1 **Metabolomic profiling reveals effects of marein on energy metabolism in**
2 **HepG2 cells**

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16 **Running Head: Metabolomic profiling reveals effects of marein**

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26 **Abstract:**

27 Previous studies have suggested that *Coreopsis tinctoria* improves insulin
 28 resistance in rats fed with high-fat diet. But little is known about the
 29 antidiabetic effects of marein which is the main component of *C. tinctoria*. This
 30 study investigated the effects of ethyl acetate extract of *C. tinctoria* (AC) on
 31 insulin resistance (IR) in rats fed a high-fat diet. High glucose and fat
 32 conditions cause a significant increase in blood glucose, insulin, serum TC, TG
 33 and LDL-C, leading to an abnormal IR in rats. However, treatment with AC
 34 protects against HFD-induced IR by improving fasting serum glucose and lipid
 35 homeostasis. High glucose conditions cause a significant decrease in
 36 glycogen synthesis and increases PEPCCK and G6Pase protein levels and
 37 Krebs-cycle-related enzymes levels, leading to an abnormal metabolic state in
 38 HepG2 Cells. However, treatment with Marein improves IR by increasing
 39 glucose uptake and glycogen synthesis and by downregulating PEPCCK and
 40 G6Pase protein levels. The statistical analysis of HPLC/MS data
 41 demonstrates that Marein restores the normal metabolic state. The results
 42 show that AC ameliorates IR in rats and Marein has the potential effect in
 43 improving IR by ameliorating glucose metabolic disorders.

44 **Keywords:** Metabolomics; Marein; insulin resistance; metabolites; Krebs
 45 cycle

46 **Abbreviations:** AC, ethyl acetate extract of *Coreopsis tinctoria*; TCA,
 47 Tricarboxylic acid; HepG2, hepatocellular carcinoma cell line; 2-NBDG, 2-(N-
 48 (7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose; G6Pase, glucose-

6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; IR, insulin resistance; HFD, high-fat diet; SDHA, succinate dehydrogenase flavoprotein subunit; ACO2, aconitase 2; IDH2, isocitrate dehydrogenase 2; CS, citrate synthase; FH, fumarate hydratase; MDH2, malate dehydrogenase; DLST, dihydrolipoamide S-succinyltransferase.

Introduction

Type 2 diabetes (T2DM) is a progressive disease characterized by deterioration of glycaemia and escalating therapeutic complexity ^[1]. In diabetes mellitus, chronic hyperglycemia develops as a consequence of decreased insulin action from impaired insulin secretion and insulin resistance (IR) ^[2, 3]. Once chronic hyperglycemia is established, it in turn aggravates IR, forming a vicious cycle that is collectively called glucose toxicity^[4, 5]. The liver is the primary organ responsible for regulating glucose homeostasis ^[6]. Hepatic IR leads to altered glucose metabolism and hyperglycemia, which is characterized by the inability of insulin to inhibit hepatic gluconeogenesis by suppressing unidirectional enzymes, namely, phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G-6Pase)^[7, 8]. G-6Pase plays a role in glucose homeostasis ^[9]. PEPCK is a key rate-limiting enzyme of gluconeogenesis. The activities of G-6Pase and PEPCK are increased significantly in the liver of diabetic rats ^[8]. Inhibition of G-6Pase and PEPCK enzymes may interfere with gluconeogenesis and can be useful in treating diabetic hyperglycemia ^[10-13]. In the liver mitochondrial PEPCK (PEPCK-M)adjoins its profusely studied cytosolic isoform (PEPCK-C) potentiating gluconeogenesis and TCA flux, so hepatic PEPCK is required to sustain the

74 Krebs cycle ^[14]. In addition, the Krebs cycle completes the oxidation of
75 glucose and plays an important role in the glucose metabolism. Therefore,
76 methods that enable simultaneous measurement of numerous cellular
77 metabolic intermediates in gluconeogenesis and the Krebs cycle are required
78 to elucidate the mechanisms of glucose metabolism disorders.

79 Metabolomics is a top-down systems biology approach whereby metabolic
80 responses to biological interventions or environmental factors are analyzed
81 and modeled ^[15]. Metabolomics measures perturbations in metabolites
82 reflecting changes of metabolism caused by environmental factors, and
83 provides insights into the global metabolic status of the entire organism by
84 monitoring the entire pattern of low molecular weight compounds rather than
85 focusing on an individual metabolic intermediate ^[16]. Deregulations of
86 metabolic processes are expected to be directly or related with relevant
87 disease end-points, which are represented by the levels of metabolites ^[17].
88 Recent longitudinal metabolomic studies have found correlations between
89 circulating metabolites and prediabetes, future development of IR, or type 2
90 diabetes in humans. For example, increasing in circulating aromatic amino
91 acids (AAAs) and branched-chain amino acids (BCAAs) are biomarkers of risk
92 [9, 18, 19].

