

## Original Article

# Application of GC/MS-based metabonomic profiling in studying the lipid-regulating effects of *Ginkgo biloba* extract on diet-induced hyperlipidemia in rats

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**Aim:** To evaluate the lipid-regulating effects of extract from *Ginkgo biloba* leaves (EGB) using pharmacological methods and metabonomic profiling in a rat model of diet-induced hyperlipidemia.

**Methods:** EGB was orally administered at a dose level of 40 mg/kg in both the EGB-prevention and -treatment groups. All rat samples obtained were examined for known and potential biomarkers and enzyme activity using commercial assay kits and GC/MS-based metabonomic profiling coupled with principal component analysis (PCA).

**Results:** The data obtained from the assay kits indicated that EGB reduced total cholesterol and low density lipoprotein cholesterol levels and increased high density lipoprotein cholesterol levels in rat plasma obtained from both the EGB-prevention and -treatment groups compared with those of the diet-induced hyperlipidemia group. EGB also increased the activities of lipoprotein lipase and hepatic lipase and excretion of fecal bile acid in rats from the EGB-prevention and -treatment groups. Using GC/MS-based metabonomic analysis, more than 40 endogenous metabolites were identified in rat plasma. PCA of rat plasma samples obtained using GC/MS produced a distinctive separation of the four treatment groups and sampling points within each group. Metabolic changes during hyperlipidemia formation and improvement resulting from EGB treatment were definitively monitored with PCA score plots. Furthermore, elevated levels of sorbitol, tyrosine, glutamine and glucose, and decreased levels of citric acid, galactose, palmitic acid, arachidonic acid, acetic acid, cholesterol, butyrate, creatinine, linoleate, ornithine and proline, were observed in the plasma of rats treated with EGB.

**Conclusion:** EGB exerts multi-directional lipid-lowering effects on the rat metabonome, including limitation of the absorption of cholesterol, inactivation of HMGCoA and favorable regulation of profiles of essential polyunsaturated fatty acid (EFA). Further experiments are warranted to explore the mechanisms of action underlying the lipid-regulating effects of EGB against hyperlipidemia.

**Keywords:** metabonomics; GC/MS; lipid-regulating effect; *Ginkgo biloba* extract; hyperlipidemia

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## Introduction

Hyperlipidemia is a metabolic disorder involving increased levels of lipids and/or lipid proteins in the blood<sup>[1]</sup>. One of the major clinical outcomes of hyperlipidemia is atherosclerosis, which is the leading cause of death in the United States and other western countries<sup>[2]</sup>. Dietary cholesterol increases low density lipoprotein (LDL) levels, which could lead to hyperlipidemia if a cholesterol-rich diet is continued<sup>[2]</sup>. Commonly used drugs to treat hyperlipidemia currently on the market are niacin, fibric acid derivatives, bile acid-binding resins

and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors<sup>[2]</sup>. Likewise, there is increasing evidence of the preventive and therapeutic effects of herbal medicines and their preparations on cardiovascular diseases, including hyperlipidemia<sup>[3–6]</sup>.

*Ginkgo biloba* is referred to as a “living fossil” because it is one of the oldest living tree species, having existed for more than 200 million years<sup>[7]</sup>. The leaves and seeds of *Ginkgo biloba* contain bioactive compounds responsible for therapeutic and preventive effects on dementia, dyslipidemia, stroke, failing memory, Alzheimer's disease, aging, cognitive speed and free-radical damage in traumatic brain and clinical applications<sup>[8,9]</sup>. Extract from *Ginkgo biloba* leaves (EGB) is the most widely used herbal supplement in recent years<sup>[3,10]</sup>. Standardized

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preparations of EGB contain 24% flavonoids, 6% terpene lactones and other substances in low amounts, including proanthocyanidins and organic acids<sup>[7, 10]</sup>. Terpene lactones in EGB include ginkgolides (*eg*, ginkgolide A, B, C, J) and biolalide. Flavonoids, ginkgolide A and B, and biolalide are bioavailable after oral or parenteral administration of EGB<sup>[7]</sup>. The diverse clinical benefits of EGB are attributed to the underlying mechanisms of action exerted by EGB. EGB has been reported to possess antioxidant properties and free-radical scavenging activities, counteract the cognitive deficits following stress or traumatic brain injury, reduce adhesion of blood cells to the endothelium, inhibit the activation of platelets and others<sup>[3, 7, 8]</sup>. Previous studies have indicated that EGB has lipid-regulating effects on hyperlipidemia<sup>[11-13]</sup>. However, the underlying mechanisms of action remain unclear as to how EGB regulates its lipid-lowering effect.

Chinese traditional medicine (CTM), composed of complex components, has multiple roles when applied in living systems. Thus, it is challenging to study the molecular mechanisms of action of TCM by exclusively using traditional pharmacology methods. Metabonomics now offers new insights into the effects of diet, drugs and diseases. As it targets a quantitative measurement of the global and dynamic metabolic responses of living systems to biological stimuli and genetic modification<sup>[14, 15]</sup>, metabonomics provides an integrated view of biochemistry in complex organisms, as well as global outcomes of system biology and environmental and lifestyle factors<sup>[15]</sup>. The approach has been applied widely and successfully to disease state diagnosis<sup>[16-19]</sup>, pharmaceutical research and development<sup>[20]</sup>, drug toxicity evaluation<sup>[14, 21]</sup>, therapeutic monitoring and other related topics<sup>[22]</sup>. As such, metabonomics may monitor metabolic responses in complex systems when TCM is applied. In addition, metabolite profiling of biological samples (*eg*, plasma) using GC/MS has been recognized to be one of the most useful metabonomic approaches in pathological diagnosis and biomarker identification.

The aim of the present study was to investigate the lipid-regulating effects of EGB using a GC/MS-based metabonomic approach combined with traditional pharmacology methods. A biochemical assessment of the lipid-regulating effects of EGB was conducted using GS/MS-based metabolic profiling of rat plasma and pattern recognition (PR) to classify the samples from rats in a control group, a diet-induced hyperlipidemia group, an EGB-prevention group and an EGB-treatment group. The results from the metabonomic approach were not only compared with those obtained with the traditional pharmacology method using commercial assay kits, but they were also analyzed to identify potential biomarkers to further explore the mechanisms of action of EGB against hyperlipidemia.

## Materials and methods

### Chemicals and reagents

EGB (Nanjing University of Technology, China) was standardized to contain 45% total flavonoids (23.6% quercetin-3-O-

rutinoside, 18.4% kaempferol-3-O-rutinoside and 3% isorhamnetin-3-O-rutinoside) and 7% terpenoids (2.2% bilobalide and 3.8% ginkgolides A, B, C), with no more than 5 parts per million ginkgolic acid. Kits for total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), lipoprotein lipase (LPL) and hepatic lipase (HL) were from Zhejiang Dongou Biological Engineering Ltd, China. Kits for fecal bile acid (FBA) were from Suzhou Aijie Biological Technology Ltd, China. [<sup>2</sup>H<sub>6</sub>]-salicylic acid (97%) used as an internal standard (IS) was purchased from Cambridge Isotope Laboratories Inc (Andover, MA, USA). Alkane series (C<sub>8</sub>-C<sub>40</sub>), *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) and trimethyl chlorosilane (TMCS) were obtained from Fluka (Buchs, Switzerland), and methoxyamine was from Supleco (Bellefonte, PA, USA). Distilled water was produced using a Milli-Q Reagent Water System (Millipore, MA, USA). The methanol was of HPLC grade (Tedia Company Inc, Fairfield, OH, USA). Pyridine and *n*-heptane were both of analytical grade and were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA) and Tedia Company Inc (Fairfield, OH, USA), respectively.

### Animals, diets, and sample preparation

Sprague-Dawley rats were obtained from Sino-British Sippr/BK Lab Animal Ltd (Shanghai, China) and housed in stainless steel cages in a controlled environment with 20 °C, 50% relative humidity and a 12 h light/12 h dark cycle for at least six days prior to the experiments. Animal experiments were carried out in accordance with the Guidelines for Animal Experimental of Nanjing University of Technology (Nanjing, China).

