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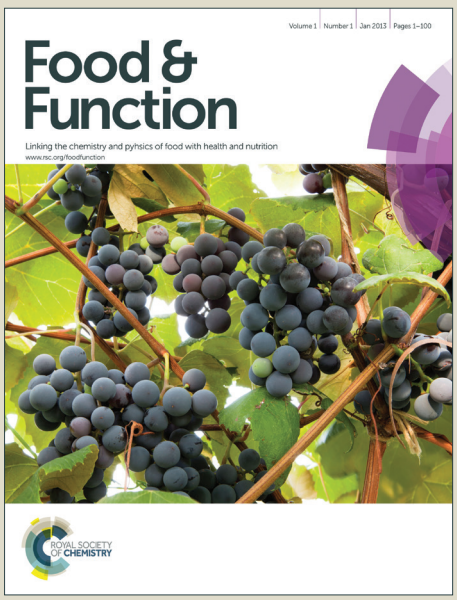

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A diet formula of *Puerariae radix*, *Lycium barbarum*, *Crataegus pinnatifida*, and *Polygonati rhizoma* alleviates insulin resistance and hepatic steatosis in CD-1 mice and HepG2 cells

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Abstract

According to the principles of traditional Chinese medicine, medicinal and edible herbs exhibit holistic effects through their actions on multiple target organs. Four herbs, namely, *Puerariae radix*, *Lycium barbarum*, *Crataegus pinnatifida*, and *Polygonati rhizoma*, were selected and combined to create a new herbal formula (PLCP). The protective effects of both aqueous extract (AE) and ethanol extract (EE) of PLCP against insulin resistance (IR) and non-alcoholic fatty liver disease (NAFLD) were evaluated in high fat and high fructose diet-fed mice. Active fractions and constituents were screened on HepG2 cells with IR or over-accumulation of triglycerides, and further identified by high-performance liquid chromatography/electrospray ionization/mass spectrometry. The results indicate that AE did not improve ($p > 0.05$) glucose tolerance after three weeks, whereas EE showed a promising effect throughout the experiment. Medium and high doses of EE were found to reduce fasting blood glucose at week 9 by 21.1% and 24.4%, respectively. In addition, their efficacies on alleviating IR were comparable with that of metformin. Compared with AE, EE effectively improved hyperlipidemia, antioxidant status, and NAFLD. By contrast, metformin did not alleviate hyperlipidemia ($p > 0.05$) or NAFLD in the mice model. Results from the cell-based study indicate that the protective effects of EE were possibly due to the actions from puerarin, 3'-methoxypuerarin, daidzin, daidzein, and ononin.

Keywords: Formula; Medicinal and edible herbs; Insulin resistance; Non-alcoholic fatty liver disease

Introduction

Insulin resistance (IR) is the most characteristic abnormality in metabolic syndrome that results from interactions between genetic and environmental factors, which include unhealthy dietary habits and sedentary lifestyle[1]. This chronic metabolic disorder causes various diseases, including obesity, dyslipidemia, hypertension, type 2 diabetes, and coronary artery disease[2]. IR is characterized by inadequate glucose transport in the skeletal muscle and fat tissue, and inadequate suppression of hepatic glucose production when stimulated by insulin, leading to an impairment in both glucose tolerance and fasting glucose[3]. Besides the alteration in glucose suppression, IR is also a major contributor to steatosis in the pathogenesis of non-alcoholic fatty liver disease (NAFLD)[4]. NAFLD comprises a disease spectrum that starts from excessive deposition of triglyceride (TG) and leads to non-alcoholic steatohepatitis, and then to fat with fibrosis or cirrhosis[5]. Several drugs are available for the treatment of IR and NAFLD, however, these drugs are also demonstrated adverse effects or drug resistance, and some drugs often work on one single target. For instance, acarbose, which act as α-glucosidase inhibitor, may cause gastrointestinal disturbances[6]. Given the safety and multiple beneficial effects of medicinal and edible herbs, more people are seeking those products as an alternative to prevent different disorders[7].

Traditional Chinese medicine (TCM) and herbal formulae have developed their own unique system in the past 3000 years. They are well documented in modern literature for treatments of different disorders[8]. A wide variety of herbal remedies are traditionally used to cure NAFLD and metabolic syndromes. The key ingredient of

Kudzu root tea is *Puerariae radix*, which is the dried root of *Pueraria lobata* (Wild) Ohwi and has been proven to be pharmacologically effective in preventing hyperglycemia and hyperlipidemia[9]. In China, wolfberry fruit (*Lycium barbarum*) is commonly consumed and is believed to possess antioxidant and hypoglycemic properties[10]. In over-the-counter medications, hawthorn (*Crataegus pinnatifida*) fruit is commonly used to treat indigestion, and also reduces blood lipid[11] and lipid deposition in liver[12]. Moreover, according to various ancient Chinese traditional prescriptions, *Polygonati rhizoma* has been widely used to treat *Xiaokezheng* (diabetes). The flavonoids of *Polygonatum odoratum* reportedly decrease serum glucose and promote insulin secretion in diabetic rats[13].

However, in practice, dietary herbs are generally combined and made into teas, soups, and porridges. A diet composed of a combination of medicinal and edible herbs might enhance their functions and affect different sites in the body in light of the principles of TCM[14]. However, herbal formulae have been rarely studied, and their effectiveness has always been questioned because of their unidentified effective compounds and obscured mechanisms. Thus, to provide theoretical support in practical applications, identifying effective components is critical. A previous finding suggested that a herbal formula containing *P. radix* and *P. rhizoma* significantly decreases the blood glucose of diabetic rats[15], but the specific functional herbs or active constituents remain unknown. Based on the different effects of herbs, this study focused on composing a new herbal formula (PLCP), which includes *P. radix*, *L. barbarum*, *C. pinnatifida*, and *P. rhizoma*, for the development of natural alternative herbal treatments. Although the

hypoglycemic and anti-NAFLD effects of these four herbs have been reported individually, the positive effects of the formula on animals have rarely been studied. Nevertheless, the bioactive components and underlying mechanisms need to be examined.

Therefore, this study aimed to investigate and compare the bioactivities of aqueous extract (AE) and ethanol extract (EE) of the PLCP formula against pre-diabetic status and NAFLD. Our previous study showed that CD-1 mice fed with high levels of fructose and fat developed hyperlipidemia, hyperglycemia, NAFLD, and IR in both liver and peripheral tissues[16]. Thus, the same animal model was adopted in this study. Blood glucose, blood lipids, IR index, oxidative stress, and histological changes were measured to examine the effects of the PLCP formula.

To validate the beneficial effects, the active constituents were purified and further screened *in vitro* and ultimately identified by high-performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS).

Materials and Methods

Chemicals

Silica gel and ADS-8 resin were purchased from Qingdao Marine Chemical Factory (Shandong, China). Cholesterol, pig bile salt and Tween 80 were supplied by Dingguo Biotech Co. (Beijing, China). Fructose was purchased from Archer Daniels Midland Company (Shanghai, China). HPLC grade acetonitrile and methanol were purchased from Mallinckrodt Baker (Phillipsburg, USA). Gallic acid (> 97.5%), oleic acid (OA),

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and bovine insulin were purchased from Sigma Chemicals Co. (St. Louis, MO., USA). Fetal bovine serum (FBS), penicillin, streptomycin and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY). All the enzymatic or quantification kits used in the study were purchased from Beijing Zhongsheng Hightech Bioengineering Company (Beijing, China).