93 *Coreopsis tinctoria* Nutt. (Asteraceae), a traditionally used preparation for
94 diabetes treatment in Portugal ^[20, 21], is a plant native to North America that
95 has spread worldwide. Our previous study showed that *C. tinctoria* increases
96 insulin sensitivity and regulates hepatic metabolism in rats fed a high-fat diet
97 ^[22]. Since these activities are closely related to metabolic regulation and IR, we
98 tried to identify the protective effect of ethyl acetate extract of *C. tinctoria* Nutt

(AC) on IR, and its possible mechanism of action. Chalcones (okanin and butein derivatives) are the main constituents of ethyl acetate extract of *C. tinctoria* and among them, identified Marein (okanin-4'-O- β -D-glucopyranoside) as the main metabolite [23]. Marein has many beneficial biological activities, including antihyperlipidemic [24], antioxidative [25], antidiabetic [26], and antihypertensive effects [27]. Previous research has also found that Marein prevented tert-Butyl-Hydroperoxide and cytokine induction in a mouse insulinoma cell line (MIN6) through the inhibition of the apoptotic signaling cascade [20]. In addition, Marein promotes pancreatic function recovery in streptozotocin-induced glucose-intolerant rats [26, 28]. Our previous studies found that Marein could be the main active compounds of *C. tinctoria* in improving IR [29]. The protective effects of marein on high glucose-induced metabolic disorder via IRS1/AKT/AS160 signal pathway in HepG2 cells [30]. These findings focused on *in vivo* anti-diabetic effect led us to further investigate the underlying mechanism, as to our knowledge no studies on the mechanisms of metabolic disorder of Marein have been reported, especially on energy metabolic level.

In this study, we employed LC/MS-based metabolomics techniques to explore the potential targets and mechanisms of Marein for the attenuating IR and type 2 diabetes. Our study revealed involvement of glucose metabolism and the Krebs cycle in the protective effects of Marein in a high-glucose and fat-induced IR model.

Materials and methods

Materials

124 The human liver hepatocellular carcinoma cell line HepG2 was purchased
125 from the Cell Bank of the Chinese Academy of Sciences (Beijing). Dulbecco's
126 modified Eagle's medium (DMEM), fetalbovine serum (FBS), and other tissue
127 culture reagents were purchased from Gibco (Life Technologies, USA). Other
128 reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA)
129 unless otherwise indicated. AC was analyzed by HPLC (Waters 2690) with a
130 diode-array detector (Waters 2487) scanning from 200–600 nm. Ethyl acetate
131 extract was separated by Shim-pack VP-ODS column (150×4.6 mm, 5 μm)
132 with the optimum condition as described previously^[28, 31]. Marein was
133 extracted from *C. tinctoria* Nutt. by our lab. And we also purchased the
134 compound Marein from Chromadex (00013126-604, California, USA), and the
135 purities exceeded 99%. Deionized water was used in all experiments. All of
136 the other chemicals and reagents were of analytical grade. The following
137 antibodies were used in immunoblotting experiments and at the indicated
138 dilutions: rabbit polyclonal anti-PCK1 (PEPCK) (ab28455, 1:1000), rabbit
139 polyclonal anti-G6Pase (ab83690, 1:1000), rabbit monoclonal anti- aconitase
140 2 (ACO2) (ab129069, 1:10000), rabbit monoclonal anti- malate
141 dehydrogenase (MDH2) (ab181857, 1:10000), mouse monoclonal anti-
142 isocitrate dehydrogenase 2 (IDH2) (ab184196, 1:1000), mouse monoclonal
143 anti- fumarate hydratase (FH) (ab113963, 1:50000), rabbit polyclonal anti-
144 citrate synthase (CS) (ab96600, 1:1000), mouse monoclonal anti- succinate
145 dehydrogenase flavoprotein subunit (SDHA) (ab14715, 1:10000), rabbit
146 monoclonal anti-SDHB (ab178423, 1:5000, abcam, UK), and anti-β-actin
147 antibody (1:1000, CW0096A, CWBio). Dihydrolipoamide S-
148 succinyltransferase (DLST)