Thirty-two male Sprague-Dawley rats, weighing around 200 g, were randomly assigned into four groups of eight rats in each group. Rats in the control group were fed with a normal diet from weeks 1-8. Rats in the diet-induced hyperlipidemia group were fed with a high-lipid diet enriched with 1% (*w/w*) cholesterol, 10% (*w/w*) lard, 0.2% propylthiouracil, 5% yolk and 1% sodium tauroglycocholate during the first four weeks, followed by a normal diet in weeks 5-8. Rats in the EGB-prevention group were fed with a high-lipid diet and EGB at a dose level of 40 mg/kg daily in weeks 1-4. Rats in the EGB-treatment group were fed with high-lipid diet in weeks 1-4 and received a normal diet and EGB (40 mg/kg daily) in weeks 5-8. Diet and EGB treatment plans for each four groups are listed in Table 1. EGB was orally administered as a suspension in water using a gavage. Before the experiment and at 8:00 am once every week, 1.5 mL of blood was collected into tubes containing EDTA via retro-orbital bleeding from each rat under fasting conditions (no food but water *ad libitum* for 12 h). Blood samples were centrifuged at 2000×*g* for 10 min at 4 °C to collect plasma and then separated into two parts. One part was used for determination of TC, TG, LDL-C, and HDL-C levels using the commercial assay kits, and the other was used for metabonomic studies. All plasma samples were stored at -80 °C until analysis.

Forty male Sprague-Dawley rats, weighing around 200 g, were randomly assigned into four groups with ten rats in each

**Table 1.** Diet and EGB treatment for all the rat groups.  $n=8$ .

Study groups	Week 1–4	Week 5–8
Control group	Normal diet <sup>a</sup>	Normal diet <sup>b</sup>
Diet-induced hyperlipidemia group	High-lipid diet <sup>a</sup>	Normal diet <sup>b</sup>
EGB-prevention group	High-lipid diet & EGB <sup>ab</sup>	N/A
EGB-treatment group	High-lipid diet	Normal diet & EGB <sup>ab</sup>

<sup>a</sup> Time for determination of lipoprotein lipase (LPL), hepatic lipase (HL), and fecal bile acid (FBA).

<sup>b</sup> Time for determination of total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and metabonomic study.

group. Diet and EGB treatment were followed as described above for the control, diet-induced hyperlipidemia, EGB-prevention and EGB-treatment groups (Table 1). A single injection of 130 U/kg of heparin was given intravenously to rats in the control, diet-induced hyperlipidemia and EGB-prevention groups after the first 4-week treatment, and to those in the EGB-treatment group after an 8-week treatment. Blood was collected after 15 min and plasma was obtained using a centrifuge under the conditions described as above. Plasma was used to determine free fatty acid (FFA), LPL, and HL. Also, rat feces were collected for each rat for three days before the end of experiment. The fecal samples were evaporated to dryness at 56 °C. Dried fecal samples were stored at -80 °C until analysis.

#### Classic pharmacological study using assay kits

##### Determination of TC, TG, LDL-C, and HDL-C

The levels of TC, TG, LDL-C, and HDL-C in rat plasma were determined using enzyme methods with commercial kits. The results obtained were compared with those gained in the metabonomic study.

##### Determination of LPL, HL and FBA

The levels of FFA were determined with copper reagents according to the kit instructions<sup>[23]</sup>. The activity of LPL and HL was calculated according to FFA levels and equations provided in the kit instructions by the manufacturer. The level of FBA in fecal samples was determined using the assay kit according to the instructions provided by the manufacturer.

#### Metabonomic study

##### Pretreatment of plasma

Four hundred microliters of methanol (including 2 µg [<sup>2</sup>H<sub>6</sub>]-salicylic acid) was added to 100 µL of plasma. The solution was extracted for 10 min (TDLSO-2B Shaker, Shanghai Anting Scientific Instrument Company, Shanghai, China) and was centrifuged at 12000×g at 4 °C for 10 min. The supernatant (100 µL) was transferred to a 1-mL GC vial and evaporated to dryness under nitrogen at 25 °C. Thirty microliters of methoxyamine in pyridine (15 µg/mL) was added to each GC vial.

The solution was then vortexed for 10 min. After the methoxyamination reaction proceeded for 16 h at room temperature, the samples were trimethylsilylated for another 1 h by adding 30 µL of MSTFA with 1% TMCS as a catalyst. Afterwards, 40 µL of heptane was added to each GC vial, and the solution was vortexed for 10 min before GC/MS analysis. The protocols of plasma extraction and derivation have been established and validated previously<sup>[24]</sup>.

##### GC/MS analysis

One microliter of derivatized sample was injected into a Finigan TRACE DSQ Gas Chromatograph (ThermoFinnigan, USA). Chromatographic separation was conducted on a fused silica capillary column (30 m×0.25 mm ID) chemically bonded with a 0.25 µm DB1-MS stationary phase (J&W scientific, Folsom, CA, USA). The injector temperature and the septum purge flow rate were controlled at 270 °C and 20 mL/min, respectively. The purge was turned on 1 min after injection. The gas flow rate through the column was 1 mL/min. The column initial temperature was kept at 70 °C for 2 min. Then temperature was increased from 70 °C to 240 °C at a rate of 20 °C/min, held for 1 min, and then elevated to 320 °C at a rate of 20 °C/min and held for 1 min. The transfer line temperature and ion source temperature were controlled at 270 °C and 200 °C, respectively. Ionization was achieved via a 70 eV electron beam at a current of 2.0 mA. Mass spectra were acquired from  $m/z$  50 to 650 at a rate of 2 s, and the acceleration voltage was turned on after a solvent delay of 4 min.

##### Validation of assay method

**Linearity** Plasma was diluted with water to relative concentrations of 0.063, 0.125, 0.250, 0.500, and 1.000 ( $v/v$ , plasma/plasma+water). One hundred microliters of the diluted plasma was then mixed with 400 µL of methanol containing 2 µg of [<sup>2</sup>H<sub>6</sub>]-salicylic acid. Extraction and derivatization were performed according to pretreatment of the plasma as described above. Peak areas of endogenous metabolites were integrated by Xcalibur version 1.3.1 (Thermo Finigan, USA). The peak-area ratio of each metabolite to IS and the linear correlation coefficients were calculated at each concentration.

**Precision** Precision was calculated using relative standard deviation (RSD) at three dilutions of plasma with 0.063, 0.250, and 1.000 ( $v/v$ , plasma/plasma+water). Intra-day precision was determined in five replicates at each dilution, and replicates were processed independently. Inter-day precision was determined at each dilution on five different days.

**Sensitivity** The limit of detection (LOD) and lower limit of quantification (LLOQ) of plasma were determined upon giving a signal-to-noise ratio of 3 and 5, respectively.

##### Data analysis

The data obtained from commercial assay kits are presented as the mean±SD. Statistical significance was assessed using the Student's *t*-test.

All of the GC/MS data were processed using Xcalibur software version 1.3.1 (Thermo Finigan, USA). Peaks with signal-to-noise (S/N) ratios lower than 5 were rejected. Retention times were corrected using IS in order to minimize run-to-run errors. The retention index for each peak/compound was calculated by comparing its retention time against those of the alkane series (C8–C40, corresponding retention index from 800–4000). To obtain accurate peak areas for IS and specific peaks/compounds, two fragment masses for each compound were specified for quantification and the data were reprocessed accordingly. Each peak area was normalized using IS before multivariate data analysis. All compounds were identified and assigned by matching both the MS spectra and retention index with those available in NIST 2.0 (2005), the Wiley library and the in-house spectra library constructed in the Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University. The Human Metabolome database (<http://www.hmdb.ca>)<sup>[25]</sup> and Lipid Maps Database (<http://www.lipidmaps.org>) were also used to search potential metabolites.

Multivariate statistical analysis (MVSA) was carried out using SIMCA-P version 11 software (Umetrics, Umeå, Sweden). The data matrix was constructed using GC/MS responses of each peak as variables, with the observations/samples in columns and the peaks in rows. Principal component analysis (PCA) was used to calculate a basic model and overview the data. Cross-validation with seven cross-validation groups was used to determine the number of components, as proposed by Wold<sup>[26]</sup>. Data was visualized using principal component scores and loadings plots. Each dot on the scores plot represents an individual sample, and each dot on the loadings plot stands for a peak/compound observed and identified in the corresponding chromatogram. Interpretation of scores plot is complemented by the corresponding loadings plot where variables responsible for the trends and patterns identified are indicated<sup>[24]</sup>. Statistically different peaks were calculated with a confidence interval of 95% and significance level of 0.05<sup>[27]</sup>.