Plant materials and preparation of extracts

Dry Gegen (*P. radix*), Wolfberry (*L. barbarum*), Hawthorn (*C. pinnatifida*), and Huangjing (*P. rhizoma*) were purchased from Beijing TongRenTang Pharmacy Store (China), cleaned, and formulated with a weight ratio of 4:3:3:4 to obtain the PLCP formula. The ratio was based on a prescription, which was determined by an Oriental medical doctor, ShunCheng Li, of the Peking University Third Hospital (Beijing, China). The combined materials were ground using a kitchen blender and passed through a 60 mesh sieve. The collected fine powder was extracted using 70% ethanol (w/v, 1:8) with 30 min of sonication at 50 °C, and then filtered. The pellet was subjected to the same procedure twice. To obtain the extract, the filtrates were pooled and concentrated using a rotary vacuum evaporator until no ethanol remained. The same procedure was performed during the preparation of AE in the filtrated mac with water (w/v, 1:8). The final volume of both extracts was made by adding water to the stock concentration of 2 g of crude PLCP/mL, and the extracts were stored at –20 °C. Furthermore, the stocks of AE and EE were diluted with water before being supplied

to animals. The total polysaccharide content in the lyophilized AE was measured by the phenol-sulfuric acid method[17].

Purification

After ethanol extraction, the solvent was removed using a rotary evaporator to yield 70% ethanol residue. The residue was suspended in water and extracted with *n*-hexane three times. The ensuing aqueous layer was then partitioned sequentially using chloroform (CH), ethyl acetate (EA), and *n*-butanol (BT). Each extraction was performed three times. The hexane, CH, EA, and BT fractions were concentrated using a rotary evaporator and dried using a freeze dryer (Four-Ring Science Instrument Plant, Beijing Co., Ltd., Beijing). All fractions were stored in –20 °C until use. The EA fraction was subsequently separated by column chromatography over silica gel (2.5 × 30 cm) with elution by chloroform-methanol (15:1, 9:1, 8:2, 7:3, and 6:4) to obtain five subfractions (F1 to F5). Furthermore, the BT fraction was separated by an ADS-8 resin column (2.5 × 20 cm). The loaded column was washed with distilled water, and eluted with 30%, 40%, and 60% ethanol. The eluent was concentrated and lyophilized to obtain F6, F7, and F8, respectively. The fractions that showed potential hypoglycemic effects on HepG2 cells were further analyzed using the HPLC program described below. Different fractions were subjected to semi-preparative HPLC to yield compounds A to G, which were all checked for impurities by thin layer chromatography. All the dried samples were dissolved in dimethyl sulfoxide (DMSO) and diluted with treatment medium to obtain the desired

concentrations for the *in vitro* assays. The final DMSO concentration in the treatment medium was less than 0.1%.

Qualitative phytochemical screening and estimation of total phenol content (TPC)

The CH fraction, EA fraction, and F6 to F8 were initially screened for the presence of phytochemicals, including phenolics, tannins, saponins, steroids, flavonoids, and anthraquinones[18]. Phenolics and tannins were identified by ferric chloride reaction, vanillin–hydrochloric acid method, and ferric chloride–potassium ferricyanide reaction. Saponins were identified by frothing test; steroids with Liebermann–Burchard test; flavonoids with aluminum chloride reaction; and anthraquinones with alkaline reaction and magnesium acetate reaction.

TPC was quantified using a gallic acid standard with Folin–Ciocalteu reagent method[19]. Values were expressed as gallic acid equivalents (GAE) per gram of each fraction based on the calibration curve.

Animals and treatment protocols

Sixty male CD-1 mice weighing 20-22 g were purchased from the Beijing Vital River Laboratory Animal Center [Certificated No. SCXK (Beijing) 2007-0001] at six weeks of age. Animals were housed in a humidity-, temperature-, and light/dark (12:12 hours) -controlled room. They were allowed to acclimate for one week on regular chow and water. From seven weeks of age, all animals were randomly divided into six groups

(n = 10). The mice model was induced by high levels of fructose and fat for 10 weeks. Briefly, six groups were classified as follows: standard diet (STD), which received a gavage of vehicle (7% Tween 80); model control (MC), which received a gavage of fat emulsion (20 mL/kg); positive control (PC), MC + 500 mg/kg metformin (MET); AE medium (AEM), MC + medium dose of AE; EE medium (EEM), MC + medium dose of EE; and EE high (EEH), MC + high dose of EE, where medium and high indicated relative extracts of 20 g of crude PLCP/kg and 30 g of crude PLCP/kg, respectively. MET, AEM, EEM and EEH were applied to mice in distilled water and fed by gavage administration, once a day for 10 weeks. The fat emulsion in water (100 mL) contained 50 g of lard, 1.5 g of cholesterol, 0.3 g of pig bile salt, and 7 mL of Tween 80. Animals were given free access to standard laboratory chow (Experiment Animal Center of Beijing, China). All groups, except STD, received fructose (15%) in their drinking water. Body weight and drinking volume were recorded, and the water intake did not differ between groups (data not shown). After the experiment, overnight-fasted animals were killed by cervical dislocation. The blood samples were centrifuged at 1500 *g* in 4 ℃ for 10 min. Total cholesterol (TC), TG, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, superoxide dismutase (SOD), malondialdehyde (MDA), and total antioxidant capacity (TAC) in serum were enzymatically determined using commercial kits. Glucokinase activity was determined using a continuous spectrophotometric assay[20]. Plasma free fatty acid (FFA) levels and hepatic glycogen content were determined using corresponding quantification kits. The liver and

abdominal adipose tissues were excised and weighed and expressed as tissue index (calculated as relative tissue weight divided by body weight). The degree of liver steatosis was determined from frozen liver sections stained with oil red O. All animal procedures were conducted in accordance with National Institutes of Health guidelines for animal care[21] and approved by the Ethics Committee of Beijing Key Laboratory of Functional Food from Plant Resources.

Oral glucose tolerance test (OGTT)

OGTT was performed during the third, sixth, and ninth weeks of treatment in overnight-fasted animals by orally administering 2 g of glucose/kg body weight. Blood samples were collected from the tail vein at 0 (just before injection) , 30, 60, 90, and 120 min after oral glucose loading. The blood glucose levels were measured using a calibrated One Touch Ultra® glucometer. The total area under the curve (AUC) was calculated as millimoles per liter per minute by trapezoidal rule. At week 10, blood samples were collected. Blood glucose and insulin levels were measured using a glucose oxidase kit and radioimmunoassay method, respectively. The *R*-value of the homeostasis model (HOMA-IR)[22] refers to the index of IR, which can be calculated using the following formula: fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5.

HPLC analysis and semi-preparative HPLC purification

The HPLC system consisted of a Shimadzu HPLC (Model LC–10ATvp two Pumps

and DGU–12A Degasser) equipped with a diode array detector (Model SPD–M10Avp) (Shimadzu, Kyoto, Japan). The analysis of F1 to 7 was performed on an Agilent ZORBAX SB–C18 column (4.6 × 250 mm, particle size 5 μm) (Agilent, Palo Alto, CA) with monitoring at 280 nm, and the column temperature was set at 30 ℃. For HPLC analysis, a 10 μL sample was injected into the column with a constant flow rate of 1.0 mL/min. The mobile phase was 0.1% formic acid in H_2O (A) and 100% acetonitrile (B). The elution conditions were as follows: 0–10 min, 5–8% (B); 10–11 min，8–15% (B); 11–31 min, 15% (B); 31–36 min, 15–22% (B); 36–46 min, 22–30% (B); 46–51 min, 30–70% (B); 51–65 min, 70% (B); 65–70 min, 70–5% (B).

Major compounds (A to G) present in the fractions were isolated by semi-preparative HPLC using a Shimadzu HPLC (Model LC–10ATvp two Pumps and DGU–12A Degasser) equipped with a diode array detector (Model SPD–M10Avp) (Shimadzu, Kyoto, Japan). HPLC separation was performed on a Kromasil C18 column (10 × 250 mm，particle size 5 μm) (Eka, Bohus, Sweden) at 30 ℃ and detected at 280 nm. The flow rate was 3 mL/min and an injection of 500 μL was employed. Solvents were 0.1% formic acid in H_2O (A) and 100% acetonitrile (B) with the following gradient: 0–5 min, 5–8% (B); 5–10 min, 8–15% (B); 10–40 min, 15% (B); 40–55 min, 15–25% (B); 55–65 min, 25–45% (B); 65–70 min, 45–70% (B); 70–75 min, 70–5% (B). Solvents were removed under vacuum and the compounds were freeze-dried.