149 ***Animals and drug treatments.***

150 Sprague Dawley rats were randomly divided into six groups of 10 animals
151 each. The control group that was given deionized distilled water to drink and
152 fed standard rat chow 32 composed of 60% vegetable starch, 12% fat, and
153 28% protein. The high-fat diet (HFD) model group^[32] that was given deionized
154 distilled water and fed a high fat diet of 60% fat, 14% protein and 26%
155 carbohydrate. The metformin group was administrated with 200 mg/kg of
156 metformin as a positive control by oral gavage and fed a HFD. The rats in the
157 AC three groups were as follows: low dose of AC (150 mg/kg of body weight +
158 HFD), middle dose of AC (300 mg/kg of body weight + HFD), high dose of AC
159 (600 mg/kg of body weight + HFD). The study was approved by the Ethics
160 Committee of the Institute of Medicinal Plant Development, CAMS&PUMC
161 (Beijing, China). All experimental procedures were performed in accordance
162 with relevant guidelines approved by the Ethics Committee of the Institute of
163 Medicinal Plant Development, CAMS&PUMC.

164

165 ***Cell culture and drug treatment***

166 HepG2 cells were cultured in low glucose DMEM (5.5 mmol/L glucose) that
167 was supplemented with 10% FBS and 1% antibiotics at 37°C in humidified air
168 containing 5% CO₂. Cells in the exponential phase of growth were used in all
169 of the experiments. HepG2 cells were grown for 3 days and then divided into
170 different groups for the treatments. The treatment groups (Marein, dissolved
171 in DMSO, not more than 0.5 percent) were as follows: (1) Control: incubated
172 in low glucose DMEM for 72 h; (2) High glucose treatment: incubated in
173 DMEM containing 55 mmol/L glucose for 72 h; (3-6) Marein and glucose

174 treatment: incubated in DMEM containing Marein (40, 20, 10, or 5 μ mol/L) or
175 0.5 mmol/L metformin (Positive control) for 24 h and then incubated in
176 DMEM containing 55 mmol/L glucose for 72 h. 100 nmol/L insulin was added
177 into the all treated group for 30 min before all experiments.

178 ***Detection of glucose uptake***

179 Glucose uptake rate was measured by adding 2-(N-(7-nitrobenz-2-oxa-1,
180 3-diazol-4-yl) amino)-2-deoxyglucose, a fluorescently labeled deoxyglucose
181 analog (2-NBDG, Cayman Chemical), as a tracer to the culture medium as
182 previously reported ^[33]. 2-NBDG uptake was then measured after stimulating
183 the cells for 15 min with 1×10^{-7} mol/L insulin as our previous described³¹. The
184 cells treated by drugs were then washed twice and incubated with 100 μ mol/L
185 of 2-NBDG in glucose-free culture medium for 20 min. The cells cultured in
186 medium without 2-NBDG were used as a negative control. The cells were
187 washed twice prior to fluorescence detection using a microplate reader
188 (Infinite 1000 M, Tecan, AUSTRIA) with fluorescence excitation at 488 nm
189 and emission detected at 520 nm.

190 ***Determination of ATP, pyruvate and lactate concentrations***

191 The cellular ATP content was detected by using an ATP content kit
192 (S0026, Beyotime Institute of Biotechnology, China). Briefly, the cell pellets
193 were collected by centrifugation at 1000 $\times g$ for 5 minutes after digestion with
194 0.25% trypsin. The homogenate was incubated in boiling water for 30
195 minutes, and the supernatant was aspirated after centrifugation at 5000 $\times g$ for
196 10 minutes. An ATP assay kit was used for all of the samples. The results
197 were analyzed with a luminometer (Fluoroskan Ascent FL, USA).

198 The cellular lactate and pyruvate concentration was detected by using a

lactate assay (A019-2, Nan Jing Jian Cheng Bioengineering Institute, Nan
Jing City, China) and pyruvate assay kit (A081, Nan Jing Jian Cheng
Bioengineering Institute, Nan Jing City, China) respectively. Briefly, cell pellets
were collected by centrifugation at 500 $\times g$ for 10 minutes after scraping the
cells with a cell scraper. Cells were washed twice with PBS, then
resuspended in PBS and lysed with a sonicator. The homogenate was
detected using a UV/Visible Spectrophotometer (Lambda 35, Perkin Elmer,
USA).

RNA extraction and quantitative real-time PCR (qPCR)

Cells treated with high glucose or Marein were then collected to isolate
total RNA. Total RNA was reverse transcribed, and *PEPCK*, *G6Pase*, *FH*,
MDH2, *SDHA*, *SDHB*, *IDH2*, *ACO2*, *CS* and *GAPDH* gene expression was
measured by QPCR using iCycler thermocycler (BioRad, Hercules, CA) as
previously described^[34]. The primers of these genes are listed in the Table 1.
All samples were analyzed in triplicate, and the gene expression levels were
normalized to control human *GAPDH* values. The relative expression of
treated samples was normalized to that of untreated cells according to ΔCt
analysis.