## Results

### Assay kit results

#### TC, TG, LDL-C, and HDL-C

The plasma lipid levels of rats in the control, diet-induced hyperlipidemia and EGB-prevention groups during the experiment were shown in Table 2. There were no significant changes among the different time points in the control group during the entire experiment. The lipid levels of the rats in the diet-induced hyperlipidemia group in the first two weeks showed no significant changes compared with those of rats before the experiment and those in the control group ( $P < 0.01$ ). After rats were administered with a high-lipid diet for three weeks, the levels of TC and LDL-C increased significantly, whereas the level of HDL-C decreased significantly compared with those of the same rats before the experiment and those of the control group ( $P < 0.01$ ). This indicated that hyperlipidemia was formed after a high-lipid diet was administered to the rats

for three weeks.

Administration of EGB with a high-lipid diet simultaneously for three weeks was found to result in a significant reduction in the level of TC and LDL-C, and an increase in the level of HDL-C in plasma compared with rats in the diet-induced hyperlipidemia group, indicating that EGB had a preventive effect against hyperlipidemia.

A diet-induced hyperlipidemia model had been set up in rats of the EGB-treatment group after a four-week administration of a high-lipid diet. With the administration of EGB and a normal diet for the following four weeks, the levels of TC and LDL-C decreased significantly, whereas the level of HDL-C increased significantly in plasma compared with the rats in the diet-induced hyperlipidemia group at the corresponding time points, indicating that EGB exhibited therapeutic effects against hyperlipidemia.

#### LPL, HL, and FBA

Tables 3 and 4 depict the findings of the high-lipid diet and the EGB prevention and treatment effects on the levels of LPL, HL and FBA. The activity levels of HL and LPL of rats in the diet-induced hyperlipidemia group were significantly decreased compared with those in the control group. Likewise, the levels of FBA in the diet-induced hyperlipidemia group were significantly decreased ( $P < 0.01$ ) compared with those in the control group.

In both the EGB-prevention and EGB-treatment groups, EGB increased the activity of HL and LPL and the excretion of FBA significantly ( $P < 0.01$ ) compared with the diet-induced hyperlipidemia group. These findings suggested that EGB, in the presence of excess cholesterol, significantly increased the activity of lipid metabolism enzymes and cholesterol catabolism in hepatic metabolism, which might be responsible for enhanced excretion of the corresponding metabolites (eg, FBA).

### Metabonomic results

#### Metabonomic profiling by GC/MS

More than 40 compounds were identified in metabonomic profiling using the GC/MS chromatographic method (Figure 1).

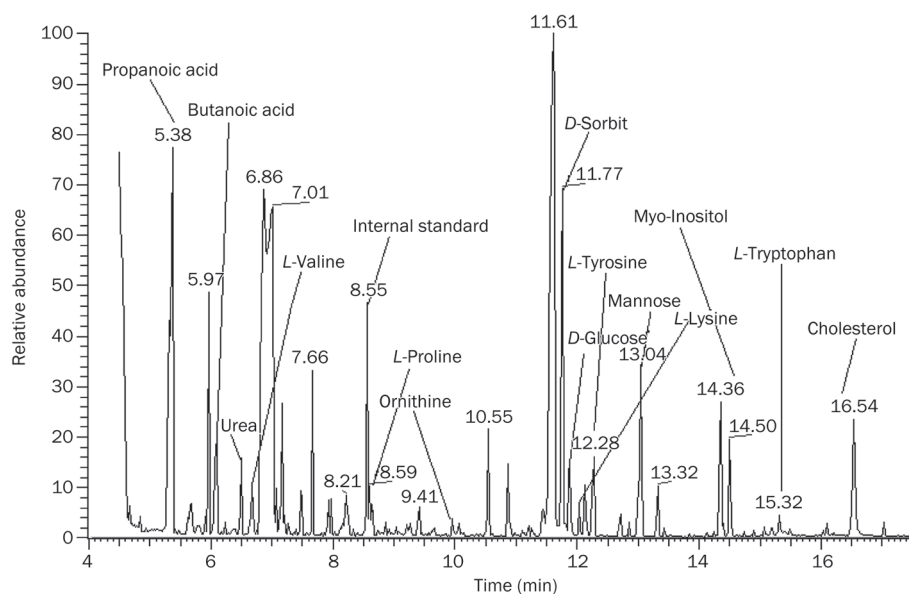
#### Validation of assay method

**Linearity** Fourteen of the endogenous compounds identified were selected to examine the linearity of the method. These compounds covered a wide span of GC retention times and represented different classes of chemicals with diverse physicochemical properties, such as organic acids, amino acids and carbohydrates. The linearity of the response was determined using five different concentrations in plasma, as shown in Table 5.

**Precision** The same fourteen endogenous compounds identified were selected to examine the precision of the method. The precision of the analysis was calculated as the relative standard deviation (RSD) of the peak area for each metabolite corrected by the peak area of the IS. The RSD values of the fourteen compounds were less than 12.5%, as

**Table 2.** Plasma lipid levels of rats in four groups during the experiment.  $n=8$ . Mean $\pm$ SD. <sup>c</sup> $P<0.01$  vs control group; <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  vs diet-induced hyperlipidemia group; <sup>h</sup> $P<0.05$ , <sup>i</sup> $P<0.01$  vs within-group control at week 0.

Group	Week	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Control	0	1.60 $\pm$ 0.42	1.19 $\pm$ 0.36	0.87 $\pm$ 0.24	1.03 $\pm$ 0.44
	1	1.58 $\pm$ 0.41	1.17 $\pm$ 0.31	0.88 $\pm$ 0.19	1.05 $\pm$ 0.42
	2	1.59 $\pm$ 0.41	1.20 $\pm$ 0.36	0.88 $\pm$ 0.23	1.03 $\pm$ 0.43
	3	1.58 $\pm$ 0.43	1.21 $\pm$ 0.36	0.95 $\pm$ 0.24	1.06 $\pm$ 0.43
	4	1.62 $\pm$ 0.40	1.20 $\pm$ 0.33	0.92 $\pm$ 0.20	1.04 $\pm$ 0.35
	5	1.61 $\pm$ 0.39	1.14 $\pm$ 0.32	0.95 $\pm$ 0.25	1.01 $\pm$ 0.42
	6	1.64 $\pm$ 0.44	1.19 $\pm$ 0.36	0.94 $\pm$ 0.22	0.97 $\pm$ 0.39
	7	1.66 $\pm$ 0.34	1.21 $\pm$ 0.32	0.91 $\pm$ 0.24	1.05 $\pm$ 0.32
	8	1.61 $\pm$ 0.44	1.15 $\pm$ 0.42	0.96 $\pm$ 0.31	1.01 $\pm$ 0.38
Diet-induced hyperlipidemia	0	1.61 $\pm$ 0.34	1.18 $\pm$ 0.28	0.88 $\pm$ 0.21	1.08 $\pm$ 0.35
	1	1.58 $\pm$ 0.29	1.18 $\pm$ 0.28	0.91 $\pm$ 0.18	1.05 $\pm$ 0.36
	2	1.63 $\pm$ 0.31	1.17 $\pm$ 0.25	0.91 $\pm$ 0.15	1.11 $\pm$ 0.37
	3	4.31 $\pm$ 0.66 <sup>ci</sup>	1.19 $\pm$ 0.25	0.64 $\pm$ 0.17 <sup>ci</sup>	1.73 $\pm$ 0.57 <sup>ci</sup>
	4	7.01 $\pm$ 1.23 <sup>ci</sup>	1.17 $\pm$ 0.26	0.51 $\pm$ 0.19 <sup>ci</sup>	4.63 $\pm$ 0.98 <sup>ci</sup>
	5	6.84 $\pm$ 1.19 <sup>ci</sup>	1.12 $\pm$ 0.35	0.55 $\pm$ 0.21 <sup>ci</sup>	4.36 $\pm$ 1.05 <sup>ci</sup>
	6	6.58 $\pm$ 1.26 <sup>ci</sup>	1.14 $\pm$ 0.29	0.58 $\pm$ 0.17 <sup>ci</sup>	4.29 $\pm$ 0.99 <sup>ci</sup>
	7	6.02 $\pm$ 1.06 <sup>ci</sup>	1.16 $\pm$ 0.33	0.61 $\pm$ 0.22 <sup>ci</sup>	3.99 $\pm$ 0.89 <sup>ci</sup>
	8	5.74 $\pm$ 1.15 <sup>ci</sup>	1.14 $\pm$ 0.39	0.66 $\pm$ 0.24 <sup>ci</sup>	3.64 $\pm$ 0.74 <sup>ci</sup>
EGB-prevention	0	1.62 $\pm$ 0.24	1.19 $\pm$ 0.23	0.86 $\pm$ 0.18	1.08 $\pm$ 0.43
	1	1.57 $\pm$ 0.24	1.18 $\pm$ 0.24	0.85 $\pm$ 0.16	1.10 $\pm$ 0.41
	2	1.61 $\pm$ 0.22	1.20 $\pm$ 0.21	0.84 $\pm$ 0.17	1.08 $\pm$ 0.41
	3	2.56 $\pm$ 0.39 <sup>fi</sup>	1.20 $\pm$ 0.22	0.81 $\pm$ 0.14 <sup>f</sup>	1.58 $\pm$ 0.60 <sup>i</sup>
	4	4.86 $\pm$ 0.53 <sup>fi</sup>	1.16 $\pm$ 0.21	0.91 $\pm$ 0.15 <sup>f</sup>	1.86 $\pm$ 0.69 <sup>fi</sup>
EGB-treatment	0	1.55 $\pm$ 0.41	1.07 $\pm$ 0.31	0.92 $\pm$ 0.34	1.11 $\pm$ 0.33
	4	7.15 $\pm$ 1.18 <sup>i</sup>	1.09 $\pm$ 0.27	0.46 $\pm$ 0.16 <sup>i</sup>	4.72 $\pm$ 1.39 <sup>j</sup>
	5	5.41 $\pm$ 1.14 <sup>if</sup>	1.11 $\pm$ 0.24	0.52 $\pm$ 0.19 <sup>i</sup>	4.23 $\pm$ 1.11 <sup>i</sup>
	6	4.93 $\pm$ 0.97 <sup>if</sup>	1.14 $\pm$ 0.24	0.68 $\pm$ 0.24 <sup>ie</sup>	3.91 $\pm$ 0.98 <sup>if</sup>
	7	4.28 $\pm$ 0.99 <sup>if</sup>	1.18 $\pm$ 0.26	0.78 $\pm$ 0.29 <sup>hf</sup>	3.27 $\pm$ 1.02 <sup>if</sup>
	8	3.85 $\pm$ 1.04 <sup>if</sup>	1.16 $\pm$ 0.27	0.89 $\pm$ 0.33 <sup>f</sup>	2.56 $\pm$ 0.84 <sup>if</sup>