HepG2 cell culture and cytotoxicity assay

The human hepatocellular carcinoma cell line (HepG2) was purchased from the Cell

Culture Center of Peking Union Medical Science (Beijing, China) and maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2. Cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Prior to experimental procedures, the HepG2 cells were seeded into 96-well plates at a concentration of 3.5×10^4 cells/mL and allowed to attach for 24 h. Fractions, subfractions, or purified compounds were dissolved in DMSO, diluted with DMEM to different concentrations, filtered, and incubated with HepG2 cells for 24 h. The untreated cells served as the control. The cytotoxicity effects of samples were tested using MTT assay[19]. In brief, cells were washed with phosphate buffered saline (PBS) and incubated with 200 μL of serum-free DMEM containing 5 mg/mL MTT. After 4 h, the supernatant was removed and 150 μL of DMSO was added to solubilize the formazan. The optical density was read at 570 nm using a microplate spectrophotometer system (SpectraMax M2^e, Molecular Devices, USA). The results were expressed as the percentage of viable cells with respect to the untreated control cells. We considered the absorbance of untreated control group as the 100% viability.

Glucose uptake in IR HepG2 cells

To evaluate the glucose uptake stimulated by different samples on IR cells, HepG2 cells were grown and induced by elevated insulin levels. The attached HepG2 cells were washed with PBS twice and induced with DMEM containing 1% FBS and 0.5 μM bovine insulin for 24 h. The medium was changed to DMEM (control), DMEM containing 1 μM insulin (MC), 1 μM insulin and the respective samples (10

and 50 μg/ml), or MET (10 and 50 μg/mL) for 36 h. Subsequently, all cells were washed with PBS, and the medium was changed to DMEM containing 10^{-3} μM insulin for an additional 24 h. The glucose concentrations in supernatants were measured by a glucose oxidase kit and normalized to total cellular protein. Uptake of extracellular glucose content (μmol/mg protein) was calculated using the following formula: [extracellular glucose content (μmol) $_{0\ h}$ − extracellular glucose content (μmol) $_{24\ h}$] /mg cell protein[23].

TG levels, glucose uptake, and glycogen content in OA-induced HepG2 cells

Cells were cultured in a 24-well plate and incubated for 24 h. The medium was then changed from 0.75 mM OA-bound to 0.75% bovine serum albumin (BSA) in DMEM containing various samples, and 0.75% BSA in DMEM was selected as the control. After 24 h, cells were lysed to detect TG and glycogen, or incubated with DMEM containing 10^{-3} μM insulin for glucose uptake test. The intracellular TG levels and glycogen contents were measured using enzymatic kits and normalized to total cellular protein. The glucose uptake by HepG2 cells were determined as described above.

ESI/MS Analysis

ESI/MS analysis was performed on an Agilent 1100 series LC/MSD SL Trap system. Samples (10 μL) were injected into the LC/MSD system through an Agilent 1100 series autosampler. Separations were carried out on a 5 μm Agilent ZORBAX SB–

C18 column (4.6 mm × 250 mm) using previous HPLC method. The tandem mass spectrometer Model micrOTOF-Q (Bruker Daltonics Inc., Germany) consisted of an electrospray ion source (ESI). The ESI voltage, capillary temperature, flow rate of dry gas, and ion sweep range were 3.9 kV, 350 °C, 10 L/min, and *m/z* 85–1500, respectively.

Statistical analysis

Data were analyzed by using SPSS 13.0 (SPSS Inc., Chicago, Ill., USA). The statistical significance comparing data between groups was assessed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. P value less than 0.05 was considered to be statistically significant, while less than 0.01 was very significant. Results are expressed as mean ± standard deviation (SD).

Results

Phytochemical screening and phenolic content of five fractions

AE contained 50.35% of the total polysaccharide content, which was determined by the phenol–sulfuric acid method. The fractions and subfractions in EE revealed the presence of phenolic compounds, tannins, saponins, steroids, and flavonoids (**Table 1**). Anthraquinones were not detectable. Among the fractions, saponins and steroids only existed in F8, which was from BT fraction.

The TPC of different fractions from EE are shown in **Table 1**. F6 from BT fraction had the highest value of 275.2 ± 2.3 GAE mg/g fresh weight, followed by EA, F8, and

CH fraction, whereas F7 had the lowest value.

Effect of AE and EE on body weight, tissue index, hepatic glycogen, and blood FFA

Pre-diabetic mice were treated for up to 10 weeks by daily gavage with AE or EE, whereas the anti-diabetic drug MET was given as PC. **Table 2** outlines the various groups and effects of treatments on body mass, liver index, and adipose index. Although body weight was insignificant ($p > 0.05$) between groups, the adipose index increased by approximately 66% in all the mice fed with fructose and fat emulsion. None of the treatments alleviated this situation. The liver glycogen content estimated in MET and EEH mice evidently increased by 1.41- and 1.26-fold, respectively (compared with MC group), whereas the hepatic glucokinase activity increased by 1.54- and 1.89-fold, respectively (compared with MC group). Moreover, the weight of livers of MET mice increased significantly ($p < 0.05$).

Effect of AE and EE on OGTT, fasting glucose, fasting insulin, and IR

The analysis of glucose tolerance and the comparison of AUC between control and experimental groups showed that MC mice developed impaired glucose tolerance after three weeks of a high-fructose and high-fat diet (23.6 ± 2.9 mmol × h/L vs. 26.5 ± 2.1 mmol × h/L, $p < 0.05$; **Table 3**). Compared with MC mice, AUC decreased in AEM mice in the third week, but this result was not observed in the sixth and ninth weeks. The data show sustained hypoglycemic effects in EEM and EEH groups with a

dose-dependent effect from the third week to the ninth week. During the ninth week, AUCs of EEM and EEH were lower by 14.4% and 23.9%, respectively, than that of MC group ($p < 0.01$). EEH prevented the development of hyperglycemia and produced a stronger effect than MET group, showing significant differences at 30, 60, and 120 min ($p < 0.01$ or $p < 0.05$, compared with MC group), whereas MET group only significantly ($p < 0.01$) inhibited the blood glucose level at 120 min. The blood glucose level of mice in MC group at 0 h increased by 28.6% in the ninth week ($p < 0.01$, compared with STD group), indicating that hyperglycemia worsened in fasting blood glucose. The fasting hyperglycemia was alleviated by MET, EEM, and EEH treatments ($p < 0.01$, compared with MC group) by 16.7%, 21.1%, and 24.4%, respectively, and the relevant glucose level reached the STD level.

HOMA-IR tests were performed to evaluate the effects against IR in the treated groups, and the data are shown in **Table 2**. At the end of the experiment, MC group yielded a HOMA-IR value of 1.62 times that of STD group, indicating the development of IR in MC mice. The IR indices of MET ($p < 0.01$), EEM, and EEH ($p < 0.05$) groups substantially improved. By comparison, MET was more effective in lowering fasting insulin, whereas EEM and EEH were more capable of reducing fasting glucose (**Table 2**). Ameliorations of IR in groups treated with EEM or EEH for 10 weeks reached similar levels to those of MET or STD group. Thus, EE showed a positive and progressive effect against hyperglycemia over time.