Western blot analysis

All immunoblots were of the cell lysate with 1×10^7 cells per sample with
RIPA cell lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% Na-
deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA and 50 mM NaF).
PEPCK and *G6Pase* were detected with anti-*PEPCK* and anti-*G6Pase*
antibodies respectively, both used at a dilution of 1:1000. *FH*, *MDH2*, *SDHA*,
SDHB, *ACO2*, *CS*, *IDH2* and *GAPDH* were detected by anti-*FH*, anti-*MDH2*,

224 anti-SDHA, anti-SDHB, anti-ACO2, anti-CS, anti-IDH2 and anti-GAPDH for 4h
225 following the instructions respectively. The secondary antibodies goat anti-
226 mouse IgG and goat anti-rabbit IgG were peroxidase-conjugated at a dilution
227 of 1:10000 and 1:20000 respectively. Immune complexes were visualized with
228 ECL chemiluminescence (GE Healthcare, Little Chalfont, UK). Densitometric
229 analyses were performed with ImageJ2 software for Microsoft Windows.

230 ***Metabolite profiling***

231 Metabolite profiling was performed as previously reported ^[35]. Targeted
232 metabolomic experiment was analyzed by TSQ Quantiva (Thermo, CA). C18
233 based reverse phase chromatography was utilized with 10mM tributylamine,
234 15mM acetic acid in water (pH ~6) and 100% methanol as mobile phase A and
235 B respectively. This analysis focused on tricarboxylic acid cycle, glycolysis
236 pathway, pentose phosphate pathway, amino acids and purine metabolism. In
237 this experiment, we used a 25-minute gradient from 5% to 90% mobile B.
238 Positive-negative ion switching mode was performed for data acquisition.
239 Cycle time was set as 1 second and a total of 138 ion pairs were included.
240 The information of partial ion pairs are listed in Table 2. The resolution for
241 quartile 1 and quartile 3 are both 0.7FWHM. The source voltage was 3500v
242 for positive and 2500v for negative ion mode. Sweep gas was turned on at
243 1(arb) flow rate. In the experiments, metabolites were extracted from 1×10^7
244 HepG2 cells and finally redissolved in 80 μ l of 80% methanol. One microliter
245 of sample was loaded for targeted metabolomics analysis. We used specific
246 sample as quality control (QC) instead of internal standard. Total ion current
247 and chromatographic patterns were evaluated. And Table 3 shows three
248 representative compounds in QC runs.

249 **Statistical analysis**

250 The MS raw data of cellular extracts was processed using Thermo
251 Xcalibur (Thermo, USA). Analyzed spectral data was transformed to contain
252 aligned peak area with the same mass/retention time pair as well as
253 normalized peak intensities and sample name. The main parameters for data
254 processing were set as follows: mass range (80-1200 Da), mass tolerance (5
255 ppm), intensity threshold (100 counts), retention time (0-20 min) and retention
256 time tolerance (0.2 min). The resulting data were mean-centered and pareto-
257 scaled prior to statistical analysis by Principal Component Analysis (PCA) to
258 differentiate each group. PCA was used to visualize the maximal difference of
259 global metabolic profiles^[36]. All data were expressed as means±standard error
260 of the mean (SEM). Statistical significance was calculated with one-way
261 ANOVAs and post hoc Turkey's tests in GraphPad Prism 5.0 (GraphPad
262 Software, San Diego, CA, USA).. Differences were considered to be
263 statistically significant when $P < 0.05$. All experiments were performed at least
264 four times.

265

266 **Results**

267 ***AC partially improves serum glucose and lipid homeostasis and*** 268 ***protects against HFD-induced IR in HFD-fed rats***

269 To explore the effect of AC in rats, we constructed the animal IR model
270 induced by high glucose and fat diet. *The results showed that* the serum total
271 cholesterol (TC) and triglyceride (TG) concentrations were lower than those in
272 the chow diet-fed animals at the end of the 8-week HFD (Fig. 1A and 1B). The
273 analysis of serum lipoproteins revealed that the hypercholesterolemia in rats

274 fed a HFD was associated with increased low-density lipoprotein-cholesterol
275 (LDL-C) and decreased high-density lipoprotein-cholesterol (HDL-C), typical
276 phenotypes of diabetic dyslipidemia (Fig. 1C and 1D). Treatment with AC for 8
277 weeks significantly improved the lipid profiles and reduced the serum levels of
278 glucose and insulin. AC or metformin (MET; 200 mg/kg) significantly reduced
279 the fasting glucose and insulin levels (Fig. 1E and 1F). Moreover, treatment
280 with Marein increased insulin sensitivity index and improved the insulin
281 tolerance (Fig. 1G and 1H). Although, HFD rats showed a clear increase in
282 body weight, there are no significantly changes in drug treated group except
283 the positive control compared with HFD group. Histological examination of the
284 liver in HFD-treated rats showed lipid accumulation and fatty degeneration in
285 the hepatocytes; however, treatment with 100 mg/kg marein significantly
286 attenuated the formation of fat vacuoles in the liver sections (Fig. 2).