**Figure 1.** A GC/MS chromatograph of plasma samples obtained from a control rat. Some typical metabolites and the internal standard are labeled.

**Table 3.** Effects of EGB on the activity levels of LPL and HL in plasma and fecal bile acid in EGB-prevention group after 4-week co-administration of EGB and high-lipid diet.  $n=10$ . Mean $\pm$ SD.  $^{\circ}P<0.01$ , diet-induced hyperlipidemia group vs control group;  $^fP<0.01$ , EGB-prevention group vs diet-induced hyperlipidemia.

Group	Dose (mg/kg)	LPL (U/mL)	HL (U/mL)	FBA ( $\mu$ mol/d)
Control	-	11.88 $\pm$ 2.10	7.24 $\pm$ 2.04	19.67 $\pm$ 5.42
Diet-induced hyperlipidemia	-	3.42 $\pm$ 1.90 $^{\circ}$	3.99 $\pm$ 1.13 $^{\circ}$	13.09 $\pm$ 2.91 $^{\circ}$
EGB-prevention	40	20.50 $\pm$ 6.91 $^f$	14.47 $\pm$ 3.30 $^f$	29.54 $\pm$ 2.65 $^f$

**Table 4.** Effects of EGB on the activity levels of LPL and HL in plasma and fecal bile acid in EGB-treatment group after 4-week administration of high-lipid diet followed by 4-week treatment of EGB.  $n=10$ . Mean $\pm$ SD.  $^{\circ}P<0.01$ , diet-induced hyperlipidemia group vs control;  $^fP<0.01$ , EGB-treatment group vs diet-induced hyperlipidemia.

Group	Dose (mg/kg)	LPL (U/mL)	HL (U/mL)	FBA ( $\mu$ mol/d)
Control	-	7.92 $\pm$ 2.11	7.24 $\pm$ 2.04	24.52 $\pm$ 3.81
Diet-induced hyperlipidemia	-	3.47 $\pm$ 1.13 $^{\circ}$	3.99 $\pm$ 3.30 $^{\circ}$	18.15 $\pm$ 2.14 $^{\circ}$
EGB-treatment	40	22.74 $\pm$ 3.21 $^f$	14.47 $\pm$ 3.30 $^f$	28.69 $\pm$ 2.72 $^f$

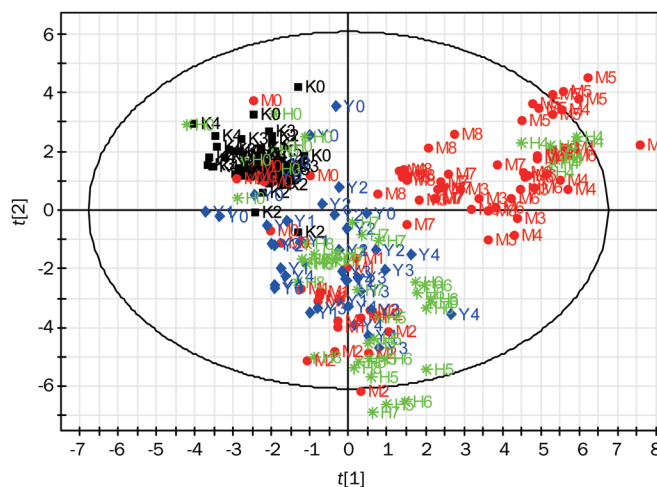
shown in Table 6.

**Sensitivity** The fourteen endogenous compounds identified were also selected to evaluate the LOD and LLOQ. These compounds were able to be detected at S/N equal to or greater than 3 when the plasma dilution was 0.02 (*v/v*, plasma/plasma+water), which was defined as the LOD. These compounds

could also be detected at S/N greater than or equal to 5 when the plasma dilution was 0.063 (*v/v*, plasma/plasma+water), which was defined as the LLOQ.

### PCA study

The PCA scores plot of the whole dataset of the four groups during the 8-week experiment is overviewed in Figure 2. In the plot, the letters represent different groups: K: control group; M, diet-induced hyperlipidemia group; Y, EGB-prevention group; H, EGB-treatment group. The numbers represent different experimental time points: 0 represents before the experiment, and numbers 1–8 correspond to



**Figure 2.** The PCA scores plot of four groups. Letters K, M, Y, and H denote different treatment groups: K (black), control group; M (red), diet-induced hyperlipidemia group; Y (blue), EGB-prevention group; H (green), EGB-treatment group. Numbers 0–8 represents different time points during the experiment: 0, before experiment; 1, week 1; 8, week 8. M4 means the point from the sample in the diet-induced hyperlipidemia group at the 4th week after the experiment.