Effect of AE and EE on serum lipid profiles and antioxidant status

353 **Table 4** describes the effect of extracts on serum lipid profiles and antioxidant status.
354 Significant elevations were observed in LDL, TC, and FFA levels of approximately
355 52.0%, 82.9%, and 43.1%, respectively, in MC group versus those in STD group
356 ($p < 0.01$ or $p < 0.05$). The abnormal changes in TC decreased in EEH group
357 compared with that in MC group after 10 weeks of administration ($p < 0.05$), whereas
358 no such decrease was detected in MET or AEM groups. The moderate increase in TG
359 levels of MC group improved by all supplementations, but only EEH group exhibited
360 a statistical significance ($p < 0.05$). The mice supplemented with EEM or EEH had
361 better control in the loss of plasma FFA by about 35% ($p < 0.05$). Moreover, MC mice
362 exhibited a significant elevation in MDA and a decrease in TAC and SOD ($p < 0.01$ or
363 $p < 0.05$). TAC significantly increased ($p < 0.05$) in AEM, EEM, and EEH groups by
364 21.8%, 25.7%, and 38.5%, respectively, and MDA concomitantly decreased by 35.5%,
365 40.1%, and 27.8%, respectively. However, an oral dose of MET (500 mg/kg/day)
366 resulted in a significant elevation in SOD by 19.3% and a reduction in MDA by 26.1%
367 compared with MC mice. Thus, EE possessed pronounced hypolipidemic effects, and
368 its improvements in antioxidant status were better than those of MET.
369
370 **Histological analysis**
371 Representative photomicrographs of liver histology for each treatment group are
372 shown in **Figure 1**. As predicted, MC group showed high lipid accumulations in the
373 cytoplasm of hepatocytes, indicating severe NAFLD in MC mice. Histological
374 evaluation revelead marked hepatic storage of lipid in MET group. In agreement with

the histological findings, the increased lipogenesis in MET group might responsible for the greater liver index as indicated above ($p < 0.05$; **Table 2**). However, AEM treatment clearly improved hepatic steatosis. Furthermore, the degree of hepatic steatosis was significantly alleviated by the daily intake of EE, as shown by the reduced surface area of steatosis of liver sections for EEM and EEH groups.

Cytotoxicity and assessment of hypoglycemic activities of partitions, subfractions, and purified compounds on IR cells

Results show that EE possessed pronounced hypolipidemic, hypoglycemic, and anti-NAFLD effects, which might be due to the presence of polyphenols in the extract. The subfractions from EA and BT fractions (which were determined to promote glucose uptake as described below), as well as compounds A to G collected by semi-separative HPLC (**Figure 2**), were used for MTT and glucose uptake assay on HepG2 cells.

To avoid cytotoxicity, the viability of HepG2 cells treated with various concentrations of fractions for 24 h was assessd by MTT assay. As outlined in **Figure 3A**, cells incubated in 50 μg/mL EA fraction or F1 caused marked cytotoxicity ($p < 0.01$ or $p < 0.05$), whereas no obvious cytotoxicities were observed in other samples at the specified concentration. Following 10^{-3} μM insulin incubation for 36 h, the uptake of extracellular glucose in IR cells with 1 μM insulin pretreatment significantly decreased than that in the control without insulin pretreatment (26.9 ± 3.2 vs. 40.4 ± 2.7, $p < 0.01$; **Figure 3B**). The medium containing 10^{-3} μM insulin, combined

with MET (10 and 50 μg/mL), CH fraction (10 and 50 μg/mL), EA fraction (10 μg/mL), F1 (50 μg/mL), F2 (10 and 50 μg/mL), F3 (10 and 50 μg/mL), F5 (50 μg/mL), F6 (10 and 50 μg/mL), or F7 (10 and 50 μg/mL), respectively, significantly increased ($p < 0.05$) uptake of extracellular glucose in IR HepG2 cells (MC cells). Given the similarity of the compounds in CH and EA fractions, focus was centered on EA fraction. Data suggest that the compounds with hypoglycemic effects might be presented in F1, F2, F3, F5, F6, and F7.

To determine which components in these fractions were the most effective, we separated compounds A to G. The chromatogram in **Figure 2** shows that the main compound in F1 was compound G. Thus, we lowered the concentration of G in the following assays to avoid cell death (caused 55.0% cell death vs. control, **Figure 3A**). As shown in **Figure 4A**, cell viability was unaffected by compounds A, B, C, D, or F, whereas E (10 and 50 μg/mL) and G (10 μg/mL) significantly reduced the number of cells ($p < 0.01$). In cells treated with A (10 and 50 μg/mL), C (10 and 50 μg/mL), and G (10 μg/mL), glucose uptake increased by 46.9%, 60.5%, 41.6%, 65.2%, 80.9%, respectively, (compared with MC cells, $p < 0.01$), and these values almost reached those in MET-treated cells.

Effects of purified compounds on OA-induced HepG2 cells

Given that hepatic steatosis and fasting glucose of MC mice were strongly inhibited by EE, the effective constituents were determined. As shown in **Figure 5A**, steatosis that appeared in OA-treated cells exhibited a dramatic TG accumulation of about

3.0-fold compared with untreated cells. Treatments with compounds A, B, C, F, and G could significantly lower the TG level ($p < 0.01$ or $p < 0.05$). Among these compounds, compound A produced the optimal effect on TG clearance, with a maximal clearance of 33.9% at 50 μg/mL. In addition, treatment with compound D resulted in a slight decline in TG levels, whereas E increased lipid deposition ($p < 0.05$).

Besides the alteration in TG, OA-induced HepG2 cells also exerted deterioration on glucose homeostasis with a marked reduction in glycogen content ($p < 0.05$; **Figure 5B**) and glucose uptake after insulin stimulation ($p < 0.01$; **Figure 5C**). C at 10 μg/mL and D or F at 50 μg/mL clearly alleviated the loss in glycogen ($p < 0.05$), as shown in **Figure 5B**. After stimulation with 10^{-3} μM insulin and their respective compounds, the results shown in **Figure 5C** illustrate that compounds A, B, C, D, and F were capable of improving glucose uptake ($p < 0.01$ or $p < 0.05$).

Identification and characterization of the compounds

In summary, compounds A to F, except E, could modulate glucose homeostasis and inhibit TG accumulation *in vitro*. Finally, the compounds were analyzed using LC/ESI/MS, and their results are presented in **Table 5**. These compounds were identified by comparing their retention time and spectral data with the values of standards or the data reported in the literature. Compound A was ascertained as puerarin, C as daidzin, G as daidzein, F as ononin, and B as 3'-methoxypuerarin[24, 25]. However, D remains unknown and is still under investigation. Although compound E

441 produced deleterious impact on TG accumulation in steatosis cell model, it was also
442 identified to unveil its structure and characteristics. Based on MS spectral data, it was
443 possibly 6”-O-acetylgenistin (M^+ *m/z* 475; MS/MS *m/z* 313, 271)[26].
444

445 ## Discussion

446 IR is defined as a marked decrease in the effectiveness of the hormone for stimulation
447 of glucose uptake and suppression of lipolysis in insulin-sensitive tissues, such as
448 muscle and fat. These metabolic abnormalities lead to the release of more FFA from
449 visceral fat and lipid metabolism alterations in liver, resulting in hepatic steatosis[4]. In
450 our previous study, we observed that a high-fructose and high-fat diet can successfully
451 induce hyperlipidemia and liver steatosis, and impair antioxidant potential and IR in
452 liver and peripheral tissues in CD-1 mice. As previously reported, *P. radix*, *L.*
453 *barbarum*, *C. pinnatifida*, and *P. rhizoma* possess hypoglycemic or anti-NAFLD
454 effects and are often used in different combinations[27, 28]. According to the principles
455 of TCM and the advice of an Oriental medical doctor, the PLCP formula of four
456 traditional edible plants was designed and administered to mice to delay the onset of
457 pre-diabetes and NAFLD progression.
458 The data in this study show that AUC of OGTT significantly increased ($p < 0.05$) in
459 the model group compared with the standard diet, which indicated that glucose
460 tolerance in peripheral tissues decreased. Moreover, the HOMA-IR index, which
461 represents IR in liver, increased by 60% in the model group. The hypoglycemic
462 properties of AE, which was rich in polysaccharides, could not be observed after three