287 ***Marein increases glucose uptake, hexokinase activity, and glycogen***
288 ***synthesis and decreases RNA and protein expression of PEPCK and***
289 ***G6Pase in high-glucose-induced IR model cells***

290 We evaluate the effect of marein (Fig. s1) extracted from *C. tinctoria* on
291 glucose consumption. 55 mmol/L glucose-induced cell model of IR was
292 successfully constructed with HepG2 cells. Glucose decreased 2-NBDG
293 uptake in a both time-dependent and dose-dependent manner (Fig. 3A). The
294 results showed that Marein had a potential effect on promoting the uptake of
295 glucose into HepG2 cells. The dose-dependent effects of Marein treatment for
296 24h were investigated from 1.25 μ mol/L to 40 μ mol/L. After treatment with 5
297 μ mol/L of Marein, the 2-NBDG uptake into HepG2 cells reached the highest
298 level, which was the plateau level (Fig. 3B). Then, the effect of Marein on IR

induced by high glucose was investigated with 2-NBDG. Pre-treatment of HepG2 cells for 24h with all concentrations of Marein obviously increased glucose uptake that approach to 90.4% of control with 10 μ mol/L Marein (Fig. 3C), although no significant difference of cell survivals were observed in HepG2 cells treatment with Marein (Fig. 3D),. We thus selected 10 and 5 μ mol/L Marein to use in the subsequent experiments. The IR model with HepG2 cells (55 mmol/L glucose) causes a decrease in the glycogen content and hexokinase (a key enzyme in glycogen synthesis) activity. Both concentrations (5 and 10 μ mol/L Marein) inhibited the effect of 55 mmol/L glucose (Fig. 3E and 3F). Marein can thus increase hexokinase activity and glycogen synthesis in a dose-dependent manner in an IR model where these were previously suppressed.

In the hepatocyte, PEPCK and G6Pase levels and gluconeogenesis are stimulated by inhibition of AKT and are restrained by the synthesis of glycogen in a situation of IR ^[37]. In this study, 55 mmol/L glucose caused an increase in protein and mRNA levels of PEPCK and G6Pase, but Marein inhibited these alterations (Fig. 3G-I).

Multivariate statistical analysis of HPLC/MS data summarizes metabolic differences in treatment groups

Measured levels of small molecule metabolites of enzymes involved in glucose metabolism and the Krebs cycle are used to verify the changes of these enzymes. Representative HPLC/MS data of cell samples from the high glucose treated cells, control cells and Marein-treated group were used in statistical analysis, as shown in Fig. 4. The three-dimensional PCA score plot

for the first three principal components (PC1, PC2 and PC3) with clustering of each group. The clear separation of the high glucose group and control group suggests that severe metabolic disturbance occurs in the IR model cells (Fig. 4A, 4B). Marein (5 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$) group was clearly separated from the model group (high glucose) and partially overlapped with control group, suggesting Marein had apparent effect on the IR. The PCA score plots of PC1, PC2 and PC3 revealed that data points, each representing one sample, were clustered in a way that allowed Model group (high glucose) to be clearly separated from control, Marein (10 $\mu\text{mol/L}$), Marein (5 $\mu\text{mol/L}$) and positive control groups along PC1 (Fig. 4C, 4D, 4E). Changes in important positions in a network more strongly impact the pathway than changes occurring in marginal or relatively isolated positions. MetaboAnalyst 3.0 revealed that differential metabolite content is important for the normal response to high glucose group, and multiple pathways are altered in high glucose and Marein group (Fig. 4F). The impact-value threshold calculated via pathway topology analysis was set to 0.10^[38], and 27 of the regulated pathways were identified as potential target pathways (Fig. 4F and Table 4) of marein in high glucose-induced model.

Furthermore, unsupervised hierarchal cluster analysis revealed the fluctuation of levels across different groups, as visualized by a heat map (Fig. 5A). Metabolic substrates of different treatment groups were changed in different degrees. Z-score plots were constructed to identify metabolic changes distinct between high glucose group and control group. 137 metabolites exclusive to cells after treatment with high glucose (red plot) were identified. However, treatment with 10 $\mu\text{mol/L}$ Marein significantly reduced the

349 levels of these metabolites (green plot, Fig. 5B).