**Table 5.** Linearity of the GC/MS method for determination of endogenous compounds in rat plasma.

Compound	Peak area ratios of plasma dilutions					Correlation coefficient
	1	0.5	0.25	0.125	0.063	
Butanoic acid	3.422	2.09	1.302	0.807	0.474	0.9951
Urea	2.627	1.586	1.015	0.726	0.462	0.9975
Valine	7.805	4.864	3.208	2.083	1.328	0.9946
Glycine	1.921	1.141	0.781	0.597	0.314	0.9932
Serine	1.473	0.807	0.558	0.343	0.211	0.9971
Threonine	3.506	1.809	0.833	0.482	0.249	0.9995
Cysteine	0.356	0.2	0.141	0.096	0.072	0.9986
Aspartic acid	0.574	0.333	0.193	0.132	0.065	0.997
Proline	0.302	0.175	0.101	0.067	0.034	0.9973
Glucose	21.63	12.522	7.449	4.196	2.429	0.9973
Tryptophan	0.177	0.145	0.126	0.112	0.108	0.9958
Inositol, myo-	1.015	0.567	0.337	0.177	0.099	0.9978
Hexanedioic acid	0.37	0.283	0.245	0.224	0.199	0.9964
Cholesterol	2.07	1.262	0.837	0.59	0.4342	0.9986

**Table 6.** Precision of the GC/MS method for determination of endogenous compounds in rat plasma.  $n=5$ .

Compound	Intra-day precision RSD (%) at dilutions (v/v) of			Inter-day precision RSD (%) at dilutions (v/v) of		
	0.063	0.25	1	0.063	0.25	1
Butanoic acid	7.58	5.33	3.35	8.26	6.22	3.31
Urea	10.12	4.58	2.78	11.20	7.12	2.89
Valine	9.15	5.86	3.79	9.85	5.78	2.81
Glycine	8.41	6.25	2.89	9.32	4.87	1.91
Serine	6.14	4.12	1.54	8.24	5.56	1.61
Threonine	11.25	7.54	3.51	12.02	7.45	3.42
Cysteine	8.69	5.84	2.36	9.23	6.32	2.38
Aspartic acid	10.25	7.52	2.57	11.44	5.41	2.58
Proline	9.85	5.21	3.32	10.32	4.52	3.31
Glucose	5.69	5.14	2.98	6.10	7.47	2.68
Tryptophan	9.58	5.68	4.17	9.63	7.58	4.19
Inositol, myo-	7.63	7.01	5.15	8.79	7.44	2.13
Hexanedioic acid	7.41	4.88	3.35	8.56	6.32	3.36
Cholesterol	6.85	5.02	2.03	7.88	5.25	2.00

weeks 1–8 after the start of the experiment, respectively. Accordingly, H6 denotes the samples in the EGB-treatment group at the sixth week after the start of the experiment.

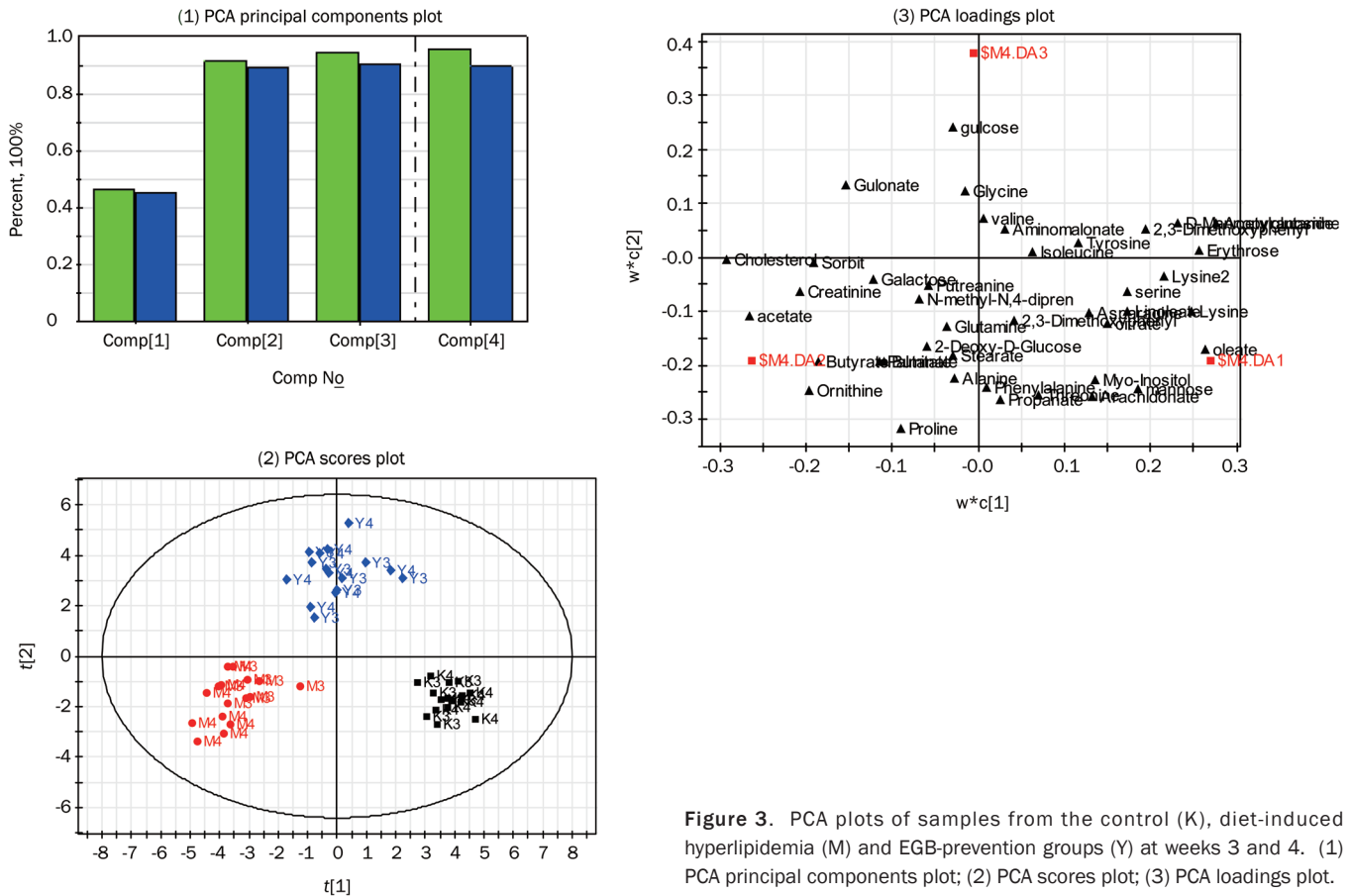
All of the samples in the control group (K0–8) clustered in the top left quadrant of the plot, suggesting a stable status of the metabolome during the eight weeks of the experiment. In the diet-induced hyperlipidemia group, the samples of M0 gathered in the same region as those in the control group. This result was in accordance with the fact that all of the samples from the same rat source fed with the same regular food exhibited similar metabolome profiles. Samples of M1 and M2 tended to gather in different regions, and other samples (M3–M8) gathered in the top right quadrant of the plot, indicating a stable rat model of hyperlipidemia after the administration of a high-lipid diet for three weeks. This finding was in accordance with the results obtained using the commercial assay kits.

There were significant differences between the EGB-prevention group and the control group on the plot. Samples Y3 and Y4 gathered in the region between the samples in the control group and samples M1 and M2, which indicated that EGB exhibited a preventive effect against hyperlipidemia. EGB showed a more remarkable therapeutic effect against hyperlipidemia in the EGB-treatment group. Samples H5 and H6 moved to the region of samples M1 and M2 after treatment with EGB for one and two weeks. Furthermore, samples H7 and H8 gathered in a different region from samples M7 and M8 in the PCA scores plot. In other words, samples H7 and H8 positioned themselves almost opposite to samples M7 and M8, indicating some dramatic differences between these two treatments. The PCA scores plot of the four groups demonstrated significant lipid-lowering effects of EGB against hyperlipidemia in the rat model.

The preventive effect of EGB was illustrated using K3, K4, M3, M4, Y3, and Y4 in Figure 3. The first plot in Figure 3, the

principal components (PCs) plot, indicated that a two-component PCA model provided goodness of fit and predictive ability over 90% of the dataset. Samples K3, K4, M3, M4, Y3, and Y4 were classified into three groups based on characteristics of the diet and treatment for each group in the PCA scores plot in Figure 3. These three groups were observed to be clearly separated from each other and gathered in three different regions in the plot. Samples K3 and K4 clustered within the right lower quadrant, whereas samples M3 and M4 gathered within the left lower quadrant. As such, the rats in the two groups had two different metabolic statuses. Samples Y3 and Y4 were located between samples K3–4 and M3–4 in the upper part of the plot, indicating that the rats in the EGB-prevention group had a metabolic status between those of the other two groups. As revealed above, the diet-induced hyperlipidemia model was completely set up at week 3 in the rats. Thus, EGB was proven to have a preventive effect against hyperlipidemia, which was in agreement with the assay kit results. From the scores plot, three groups were located distinctly away from each other. Likewise, in the PCA loadings plot, variables responsible for the classification in the scores plot were identified to explain the biochemical significance of the clustering. In the corresponding loadings plot, the metabolites responsible for the classification in the scores plot were position-correlated. When the rat hyperlipidemia model was set up, the elevated levels of cholesterol, butyrate, creatinine, acetate, ornithine, proline and others, and the decrease of erythrose, *D*-mannopyranoside, lysine, tyrosine, oleate, mannose and others were observed in the loadings plots when compared with those of the control rats. In the EGB-prevention group, the levels of cholesterol, creatinine, acetate and other metabolites were prevented from increasing. Changes of metabolite levels are shown in Table 7.