463 weeks. EE successfully inhibited AUC and HOMA-IR value throughout the
464 experiment and achieved similar effects with MET, indicating an eminent
465 improvement in glucose disposal (**Tables 2** and **3**). Theoretically, the polyphenols in
466 EE were highly anticipated as hypoglycemic molecules. Hepatic glucose production,
467 which includes gluconeogenesis and glycogenolysis, is the major contributor to
468 hyperglycemia in diabetes and has an essential function in maintaining fasting blood
469 glucose levels[29]. The decline in glucokinase (glucose-phosphorylating enzyme)
470 activity in liver results in decreased glucose utilization and glucose uptake[30]. Thus, the
471 loss in glycogen caused by reduced glucokinase activity may account for elevated
472 fasting glucose and postprandial glucose in MC and AE mice. However, all these
473 alterations in glucose tolerance, glucokinase, glycogen, and fasting glucose were
474 reversed by EE supplementation and reached normal levels with those of STD mice.
475 In this study, the results from animal experiments could be explained by the data
476 obtained from *in vitro* experiments. Incubation with high insulin levels resulted in a
477 decrease in extrahepatic glucose disposal of HepG2 cells, whereas three compounds
478 (puerarin, daidzin, and daidzein) significantly inhibited the development of IR as
479 effectively as MET (**Figure 4**). HepG2 cells incubated with OA revealed a reduction
480 in glucose uptake. Puerarin, 3'-methoxypuerarin, daidzin, and ononin alleviated the
481 OA-induced IR by increasing insulin activity, and daidzin and ononin could restore
482 the intracellular glycogen (**Figure 5**). Meezman *et al.*[31] suggested that puerarin
483 improves glucose tolerance and inhibits the high levels of blood glucose in mice.
484 When applied to C57BL/6J lean mice, puerarin inhibits glucose uptake and glycogen

formation, whereas daidzin and its hydrolyzed compound daidzein stimulate glucose uptake[31]. Although the molecular mechanism underlying the hypoglycemic effect of puerarin may differ from that of daidzin and daidzein, their combination may work together to control glucose homeostasis in pre-diabetic status.

After 10 weeks of eating a high fructose and high fat diet, mice that developed severe hepatic steatosis were profoundly intervened by EE. Oxidative stress and IR are believed to be major contributors in the pathogenesis of NAFLD, and reactive oxygen species (ROS) have a causal function in multiple forms of IR[4]. Thus, clearance of ROS and/or attenuation of IR are theoretically effective techniques in treating NAFLD. Besides the ameliorating effect of IR, our data suggest that EE might scavenge ROS because of its abundant polyphenols (**Table 1**), leading to a significant increase in antioxidant status (**Table 4**) and a reduction in the lipid deposits in liver. EE showed better anti-NAFLD effects over AE or MET, which could be attributed to more positive outcomes (e.g., lowered serum cholesterol and TG), and less delivery of FFA to liver because of better control of adipose IR (**Tables 2** and **4**), as previously shown in animals[15, 32]. Our *in vitro* data indicate that puerarin, daidzin, 3'-methoxypuerarin, ononin, and daidzein were possibly responsible for the TG reduction abilities of EE. Besides the possible ROS scavenging effects of these polyphenols[33, 34], the mechanism underlying TG clearance could be attributed to the activation of peroxisome proliferator-activated receptors (PPARs). Puerarin significantly ($p < 0.05$) promotes PPARγ mRNA expression[35], and daidzein upregulates PPARα gene expression[36]. PPARs regulate lipid metabolism by inducing

FFA catabolism. Thus, puerarin and daidzein possibly alleviated liver steatosis directly by increasing the β-oxidation of FFA. Fructose, which can bypass the control step of glucose metabolism, is a strong inducer of *de novo* lipogenesis by activating the carbohydrate-responsive element-binding protein (ChREBP) pathway[37]. In addition, fructose metabolism cannot be properly controlled by insulin. Nevertheless, polyphenols, such as daidzein, can lower *de novo* hepatic lipid synthesis via the ChREBP pathway[36]. Thus, EE could reduce TG deposits in liver after a long-term high fructose diet. Whatever pathway was involved, EE clearly had an effect on lipid metabolism in mice fed with high fructose and high fat, and our study also confirmed such effects on HepG2 cells.

The effects of MET in liver fat in this study differed from those in several studies[38, 39]. Our data show that MET did not improve lipid profiles in serum or hepatic fat content. The difference in results might be due to the different animal strains or diets that we used. In addition, some animal[40] and human studies[41-43] showed that MET has no protective properties against liver steatosis. Although MET prevented IR induced by acute lipid load by activating adenosine 5'-monophosphate -activated protein kinase, it lacked effects on the partitioning of fatty acids, which deposit into adipose tissue and are carried away from the liver and muscle[40]. However, the distinct results in our study for MET require further investigation.

The polyphenolic constituents of PLCP exhibited promising anti-diabetic and anti-NAFLD characteristics both in mice and cell-based bioassays, which implies that this formula could be used as an herbal treatment. The active constituents identified in

present study are mainly presented in *P. radix* and *P. rhizoma*[24, 44]. However, in our formula, these two plants were not the sole reason for the decrease in blood glucose and anti-NAFLD properties because the polysaccharides presented in four plants also had important functions. Other than the active compounds screened on HepG2 cells, there might be more hypoglycemic constituents which could be screened on other tissue cells, such as adipocytes or muscle cells. This study was the first to demonstrate that 3'-methoxypuerarin and ononin had direct effects on enhancing glucose utilization and preventing TG accumulation in HepG2 cells, and 6"-O-acetylgenistin possessed significant cytotoxicity and deterioration of steatosis. More precise mechanisms underlying the effect of EE of PLCP on both IR and NAFLD require further investigation.

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Abbreviations

AUC, area under the curve; BT, *n*-butanol; CH, chloroform; ChREBP, carbohydrate responsive element binding protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EA, ethyl acetate; ESI /MS, electrospray ion source/mass

spectrometry; FBS, fetal bovine serum; FFA, free fatty acid; GAE, gallic acid equivalents; HDL, high-density lipoprotein; HOMA-IR, R-value of homeostasis model for insulin resistance; HPLC, high-performance liquid chromatography; IR, insulin resistance; LDL, low density lipoprotein; MDA, malondialdehyde; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide;NAFLD, non-alcoholic fatty liver disease; OA, oleic acid; OGTT, oral glucose tolerance test; PBS, phosphate buffered saline; PPARs, peroxisome proliferator-activated receptors; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity; TC, total cholesterol; TCM, traditional Chinese medicine; TG, triglycerides; TPC, total phenol content

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Figure captions

Figure 1. Histological structure of liver in each group (Stain: Oil red O and hematoxylin; original magnification: ×200). Arrows indicate the lipid droplets stained by oil red O. STD: standard diet; MC: model control; MET: metformin; AEM: aqueous extract in medium dose; EEM: ethanol extract in medium dose; EEH: ethanol extract in high dose. Medium and high dosages indicate extracts of 20 g of crude PLCP/kg and 30 g of crude PLCP/kg, respectively.

Figure 2. Chromatograms of F1 to F5 from EA fraction and F6 and F7 from BT fraction. HPLC conditions are described in the Methods section.

Figure 3. Cytotoxic effects of various fractions on HepG2 cells (A) and their hypoglycemic activities on IR HepG2 cells (B). Cells were incubated with various fractions on HepG2 cells for 24 h, and cell viability was determined by MTT assay. IR cells were induced by 0.5 μM insulin for 24 h, followed by changing the medium to different treatments for 36 h. Medium was then changed to DMEM containing 10^{-3} μM insulin for 24 h. The glucose concentration in supernatants was measured, and glucose uptake levels were calculated. Data are expressed as the means ± SD (n = 8). ** $p < 0.01$, * $p < 0.05$ compared with MC; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared with Control; MC: model control; MET: metformin; CH: chloroform fraction; EA: ethyl acetate fraction. Low and high concentrations represent 10 and 50 μg/mL, respectively. F1 to F5 were subfractions from EA fraction, whereas F6 to F8 were from BT fraction.