350

351 ***Effects of Marein on energy metabolism intermediates in high glucose-***
 352 ***induced IR model cells.***

353 High glucose-treated cells exhibited changes in the levels of metabolic
 354 intermediates that participate in energy metabolism, including the glycolytic
 355 pathway and the Krebs cycle. Concentrations of major intermediates involved
 356 in glycolysis, such as dihydroxyacetone phosphate (DHAP; 0.48-fold, $p=$
 357 8×10^{-3}), and 3-phosphoglycerate (0.61-fold, $p= 0.04$), were significantly
 358 decreased in the high glucose group, compared with control group. However,
 359 10 $\mu\text{mol/L}$ Marein reduced the effect of high glucose on these two metabolites
 360 (Fig. 6).

361 The high glucose condition clearly increased levels of other measured
 362 Krebs cycle intermediates compared with control group, including citrate
 363 (5.09-fold, $p= 1.5 \times 10^{-7}$), succinate (6.46-fold, $p= 1.7 \times 10^{-4}$), aconitate (3.11-
 364 fold, $p= 5.2 \times 10^{-6}$), and malate (4.08-fold, $p= 8.8 \times 10^{-6}$). 10 $\mu\text{mol/L}$ and 5 $\mu\text{mol/L}$
 365 Marein partially decreased the effect of high glucose on these Krebs cycle
 366 intermediates.

367 ***Marein reduces mRNA and protein expression of Krebs related enzymes***
 368 ***SDHA, ACO2, IDH2 and CS in high-glucose-induced IR model cells.***

369 To evaluate the effect of Marein on Krebs cycle metabolism of human
 370 hepatocyte exposed to high glucose, the RNA and protein expression of
 371 Krebs related enzymes were detected with qPCR and western blot assays. As
 372 shown in figure 7A, treatment with 55 mmol/L glucose caused to an increase
 373 of some Krebs cycle mRNA levels, including *SDHA*, *ACO2*, *IDH2* and *CS*,

whereas this effect was inhibited by Marein. There were no detectable differences in mRNA levels of *FH*, *MDH2* and *DLST* in the high-glucose-induced IR model cells. In accordance with the results of mRNA expression, the protein levels of SDHA, ACO2, IDH2 and CS were also increased in the treatment group with 55 mmol/L glucose, but FH, MDH2 and SDHB were not altered. Marein reduced the effect of high glucose on the protein expression of SDHA, ACO2, IDH2 and CS in a dose-dependent manner (Fig. 7B and 7C).

Marein reduces ATP levels, pyruvate and lactate concentration in high glucose-induced IR model cells.

As exposure to high glucose will decrease glycogen synthesis, the effect of high glucose on gluconeogenesis was evaluated by detecting the pyruvate and lactate concentration and ATP levels. Under the IR condition, cells produced more ATP to supply energy due to a decline in glucose uptake. Treatment with high glucose also caused the elevation of pyruvate and lactate concentration, whereas this was inhibited by 10 and 5 μ mol/L Marein (Fig. 7D-F). This result indicates that increased gluconeogenesis plays a key role in the damage caused by high glucose in HepG2 cells, and that Marein can ameliorate that effect.

Discussion

Previous study showed that the flower tea *C. tinctoria* increases insulin sensitivity and regulates hepatic metabolism in rats fed a high-fat diet^[22]. In this study, it had been showed that AC strongly improves glucose and lipid homeostasis disrupted by HFD in rats. As the main components of AC, Marein protects HepG2 cells from high glucose-induced glucose metabolism disorder. Metabolomics analyses showed that Marein improves the multitude metabolic

399 pathway including TCA cycle, gluconeogenesis and amino acid metabolism in
400 HepG2 cells. Our results demonstrate that pretreatment of HepG2 cells with
401 Marein significantly reversed high glucose-induced the changes of the key
402 substrates and enzymes in gluconeogenesis and Krebs cycle.

403 The liver plays an important role in maintaining blood glucose
404 concentration both by supplying glucose to the circulation via glycogenolysis
405 and gluconeogenesis and by removing glucose from the circulation to
406 increase glycogen synthesis ^[37]. We found that glucose utilization and
407 glycogen synthesis were significantly decreased but the activity of PEPCK
408 and G6Pase, the key enzymes in the metabolic pathway of gluconeogenesis
409 were obviously increased in cells treated with high glucose (Fig. 3). These
410 results are in agreement with the previous findings that hepatic IR can be
411 attributed mostly to decreased stimulation of glycogen synthesis by insulin
412 while gluconeogenesis is abnormally enhanced due to the inefficient utility of
413 glucose ^[39]. Our data showing that pretreatment with Marein significantly
414 reversed the high-glucose-induced decrease in glucose utilization and
415 glycogen synthesis, increasing in the activity of PEPCK and G6Pase.
416 Consistent with the protein expression of gluconeogenesis enzymes, the
417 metabolites of glycolytic and gluconeogenesis pathway were significantly
418 decreased in the high glucose group (Fig.. 3G), which could be reversed by
419 pretreatment with Marein. In accordance with previous reports, pyruvate and
420 lactate were clearly increased in the high glucose treatment cells ^[40, 41]. These
421 metabolites were the end product of glycolysis, thus their levels elevated in
422 response to an increased glycolytic activity. This is different from the liver
423 tissues with type 2 diabetes ^[42]. One possible explanation for these results is