PCA plots of the EGB-treatment group at different time points during the eight-week experiment are shown in Figure



**Figure 3.** PCA plots of samples from the control (K), diet-induced hyperlipidemia (M) and EGB-prevention groups (Y) at weeks 3 and 4. (1) PCA principal components plot; (2) PCA scores plot; (3) PCA loadings plot.

**Table 7.** Significant alterations of endogenous metabolites detected by GC/MS.

Molecule	Biological role	Fold change		
		A	B	C
Ornithine	Amino acid produced in the urea cycle from arginine	-	0.6	-
Tyrosine	A precursor for epinephrine	0.58	1.72	-
Proline	Non-essential amino acid synthesized from glutamic acid	-	0.7	-
Citric acid	Tricarboxylic acid cycle	0.68	0.31	-
Glucose	Primary source of energy, available from glycogenolysis and gluconeogenesis or food	1.8	1.5	-
9,12-Octadecadienoic acid (Linoleate, LA)	Essential fatty acid	0.72	0.13	-
D-Sorbitol	Component of fructose and mannose metabolism	1.37	1.60	-
Galactose	Component of glycerolipid and glycosphingolipid metabolism	1.71	0.64	-
Hexadecanoic acid (palmitic acid)	Saturated fatty acids	1.81	-	0.40
Creatinine	An indicator of renal function	4.10	0.48	0.48
Acetic acid	A major source of acetyl group	5.52	-	0.30
Cholesterol	Biomarker of hyperlipidemia	8.25	0.70	0.55
Butyric acid		-	0.4	0.3
Arachidonic acid (AA)	Precursor of prostaglandins	-	0.53	0.44

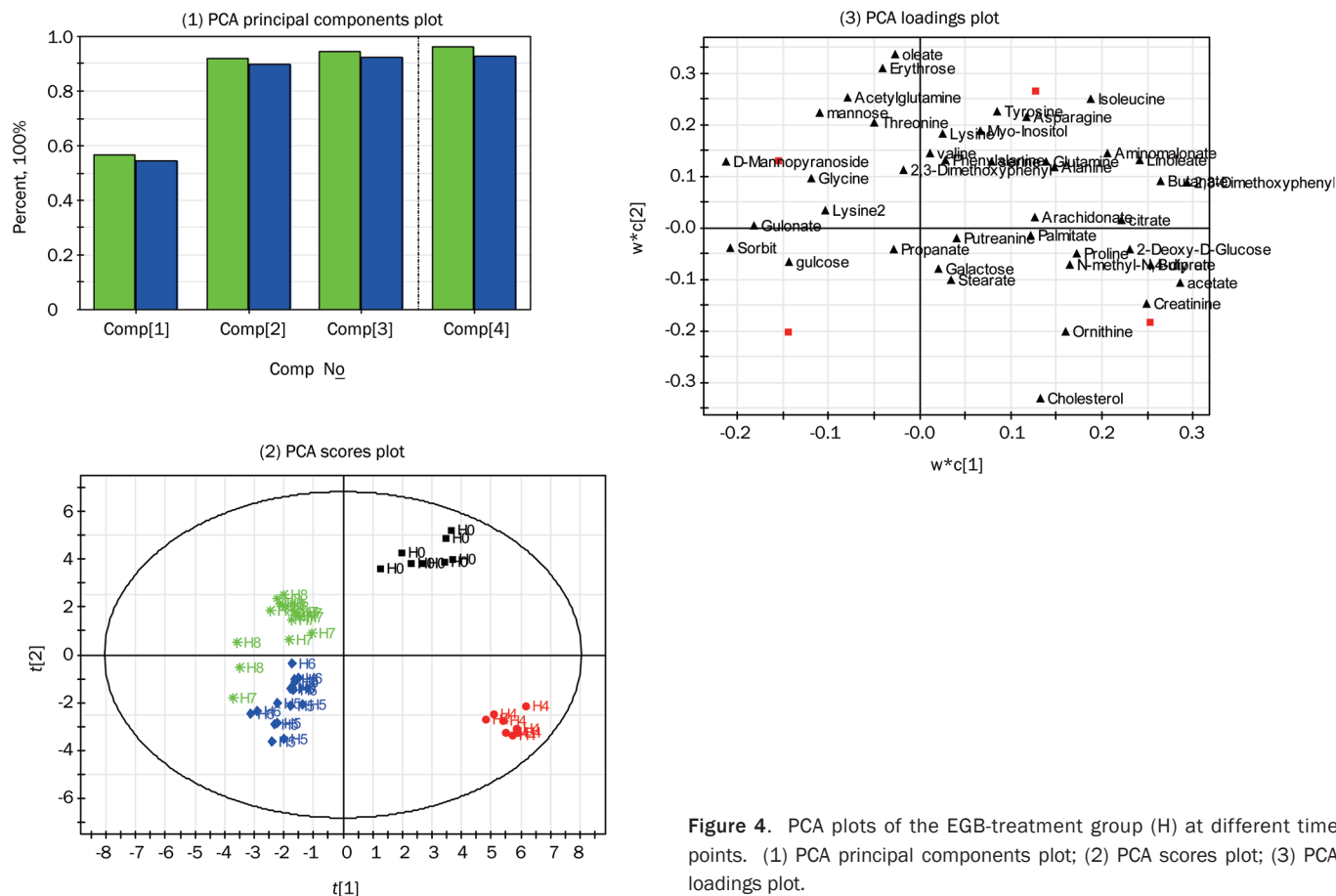
A: Ratio of abundance of metabolites in plasma samples between diet-induced hyperlipidemia group and control group at week 4.

B: Ratio of abundance of metabolites in plasma samples between EGB-prevention group and diet-induced hyperlipidemia group at week 4.

C: Ratio of abundance of metabolites in plasma samples between EGB-treatment group and diet-induced hyperlipidemia group at week 8.

All molecules differ significantly ( $P < 0.05$ ) between treatment groups. Unless otherwise noted, biological role annotations in this table were found using the Human Metabolome Database: www.hmdb.ca





**Figure 4.** PCA plots of the EGB-treatment group (H) at different time points. (1) PCA principal components plot; (2) PCA scores plot; (3) PCA loadings plot.

4. In the PCA scores plot, the fact that H4 samples moved far away from H0 samples indicated that the hyperlipidemia rat model exhibited a distinct metabolic profile compared with that of normal rats. After EGB treatment, the position of sample H4 had changed from the right lower quadrant to the left lower one, where samples H5, H6, H7, and H8 were placed. Clearly, the positions of samples H5, H6, H7, and H8 moved slowly toward and approached those of sample H0, indicating that the metabolic profile of the rats in EGB-treatment group gradually reverted to normal status through EGB treatment. In the corresponding PCA loadings plot, the decrease of cholesterol, butyrate, creatinine, acetate, ornithine and proline and the increase of erythrose, *D*-mannopyranoside, tyrosine, lysine, oleate and other metabolites were observed after a 4-week EGB treatment. Interestingly, the changes of these endogenous metabolites in diet-induced hyperlipidemia rats were in the opposite directions. These endogenous metabolites may exert a direct or indirect relationship with the lipid-regulating effects of EGB.

## Discussion

### Mechanism of action of EGB against hyperlipidemia

One of the major therapeutic indications of EGB is its effects on improving central nervous system (CNS) impairments, including age-associated impairments of memory, attention

and other cognitive functions<sup>[7, 28, 29]</sup>. The common pathogenetic mechanisms of both dementia and cardiovascular diseases (eg, inflammation, generation of free radicals) indicate a causal link<sup>[30, 31]</sup>. A standardized garlic-ginkgo preparation was found to lower total cholesterol under dietary conditions in a randomized, placebo-controlled, double blind clinical trial<sup>[11]</sup>. EGB treatment was reported to decrease the capacity of LDL to carry free cholesterol to various tissues without affecting the capacity of HDL to carry cholesterol back to the liver in rats<sup>[9]</sup>. In addition, EGB treatment can partially reverse ethanol-induced dyslipidemia at dose levels of 48 and 96 mg/kg in rats by reducing the lipid peroxidation induced by ethanol<sup>[13]</sup>.