Figure 4. Cytotoxic effects of purified compounds on HepG2 cells (A) and their hypoglycemic

activities on IR HepG2 cells (B). Cells were incubated with various purified compounds on HepG2 cells for 24 h, and cell viability was determined by MTT assay. IR cells were induced by 0.5 μM insulin for 24 h, followed by changing the medium to different treatments for 36 h. The medium was then changed to DMEM containing 10^{-3} μM insulin for 24 h. The glucose concentration in supernatants was measured, and glucose uptake levels were calculated. Data are expressed as the means ± SD (n = 8). ** $p < 0.01$, * $p < 0.05$ compared with MC; $^{\#\#}$ $p < 0.01$, $^{\#}p < 0.05$ compared with Control; MC: model control; MET: metformin; A: puerarin; B: 3'-methoxypuerarin; C: daidzin; D: unidentified; E: 6"-O-acetylgenistin; F: ononin; G, daidzein. Low and high concentrations represent 10 and 50 μg/mL, respectively. Low and high concentrations for G represent 5 and 10 μg/mL, respectively.

Figure 5. Effects of purified compounds on TG accumulation (A), glycogen content (B), and glucose uptake (C) on OA-induced HepG2 cells. HepG2 cells were incubated with 0.75 mM OA alone and 0.75 mM OA in different compounds for 24 h. Cells were lysed for TG and glycogen determination or treated with DMEM containing 10^{-3} μM insulin for 24 h. The glucose concentration in supernatants was measured, and glucose uptake levels were calculated. Data are expressed as the means ± SD (n = 8). ** $p < 0.01$, * $p < 0.05$ compared with MC; $^{\#\#}$ $p < 0.01$, $^{\#}p < 0.05$ compared with Control; MC: model control; MET: metformin; CH: chloroform fraction; EA: ethyl acetate fraction; A: puerarin; B: 3'-methoxypuerarin; C: daidzin; D: unidentified; E: 6"-O-acetylgenistin; F: ononin; G, daidzein. Low and high concentrations represent 10 and 50 μg/mL, respectively. Low and high concentrations for G represent 5 and 10 μg/mL, respectively.

Table 1. Phytochemical screening and phenol content of different fractions

Phytochemical	CH fraction	EA fraction	BT fraction		
			F6	F7	F8
Phenolics & tannins	+	+	+	+	+
Saponins & steroids	—	—	—	—	+
Flavonoids	+	+	+	+	+
Anthraquinones	—	—	—	—	—
TPC (GAE mg/g FW)	104.1 ± 2.4	198.8 ± 5.1	275.2 ± 2.3	20.5 ± 1.9	182.1 ± 2.0

CH: chloroform; EA: ethyl acetate; BT: *n*-butanol; TPC: total phenol content.

Table 2. Effects of AE and EE on body mass, relevant tissue weight index, fasting glucose, fasting insulin , HOMA-IR index, hepatic glycogen and hepatic glucokinase

	STD	MC	MET	AEM	EEM	EEH
Weight (g)	34.30±2.70	33.30±5.00	33.20±3.20	34.90±4.30	35.40±4.40	34.90±3.20
Tissue index [a]						
Liver (g/100 g bw)	3.58±0.23	3.73±0.35	3.97±0.48 *	3.58±0.29	3.48±0.31	3.84±0.57
Adipose (g/100 g bw)	1.43±0.80	2.38±1.42 #	2.22±0.77	2.37±1.02	2.38±0.71	2.30±0.73
Fasting glucose (mmol/L)	6.98±0.63	8.95±0.92 ##	7.45±1.45	7.50±0.90	6.68±1.38 **	6.40±0.60 **
Fasting insulin (mU/L)	6.62±1.28	8.32±1.43	6.09±1.47 *	7.31±1.16	6.85±0.70	7.29±1.80
HOMA-IR [b]	2.05±0.45	3.32±0.66 #	2.02±0.67 **	2.47±0.64	2.05±0.54 *	2.09±0.70 *
Hepatic glycogen (mg/g liver)	14.05±2.89	10.29±2.45 #	14.55±2.88 **	9.47±1.57	10.38±3.83	12.92±1.92 *
Hepatic glucokinase (nmol/min/mg protein)	4.53±0.87	2.78±0.78 #	3.52±1.50	2.62±0.20	3.01±0.85	4.31±0.53 *

Values are means ± SD (n = 10 animals per group). * * $p < 0.01$, * $p < 0.05$ compared to MC; ## $p < 0.01$, # $p < 0.05$ compared to STD; STD: standard diet; MC: model control; MET: metformin; AEM: aqueous extract in medium dose; EEM: ethanol extract in medium dose; EEH: ethanol extract in high dose. Medium and high dosages indicate extracts of 20 g of crude PLCP/kg and 30 g of crude PLCP/kg, respectively.

[a] The liver index and adipose index were calculated as relative tissue weight divided by body weight.

[b] HOMA-IR = fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5

Table 3. Plasma glucose and the area under the curve (AUC) responses of OGTT after 3, 6 and 9 weeks

Groups	Blood glucose (mmol/L)				AUC
	0 min	30 min	60 min	120 min	(mmol × h / L)
3 weeks					
STD	5.5±0.5	18.5±3.0	12.9±2.2	6.5±0.7	23.6±2.9
MC	5.7±0.8	21.4±1.8$^{\#}$	14.3±2.8	7.3±1.0	26.5±2.1$^{\#}$
MET	6.0±0.8	18.7±3.4*	11.4±1.9**	6.3±0.6	22.5±2.9**
AEM	5.6±1.0	18.2±4.0*	12.3±1.5	6.9±1.3	23.2±2.0*
EEM	6.5±1.2	20.6±1.8	14.6±1.8	7.8±2.1	26.7±2.7
EEH	5.2±0.9	17.7±3.0**	11.5±1.8**	6.0±0.8*	21.7±2.7**
6 weeks					
STD	4.7±0.8	15.8±2.5	10.0±0.9	6.2±0.8	19.7±1.5
MC	4.9±0.7	19.5±3.9$^{\#\#}$	12.0±3.3$^{\#}$	6.8±1.2	23.4±4.4$^{\#\#}$
MET	4.2±0.8	16.7±2.2*	9.7±1.0*	6.3±0.8	20.4±1.6*
AEM	4.3±0.7	17.3±2.0	10.9±0.9	7.6±0.8	21.7±0.9
EEM	5.0±0.7	18.6±3.4	11.8±2.4	6.2±1.2	22.5±4.0
EEH	4.6±0.9	14.6±3.1**	11.0±1.9	6.4±1.0	19.9±1.8**
9 weeks					
STD	7.0±0.6	16.3±2.0	11.6±1.5	7.7±0.7	22.4±1.8
MC	9.0±0.9$^{\#\#}$	19.1±2.0$^{\#}$	12.8±1.4	8.8±1.2$^{\#}$	25.7±1.4$^{\#\#}$
MET	7.5±1.4**	17.0±2.1	11.4±1.3	7.2±1.1**	22.5±2.0*

AEM	7.9±1.5	16.9±3.7	13.1±3.0	9.2±1.7	24.9±4.8
EEM	7.1±1.2**	15.1±2.3**	11.5±1.5	7.9±1.2	22.0±2.8**
EEH	6.8±1.4**	13.5±2.9**	10.3±2.0*	7.1±1.6**	19.8±3.5**

Values are means ± SD (n = 10 animals per group). * * $p < 0.01$, * $p < 0.05$ compared to MC; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to STD; STD: standard diet; MC: model control; MET: metformin; AEM: aqueous extract in medium dose; EEM: ethanol extract in medium dose; EEH: ethanol extract in high dose. Medium and high dosages indicate extracts of 20 g of crude PLCP/kg and 30 g of crude PLCP/kg, respectively.