424 that HepG2 cell is a kind of tumor cell line with a high rate of aerobic
425 glycolysis, known as the Warburg effect, which is a hallmark of cancer cell
426 glucose metabolism ^[43]. Our results demonstrated that pretreatment with
427 Marein before high glucose treatment significantly reduced the high glucose-
428 induced increase in pyruvate and lactate generation and decrease in
429 glycolytic activity in HepG2 cells. It is widely accepted that pyruvate is
430 converted to acetyl-CoA and then enter the Krebs cycle in the mitochondria.
431 Previous reports have demonstrated that the key enzyme activities of the
432 Krebs cycle are significantly altered in type 2 diabetes ^[35, 44, 45].

433 In this study, mRNA expression and protein levels of these enzymes
434 were measured, and it was found that treatment with high glucose caused
435 an increase in mRNA and protein levels of some key enzymes including
436 ACO2, IDH2, CS and SDHA, and affected upstream reactions of the Krebs
437 cycle, findings which are in agreement with the previous findings that are
438 mostly focused on the key enzymes activities of Krebs cycle ^[35, 44, 45]. The
439 high-glucose condition did not affect the enzymes downstream of the Krebs
440 cycle, such as FH and MDH2. *SDHB* gene expression was not affected by
441 high glucose, which may be because SDHB is not the catalyzing subunit
442 (Fig. 7). However over-activation of these enzymes as induced by high
443 glucose may be suppressed by pretreatment with Marein.

444 A recent report by Choi et al. ^[46] implicates glucolipotoxicity as a cause for
445 impaired glucose metabolism, leading to a decrease in Krebs cycle
446 intermediates that is consistent with our current findings that the levels of
447 Krebs cycle intermediates, such as succinate, citrate, aconitate, and malate
448 are significantly increased in the high glucose group. On the other hand,

449 pretreatment with Marein before high glucose significantly increased the
450 above Krebs cycle intermediates, which suggested that Marein could
451 significantly increase the rate of the Krebs cycle and the accumulation of
452 these intermediates, and thus reduce the toxicity of glucose. However, our
453 study also found that mRNA and protein levels of Krebs cycle-related
454 enzymes, such as SDHA, CS, ACO2 and IDH2, significantly increased in high
455 glucose group and decreased in Marein pretreatment group, which may
456 reverse the stress response to these substrates. The increase in these
457 enzymes may degrade the related substrates.

458 In summary, we evaluated the effect of marein extracted from on IR. And
459 then we have demonstrated that Marein attenuated the IR induced by high
460 glucose in vitro. Our results show that Marein's improvement of the IR effect
461 can be attributed to it ameliorating numerous high glucose-induced processes
462 such as decrease in glucose uptake and glycogen synthesis, inhibition the
463 activity of hexokinase, perturbation of the glyconeogenesis and Krebs cycle
464 homeostasis, and an increase in the levels of ATP, pyruvate and lactate.
465 Meanwhile, Marein also improves glucose and lipid metabolism disorder
466 disrupted by HFD in rat IR model. These findings suggest that Marein may
467 have considerable potential for preventing high glucose-induced glucose
468 metabolism disorder and IR.

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621 **Figures and Legends**

622

623 **Figure 1** AC improves serum glucose and lipid homeostasis and protects
624 against HFD-induced IR. The serum levels of TC (A), TG (B), LDL-C (C),
625 HDL-C (D), fasting blood glucose (E), fasting insulin (F) were measured. G,
626 Insulin sensitivity index [ISI =1/(fasting insulin × fasting plasma glucose)]. H,
627 insulin tolerance tests (ITT). I, body weight Values are the means ± SEM (n =
628 10). . * $P<0.05$ vs the control group; ** $P<0.01$ vs the control group; # $P<0.05$
629 vs HFD-treated group; ## $P<0.01$ vs HFD-treated group.

630

631 **Figure 2** Effects of AC on lipid accumulation and steatosis. Representative
632 hematoxylin and eosin staining of the liver (magnifications, ×200).