In our study, EGB treatment successfully prevented hyperlipidemia in the levels of TG, HDL-C, and LDL-C caused by a high-lipid diet in rats. Furthermore, EGB reversed TC levels and partially improved HDL-C and LDL-C levels in rats with diet-induced hyperlipidemia mediated by a 4-week EGB treatment in our study. As such, EGB may have preventive and therapeutic effects against hyperlipidemia and thus atherosclerosis. The inhibitory effects of EGB on atherosclerosis have been attributed to the upregulation of radical scavenging enzymes and attenuation of the risk factors of oxidized LDL/LDL and lipoprotein(a)<sup>[32, 33]</sup>. Quercetin, a major flavonoid component of EGB, was identified to promote an increase of

fecal sterols, thereby leading to limitations in the absorption of dietary cholesterol, and to lower plasma and hepatic cholesterol levels by inactivating  $\beta$ -hydroxy-methyl-glutaryl CoA (HMG CoA) reductase<sup>[34]</sup>.

#### EGB effect on LPL, HL, and FBA

The plasma activity of lipoprotein lipase (LPL) and hepatic lipase (HL) were decreased significantly in rats with diet-induced hyperlipidemia, while they were increased significantly in rats simultaneously or sequentially fed with a high-lipid diet and EGB. HL and LPL hydrolyze triglycerides and phospholipids present in circulating plasma lipoproteins. LPL hydrolyzes triglycerides from chylomicrons and very low-density lipoproteins, whereas HL catalyzes the metabolism of chylomicron remnants, intermediate density lipoproteins and high-density lipoproteins<sup>[35, 36]</sup>. Increased activity of LPL and HL would promote the metabolism of total cholesterol, including triglycerides. In addition, the excretion of FBA was observed to be increased significantly in rats simultaneously and sequentially fed with a high-lipid diet and EGB. This increased excretion of bile acid in feces might be associated with EGB activating the important enzyme,  $7\alpha$ -hydroxylase, in the conversion of cholesterol into bile acids<sup>[37, 38]</sup>.

Decreased FBA excretion has been found in patients with coronary atherosclerosis, even with plasma cholesterol levels comparable to healthy controls<sup>[39]</sup>. Patients with coronary atherosclerosis are unable to excrete adequate amounts of bile acids to reduce cholesterol overload. Based on the findings in our experiments, EGB has the potential to help produce and excrete large amounts of bile acids to get rid of excess cholesterol and protect against atherogenesis in humans.

#### Effects on energy metabolism

High levels of cholesterol could enhance amyloid beta ( $A\beta$ ) peptide production and deposition<sup>[12, 40]</sup>.  $A\beta$  deposition, in turn, increases extracellular glutamate levels mediated by inducing microglia to release glutamate and by impeding glutamate uptake and conversion to glutamine by astrocytes<sup>[40]</sup>. Excessive glutamate, if accumulated outside cells, could cause excitotoxicity and initiate irreversible cell necrosis<sup>[41]</sup>. EGB has been found to inhibit  $\beta$ -amyloid production and amyloidogenesis by lowering free cholesterol levels<sup>[9]</sup>, which indirectly reduces glutamate excitotoxicity. In our study, n-glutamine and glucose levels were increased, whereas ornithine and proline levels were decreased in rat plasma in the EGB-prevention group compared with the diet-induced hyperlipidemia group. This indicates that EGB might promote conversion of glutamate to n-acetylglutamine via glutamine. Decreased levels of proline and ornithine revealed that EGB might inhibit the glutamate/P5C synthase pathway via inactivating P5C synthase or other enzymes involved<sup>[42-44]</sup>. In addition, elevated glucose levels might be caused by EGB regulating the oxidation of excessive glutamate to  $CO_2$  or glucose and the synthesis of hexosamines by inhibiting the transfer of the amide nitrogen of glutamine to fructose 6- $PO_4$  required by glucosamine 6- $PO_4$ <sup>[42, 45, 46]</sup>.

The D-sorbitol level was increased in rats both from the diet-induced hyperlipidemia group compared with the control group and from the EGB-prevention group when compared with the diet-induced hyperlipidemia group. Sorbitol is a carbohydrate that can be found in fruits and be synthesized from glucose and converted to fructose during metabolism in human liver<sup>[27]</sup>. Although sorbitol is one of the minor components in *Ginkgo biloba*<sup>[10]</sup>, the fold change observed for sorbitol levels in the diet-induced hyperlipidemia group compared with the control group indicated that elevated levels might be produced from glucose. Elevated sorbitol pathway activity can result in oxidative stress inside cells and tissues, leading to diabetic and atherogenic complications<sup>[47-49]</sup>. Elevated sorbitol levels in rat plasma were found without the increased levels of fructose and uric acid observed in the EGB-prevention group. EGB may inactivate NAD-dependent sorbitol dehydrogenase to inhibit sorbitol conversion to fructose, which is a 10 times more potent glycosylation agent than glucose. Fructose can increase uric acid levels by activating the enzymes involved in the degradation of purine nucleotides<sup>[50, 51]</sup>. Serum uric acid is an independent predictor for death in patients with cardiovascular disease<sup>[52]</sup>. EGB could prevent elevated levels of fructose and uric acid by accumulating the relatively less toxic sorbitol in plasma when EGB was co-administered with a high-lipid diet in rats. When EGB was given with a normal diet in the EGB-treatment group, no significant changes in sorbitol concentrations were observed compared with the hyperlipidemia group. EGB exhibited different modulating effects at different stages of this metabolic syndrome.

Galactose levels were increased in rat plasma in the diet-induced hyperlipidemia group compared with the control group, reduced to normal levels in the EGB-prevention group and decreased further in the EGB-treatment group. This indicated that EGB had a galactose-lowering effect. Galactose and glucose are associated with the intestinal absorption of cholesterol by regulating the intestinal cholesterol transporter hSR-BI<sup>[53]</sup>. There are two sources of galactose available to humans<sup>[54]</sup>. One is nutritional supply from EGB, where galactose is a component<sup>[55, 56]</sup>. The other source is from glucose. EGB may regulate enzymes involved in galactose metabolism to amino acids to lower galactose levels<sup>[54]</sup>. This was evidenced by changes of citric acid levels parallel to those of galactose, but changes of alanine levels opposite to those of galactose. Further experiments are warranted to identify the underlying mechanisms of the EGB lipid-lowering and galactose-lowering effects.

#### Fatty acid metabolism

Linoleic acid (ie linoleate, LA) is an essential polyunsaturated fatty acid (EFA) *in vivo* because it cannot be synthesized by humans<sup>[57]</sup>. It is a precursor of prostaglandins (PGs) and leukotrienes via the arachidonic acid (AA) pathway<sup>[43, 48]</sup>. Linoleic acid levels were found to be decreased in the EGB-treatment group when compared with the diet-induced hyperlipidemia group. The optimal levels of AA and eicosapentaenoic acid and docosahexaenoic acid derived from linolenic acid are

needed in tissue in order to maximize the beneficial formation of eicosanoids, lipoxins and resolvins to prevent atherosclerosis<sup>[58]</sup>. PGs (*eg*, PGL<sub>2</sub>, TXA<sub>2</sub>) derived from AA show multiple beneficial effects against cardiovascular risks, including reducing blood pressure and inhibiting platelet aggregation<sup>[59]</sup>. The decreased level of LA can be explained via the mechanism of LA being converted to PGs to enhance cardiovascular protection. However, LA is the most potent EFA in terms of reducing plasma TC and LDL-C<sup>[60]</sup>. Drieu *et al* claimed that EGB treatment might reduce lipid peroxidation and thereby increase circulating LA and AA levels in rats after a 10-day treatment with EGB<sup>[61]</sup>. Our findings on the decreased levels of LA in this study suggest that EGB treatment promoted LA being converted to PGs via the AA pathway after a 4-week high-lipid diet followed by EGB treatment. The effect exerted from EGB in the body is not a specific unidirectional action, but rather regulatory, in which it helps the body adapt to circumstances<sup>[62]</sup>. Likewise, homeostasis of cholesterol and LA metabolism could be reached via optimizing the beneficial effects of endogenous metabolites in the body when EGB is administered. This homeostasis revealed in our study favors a decreased level of circulating LA.