Table 4. Lipid profiles and antioxidant status in serum of mice fed with AE or EE at 10 weeks

Groups	STD	MC	MET	AEM	EEM	EEH
LDL-C (mmol/L)	0.25±0.07	0.38±0.11$^{\#\#}$	0.52±0.09	0.47±0.11	0.38±0.09	0.37±0.08
HDL-C (mmol/L)	4.20±0.96	4.96±1.11	4.72±1.34	4.31±1.30	3.83±0.92*	3.84±0.96
TC (mmol/L)	5.78±1.00	10.57±1.88$^{\#\#}$	12.71±1.83	10.72±1.35	9.78±2.79	8.56±1.98*
TG (mmol/L)	1.33±0.42	1.49±0.78	1.23±0.43	1.23±0.45	1.31±0.48	1.23±0.30*
FFA (mmol/L)	0.51±0.14	0.73±0.26$^{\#}$	0.55±0.14	0.67±0.18	0.46±0.16*	0.48±0.19*
TAC (U/ml)	15.19±2.36	11.96±2.15$^{\#\#}$	14.01±2.41	14.57±1.77*	15.03±1.27*	16.57±2.05**
SOD (U/ml)	182.3±23.6	152.9±19.4$^{\#}$	182.4±56.4*	153.1±23.0	166.2±24.9	170.5±12.4
MDA (nmol/ml)	5.60±1.71	11.85±3.53$^{\#\#}$	8.76±2.36**	7.64±2.61**	7.10±1.78**	8.56±2.75**

Values are means ± SD (n = 10 animals per group). ** $p < 0.01$, * $p < 0.05$ compared to MC; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to STD; STD: standard diet; MC: model control; MET: metformin; AM: aqueous extract in medium dose; EM: ethanol extract in medium dose; EEH: ethanol extract in high dose. Medium and high dosages indicate extracts of 20 g of crude PLCP/kg and 30 g of crude PLCP/kg, respectively.

Table 5. Characterization of compounds in EE of PLCP by LC/ESI/MS analysis

Peak[a]	RT[b] (min)	$[M+H]^+$	MS^2	Identification
A	20.0	417	399, 351	puerarin[d]
B	20.7	447	429, 381	3'-methoxypuerarin[c]
C	24.1	417	255, 199, 137	daidzin[d]
D	41.9	525	481,455,255	unknown
F	47.2	431	269	ononin[d]
G	49.8	255	227, 199, 137	daidzein[d]

[a] Peak name was as in **Figure 4**. [b] Retention time. [c] Tentatively identified on the basis of literature data and high resolution *m/z* values of $[M + H]^+$ ions. [d] Identified by comparison with LC/MS spectra and retention times of standards.