633

634 **Figure 3** Marein inhibits the decrease of glucose uptake and the imbalance of
635 glucose metabolism induced by high glucose in HepG2 cells. (A) Dose-
636 dependent and time-dependent effect of glucose on 2-NBDG uptake. (B)
637 Dose-dependent effect of Marein on 2-NBDG uptake. HepG2 cells were
638 incubated for 24h. (C) Glucose uptake expressed as a percent of control
639 group are means ± SD of at least 4 different samples per condition. (D) Cell
640 viability shows the toxicological effect of Marein in HepG2 cells. (E) Effect of
641 Marein on glycogen content. (F) Effect of Marein on Hexokinase activity. (G)

642 Bands of representative experiments for PEPCK and G6Pase. (H)
 643 Densitometric quantification of PEPCK and G6Pase. (I) Effect of Marein on
 644 mRNA expression of PEPCK and G6Pase. All experiments were performed at
 645 least 4 times. * $P<0.05$ vs the control group; ** $P<0.01$ vs the control group;***
 646 $P<0.001$ vs the control group; # $P<0.05$ vs 55 mmol/L glucose-treated group;
 647 ## $P<0.01$ vs 55 mmol/L glucose-treated group.

648

649 **Figure 4** PCA scores plots for HPLC/MS data of all groups. (A) The three-
 650 dimensional PCA score plot of metabolic states of normal control group (red
 651 ●), high glucose group (green ●), 10 μ mol/L Marein group (blue ●), 5 μ mol/L
 652 Marein group (bright blue ●) and Positive control group (pink ●). (B) The
 653 three-dimensional PCA score plot of metabolic states of normal control group
 654 (red ●) and high glucose group (green ●). (C) The three-dimensional PCA
 655 score plot of metabolic states of high glucose group (red ●) and Positive
 656 control group (green ●). (D) The three-dimensional PCA score plot of
 657 metabolic state of high glucose group (red ●) and 5 μ mol/L Marein group
 658 (green ●). (E) The three-dimensional PCA score plot of metabolic state of
 659 high glucose group (red ●) and 10 μ mol/L Marein group (green ●). (F)
 660 Summary of pathway analysis with MetPA. Positive control is 0.5 mmol/L
 661 metformin.

662

663 **Figure 5** The variance analysis of metabolites based on the data of HPLC/MS
 664 of cells. (A) Heat map visualization of the correlation analysis of all
 665 metabolites. Row: groups (red: normal control; green: high glucose; blue: 10
 666 μ mol/L Marein; bright blue: 5 μ mol/L Marein; pink: Positive control); columns:

667 metabolites; Cell color key indicates the cluster score, green: Lowest, red:
668 highest. (B) High glucose-based z-score plot of named metabolites for
669 comparison between normal control (black), high glucose (red) and 10 μ mol/L
670 Marein (green). Data are shown as standard deviation from the mean of
671 respective sham. Each dot represents a single metabolite in 1 sample; n=4
672 per group.

673

674 **Figure 6** Metabolome pathway map of quantified metabolites, including
675 components of the glycolytic or gluconeogenesis pathway and Krebs cycle in
676 all groups. The red box shows the enzymes increased by high glucose
677 treatment. * $P<0.05$ vs the control group; ** $P<0.01$ vs the control group;***
678 $P<0.001$ vs the control group; # $P<0.05$ vs 55 mmol/L glucose-treated group;
679 ## $P<0.01$ vs 55 mmol/L glucose-treated group. F6P, Fructose 6-phosphate;
680 F1,6P, Fructose 1,6-bisphosphate; DHAP, Dihydroxyacetone phosphate; PEP,
681 Phosphoenolpyruvate;

682

683 **Figure 7** Marein inhibits the increase of mRNA expression and protein levels
684 of Krebs cycle enzymes induced by high glucose in HepG2 cells. (A)Effect of
685 Marein on mRNA expression of Krebs cycle genes. (B) Bands of
686 representative experiments for FH, MDH2, SDHA, SDHB, IDH2, ACO2 and
687 CS. (C) Densitometric quantification of Krebs cycle enzymes. (D) ATP levels
688 expressed as a percent of control group are means $\pm$ SD of at least 4 different
689 samples per condition. (E) Effect of Marein on pyruvate concentration. (F)
690 Lactate concentrations expressed as a percent of control group are means $\pm$
691 SD of at least 4 different samples per condition.* $P<0.05$ vs the control group;

692 ** $P < 0.01$ vs the control group; # $P < 0.05$ vs 55 mmol/L glucose-treated group;

693 ## $P < 0.01$ vs 55 mmol/L glucose-treated group.

694

695

A **B** **C** **D** **E** **F** **G** **H** **I**