AA levels were observed with no significant alterations in rat plasma when comparing the diet-induced hyperlipidemia group with the control group, whereas AA levels were reduced in the EGB-prevention and treatment groups compared with the diet-induced hyperlipidemia group. Harris *et al* found that AA concentrations were 8.5% lower in tissues in patients with coronary heart disease compared with control subjects<sup>[63]</sup>. Loustarinen *et al* observed that the percentage of palmitic acid and LA were significantly higher, but the percentage of AA was significantly lower in the total phospholipid fraction of human coronary arteries of those with sudden cardiac death caused by coronary heart disease<sup>[64]</sup>. Therefore, a deficiency of AA levels, not an excess of AA levels, may contribute to coronary heart disease<sup>[65]</sup>. Kudolo *et al* claimed that EGB reduced lipid peroxidation mediated by inhibiting cyclooxygenase-1-mediated AA oxygenation or reducing the AA pool<sup>[66]</sup>. In our study, EGB decreased free AA concentrations in rat plasma in both the EGB-prevention and treatment groups compared to the diet-induced hyperlipidemia group. Also, EGB decreased palmitic acid concentrations in rat plasma in the EGB-treatment group when compared with the diet-induced hyperlipidemia group. Palmitic acid was shown to increase LDL-C levels in hamsters<sup>[67]</sup>. EGB regulated cholesterol plasma profiles in rats mediated partly by decreasing palmitic acid levels to lower LDL-C concentrations.

EGB exerted preventive and therapeutic effects against hyperlipidemia and related cardiovascular and diabetic diseases by favorably regulating profiles of EFA (*eg*, LA, AA, palmitic acid). Further experiments are warranted to explore the underlying mechanisms of action of EGB on EFA.

#### Relationship between lipid disorder and renal damage

Moorhead *et al* has proposed that the progression of renal disorders depends partially on the damage inflicted on the glom-

erulus by lipoproteins<sup>[68]</sup>. Dyslipidemia could contribute to kidney damage (*eg*, glomerulosclerosis)<sup>[69,70]</sup>. Creatinine is usually produced at a relatively constant rate by the human body. Although serum creatinine is a commonly used indicator of renal function, a rise in serum creatinine levels is observed only when marked damage happens in functioning nephrons. Therefore, increased levels of plasma creatinine observed in the diet-induced hyperlipidemia group might indicate renal damage caused by hyperlipidemia. Likewise, decreased plasma levels of creatinine in the EGB-treatment group might indicate that EGB prevented renal damage caused by lipid disorders in our study.

#### Biomarkers of hyperlipidemia identified in our metabonomic study

The metabolites identified in our metabonomic study might further explain the mechanisms of action of EGB against hyperlipidemia. Cholesterol is a putative biomarker and risk factor in hyperlipidemia. After the hyperlipidemia rat model was built with a 4-week high-lipid diet, the cholesterol levels were significantly increased in rats from the diet-induced hyperlipidemia group compared with those of the control group. With EGB administration in either the prevention or treatment group, the cholesterol levels were significantly decreased when using both the assay kit and the metabonomic study.

Tyrosine might be another biomarker for hyperlipidemia. As epinephrine is known to promote lipid metabolism, epinephrine consumption would be increased when a high-lipid diet is ingested. Epinephrine is derived from phenylalanine and tyrosine. Likewise, tyrosine consumption might be increased when epinephrine is demanded by high lipid metabolism. However, *in vivo* tyrosine is obtained only from the diet. As a result, the tyrosine levels were observed to be decreased in the diet-induced hyperlipidemia group, indirectly due to excessive lipid metabolism. Conversely, the tyrosine level was increased after the administration of EGB. This indicated that EGB might activate other pathways for lipid metabolism (*eg*, cholesterol metabolized into bile acid excreted in feces) instead of the pathway using epinephrine. This is evidenced by increased levels of tyrosine in healthy human urine after a daily 240 mg dose of EGB 761 was administered for three weeks in a randomized, placebo-controlled, double-blind clinical trial<sup>[71]</sup>. EGB was hypothesized to inhibit catecholamine degradation to decrease the blood flow measured in the trial. Catecholamines, including norepinephrine, epinephrine and dopamine, are derived from tyrosine<sup>[71]</sup>. Thus, inhibiting catecholamine degradation by EGB might cause elevated levels of tyrosine in excretion<sup>[71]</sup> and circulation, as evidenced in our study.

Excessive triglycerides ingested through diet are hydrolyzed to glycerin and fatty acids. Glycerin is oxidized to dihydroxyacetone phosphate (DHAP), and DHAP is isomerized to glyceraldehyde phosphate. Further, glyceraldehyde phosphate can be converted to glucose by glyconeogenesis, which might explain the high levels of glucose in plasma in the diet-

induced hyperlipidemia group. The flavonoid fraction in EGB has been found to lower blood glucose in rats<sup>[72]</sup>. Cholesterol synthesis could be inhibited by high levels of exogenous cholesterol, due to the fact that HMG-CoA reductase (the rate-limiting enzyme in cholesterol synthesis) is limited by high concentrations of cholesterol. As such, acetyl-CoA used for cholesterol synthesis *in vivo* might be excessive. Meanwhile, acetyl-CoA is also generated via a  $\beta$ -oxidation route from fatty acids. The excessive acetyl-CoA might be converted to ketone bodies, such as acetoacetate, D- $\beta$ -hydroxybutyric acid and acetone. D- $\beta$ -hydroxybutyric acid in rat plasma in the diet-induced hyperlipidemia group was found to increase, which might be associated with this metabolic process. Acetyl-CoA can be converted to acetic acid as catalyzed by acetyl-CoA hydrolase. This explains the fact that acetic acid levels were increased in the diet-induced hyperlipidemia group. In addition, the acetate level was high in the diet-induced hyperlipidemia group, which suggested a decrease in its metabolism to acetyl-CoA<sup>[73]</sup>. Acetate is also produced from glycerolphospholipid and pyruvate metabolism<sup>[73]</sup>. This might cause decreased levels of threonine and glycine in the diet-induced hyperlipidemia group. However, the acetone generated in such a process was not observed, which should be evaluated in a future study.

## Conclusions

The findings in this study revealed the preventive and therapeutic efficacies of EGB against hyperlipidemia by employing GC/MS-based metabolomic profiling combined with multivariate statistical analysis, as well as classical pharmacology studies using assay kits. Additionally, several potential biomarkers identified in this study might help explain the metabolic effects of hyperlipidemia and the mechanisms of action of EGB against hyperlipidemia. Overall, EGB exhibited regulatory and adaptive effects when used to treat hyperlipidemia. CTM often contains compounds that act synergistically, or opposite in ways, which interactively regulate effects at safe and effective levels in humans. When TCM is introduced in the body, metabolic homeostasis will be reached *in vivo* according to the combined effects of each molecule in the complex natural mixture. As such, TCM might have effects on numerous metabolic pathways based on the molecules contained in the mixture. In addition to conventional pharmacology methods used to assess an isolated single compound, the preventive and therapeutic effects of TCM need to be fairly and fully evaluated using methods of systems biology, including metabolomics. State-of-the-art mass spectrometry (*eg*, LC-MS/MS, GC-MS/MS) and/or NMR and multivariate analysis were incorporated to provide suitable means to explore the systemic mechanisms of action of TCM.

The lipid-lowering effect of EGB may provide treatment or prevention solutions for not only hyperlipidemia, but also other diseases (*eg*, dementia). Translational research bridging animals and humans is warranted to fully evaluate EGB's lipid-lowering effects in naïve and diseased states. Understanding how each and multiple active agents in EGB contribute to

its combined pharmacological effectiveness would facilitate applying EGB in the clinic.

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## Author contribution

Qi ZHANG designed research and wrote the paper; Guang-ji WANG designed research; Ji-ye A analyzed data; Di WU revised and wrote the paper; Ling-ling ZHU, Bo MA, and Yu DU performed research.

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