Figure 1

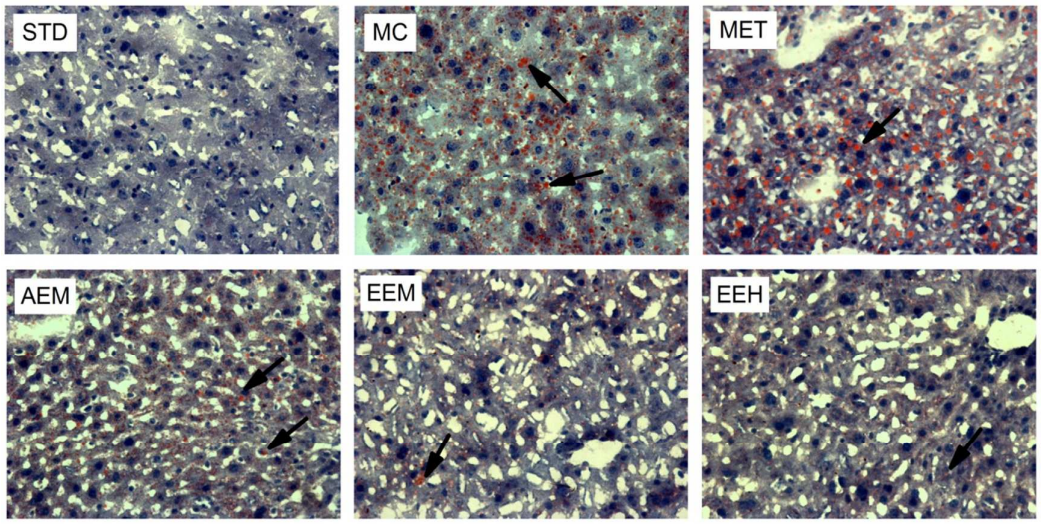


Figure 2

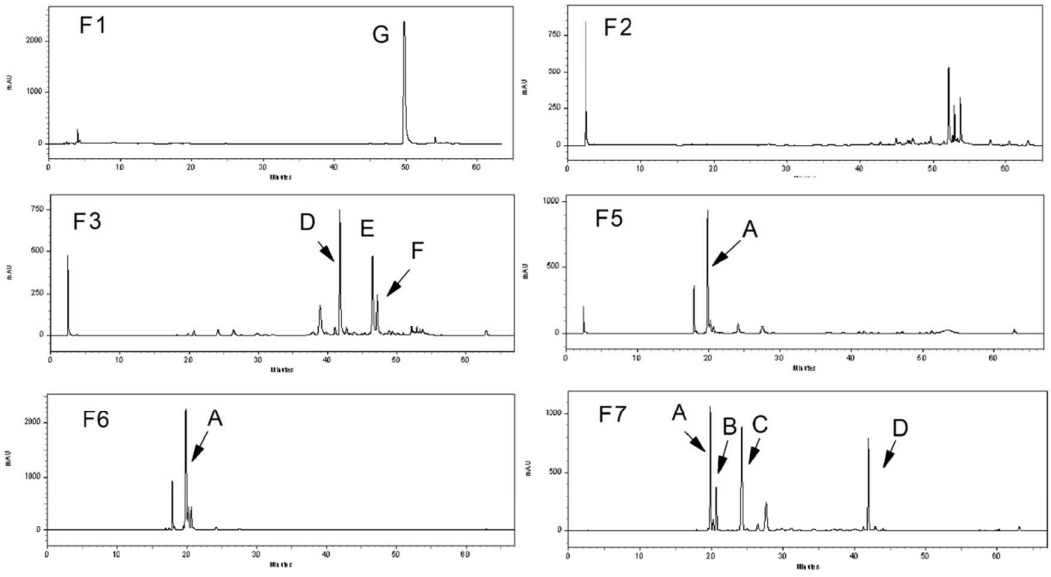


Figure 3

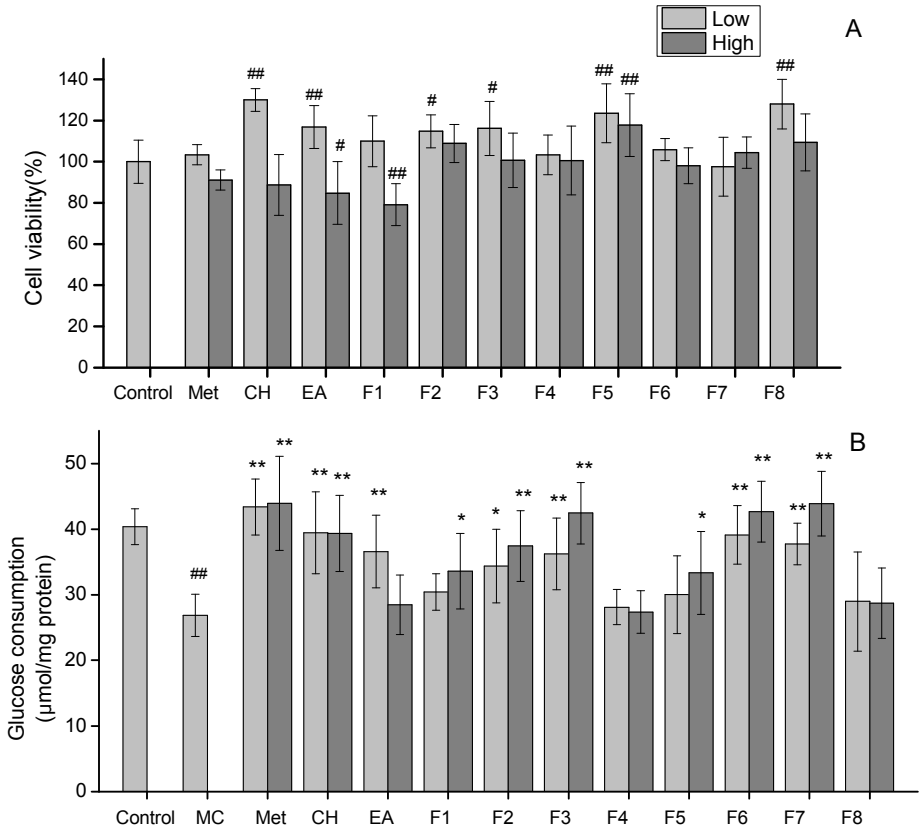


Figure 4

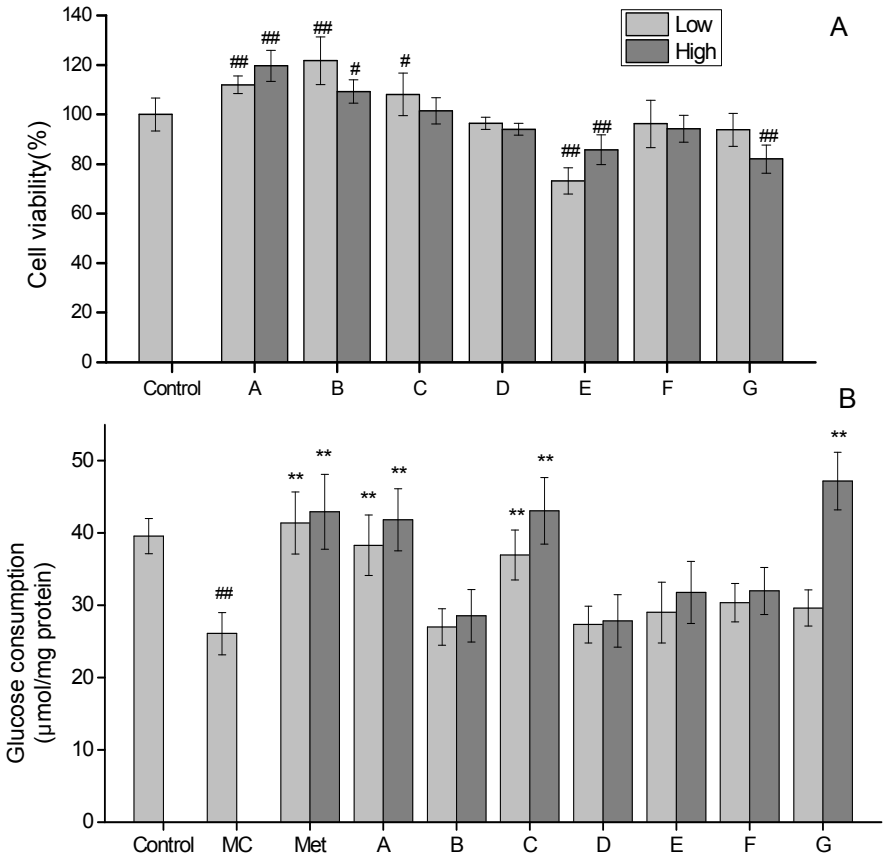
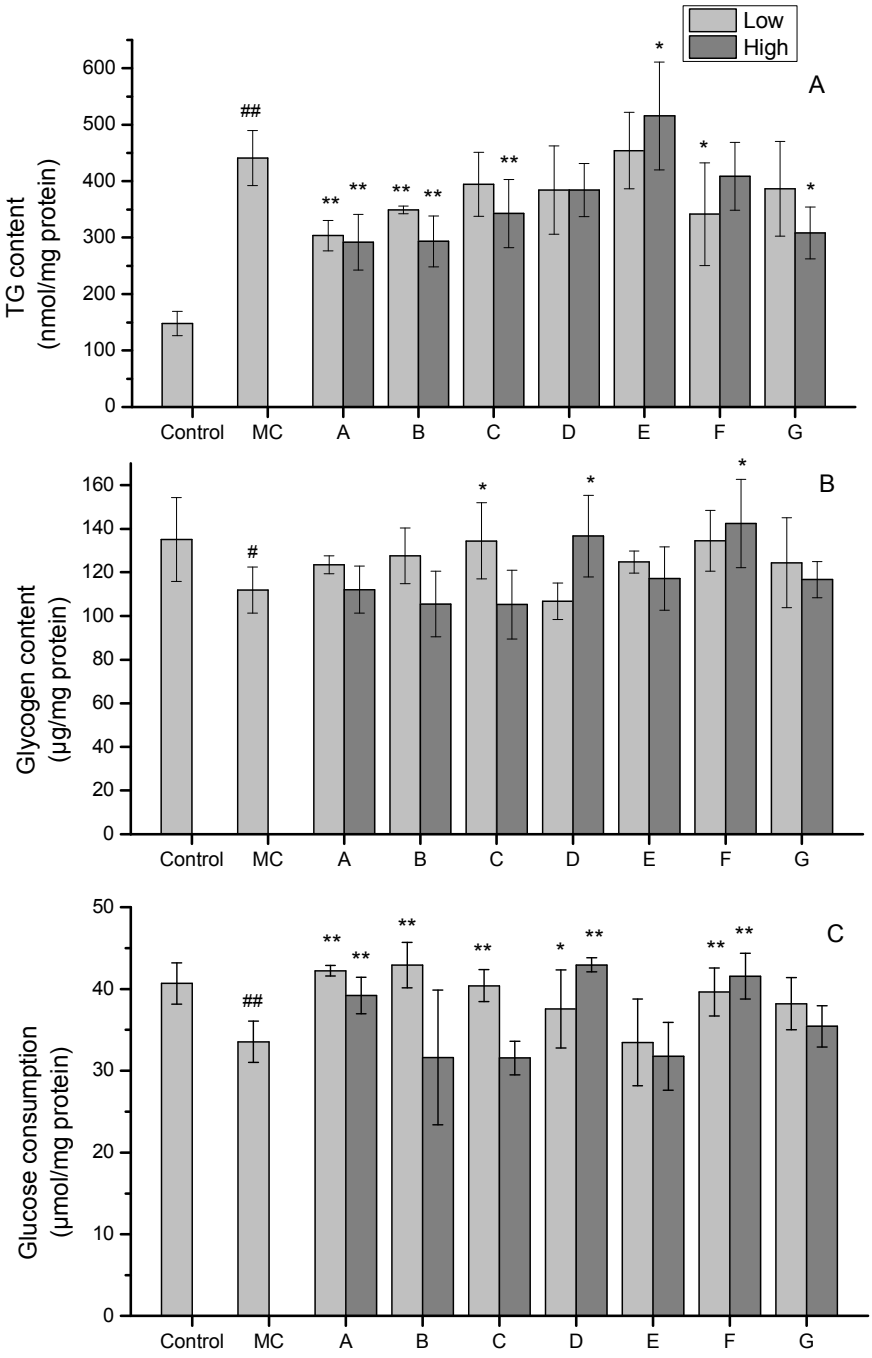


Figure 5



TOC graphic