, GSH, and antioxidant enzymes in diabetic kidney. Evid Based Complement Alternat Med 2012: 580104, 2012.
39. Suzuki M, Takeuchi H, Kakita T, Unno M, Katayose Y and Matsuno S: The involvement of the intracellular superoxide production system in hepatic ischemia-reperfusion injury. In vivo and in vitro experiments using transgenic mice manifesting excessive CuZn-SOD activity. Free Radic Biol Med 29: 756-763, 2000.
40. Mateos R, Lecumberri E, Ramos S, Goya L and Bravo L: Determination of malondialdehyde (MDA) by high-performance liquid chromatography in serum and liver as a biomarker for oxidative stress. Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruits. J Chromatogr B Analyt Technol Biomed Life Sci 827: 76-82, 2005.
41. Cheng N, Ren N, Gao H, Lei X, Zheng J and Cao W: Antioxidant and hepatoprotective effects of Schisandra chinensis pollen extract on CCl4-induced acute liver damage in mice. Food Chem Toxicol 55: 234-240, 2013.
42. Heck DE, Shakarjian M, Kim HD, Laskin JD and Vetrano AM: Mechanisms of oxidant generation by catalase. Ann N Y Acad Sci 1203: 120-125, 2010.