

RESEARCH ARTICLE



UHPLC/QTOF-MS-based metabolomics reveal the effect of *Melastoma dodecandrum* extract in type 2 diabetic rats

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ABSTRACT

Context: *Melastoma dodecandrum* Lour. (Melastomataceae) is a traditional Chinese medicine. This is the first study to report a protective effect of the ethanol extract from *M. dodecandrum* (MDE) in type 2 diabetic (T2DM) rats.

Objective: To investigate the therapeutic mechanism of MDE in T2DM rats.

Materials and methods: Sprague-Dawley rats were fed a high-fat diet for 6 consecutive weeks, followed by intraperitoneal injection of streptozotocin (STZ) (30 mg/kg) to induce diabetes. T2DM rats were divided into untreated diabetic, metformin-treated and MDE-treated groups. Additionally, normal rats without treatment served as the control group ($n=6$). Metformin (250 mg/kg) and MDE (600 mg/kg) were intragastrically administered to T2DM rats for 5 consecutive weeks. Serum samples were evaluated via ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS), followed by principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA).

Results: The 17 identified potential biomarkers were mainly involved in lipid, amino acid, arachidonic acid, taurine and nicotinic acid metabolism. MDE also significantly reduced the level of fasting blood glucose (FBG), oral glucose tolerance, insulin, total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), malondialdehyde (MDA), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and urea nitrogen (BUN) in T2DM rats. The high-density lipoprotein (HDL), serum creatinine (Scr), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) levels were elevated in MDE-treated group.

Discussion and conclusion: MDE possesses substantial antidiabetic activity, especially in lipid disorder regulation. This suggests that the use of MDE can be generalized to broader pharmacological studies, such as obesity and hyperlipidaemia.

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Flavonoid; blood lipid; blood glucose; oxidative stress; streptozotocin

Introduction

Diabetes mellitus (DM) is a syndrome of glucose, protein and lipid metabolism disorders caused by insulin deficiency or resistance. In 2015, an estimated 415 million individuals worldwide were identified as diabetics, and the total global health expenditure related to diabetes was estimated at 673 billion U.S. dollars (Ogurtsova et al. 2017). Type 2 diabetes mellitus (T2DM) is often associated with obesity, physical inactivity and genetics, accounting for 90% of all diabetic patients (Pasquali et al. 2014). Due to the rapid increase in morbidity and mortality, T2DM has become a tremendous threat to human health and one of the greatest challenges in current medicine. Herbal medicine has been used extensively by practitioners for thousands of years due to its feasibility for daily use, easy availability and relatively low cost (Chan 1995). Herbs are an important source of drugs, and various examples have demonstrated the potential to treat human diseases, especially complex and chronic diseases such as T2DM (Huang et al. 2019).

Melastoma dodecandrum Lour. (Melastomataceae) is extensively distributed throughout the southern provinces of China. According to the classic Chinese herbology volume

‘Compendium of Materia Medica’, *M. dodecandrum* has been used for several centuries to treat dysmenorrhoea, postpartum abdominal pain, hematochezia and dysentery. Modern pharmacological studies have verified that *M. dodecandrum* exerts hypoglycaemic, hypolipidemic, antioxidant and liver protective activities (Yu et al. 2011; Li et al. 2013; Weng et al. 2019). *M. dodecandrum* contains 2–3% flavonoids, more than 30 flavonoids were characterized, including flavones, flavanone, flavan-3-ols, anthocyanidins and flavonols; mainly including rutin, quercetin, proanthocyanins, kaempferol and luteolin (Wang et al. 2017). Since flavonoids are considered the potentially active antidiabetic components of *M. dodecandrum*, their effects and metabolic mechanism should be confirmed experimentally. We hypothesized that the therapeutic effect of MDE on T2DM is related to changes in endogenous molecules; therefore, it is necessary to establish a method with which potential biomarkers and metabolic pathways can be identified, in order to understand the manner by which MDE plays an integrated role in the therapeutic effect on T2DM.

Metabolomics is a relatively recently developed technology following genomics and proteomics, which involves the high-throughput identification and quantitation of small components

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(<1500 Da) of the metabolome (German et al. 2005). Through analysis of the content variation in metabolites, metabolomics can reveal the metabolic activities in an organism following stimulation with internal and/or external factors (Shyur & Yang 2008; Shi et al. 2016). Currently, ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS) is extensively used in metabolomic research due to its high reproducibility of measurements and high sensitivity (Dunn et al. 2011). UHPLC/QTOF-MS-based serum metabolomics could reflect systemic physiological changes that will help clarify the antidiabetic mechanism of MDE.

In the present study, a rat model fed high-fat diet was used to induce diabetic conditions with a low-dose of STZ. The antidiabetic effect of MDE was investigated by biochemical parameters evaluating and examining pancreatic histopathology. In addition, a metabolomic method was established to investigate the modulatory effects of MDE on endogenous metabolites in T2DM rats. Principal components analysis (PCA) was used for cluster analysis, and orthogonal partial least squares discriminant analysis (OPLS-DA) was carried out to estimate the changes in metabolite levels. The underlying regulations of metabolic pathways are discussed according to the identified metabolites. The present study is the first to report the protective effects of MDE in T2DM rats based on a metabolomic approach.

Materials and methods

Chemicals and reagents

HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Streptozotocin (STZ), formic acid and 2-chloro-L-phenylalanine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Metformin was purchased from Sino-American Shanghai Squibb Co., Ltd. (No. 1805089, Shanghai, China).

Preparation of MDE

The aerial part (leaves and branches) of *M. dodecandrum* was collected from Guangxi Zhuang autonomous region (southwestern China) in May 2018. The plant was botanically identified by Dr. Zi-ning Liang, Professor of Pharmacognosy at Guangxi University of Chinese Medicine, China. The specimen was stored in the Department of Pharmacology, Guangxi University of Chinese Medicine (No. 20180208).

The plant material was dried at room temperature, powdered, and stored in a dry place until use. For extraction, 1 kg powder was refluxed three times in 70% ethanol (10 L) for 1 h. The extracts were filtered and concentrated at reduced pressure, then purified using AB-8 macroporous adsorption resin with 50% ethanol. Following purification, the polysaccharides, phenolic acids and proteins were removed; the proportion of flavonoids in the extract was more than 80%.

Animals and treatment

Male Sprague-Dawley rats, weighing 180–200 g, were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). All rats were kept under controlled environmental conditions: 12 h light/dark cycle; a temperature of 25 °C; and relative humidity of 30–45%. In accordance with the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals, all experiments were carried out using protocols approved by the

Laboratory Animal Ethics Committee at Guangxi University of Chinese Medicine.

Following a 1-week acclimation, 24 rats were randomly allocated to 4 groups ($n = 6$). The control group was fed standard rat chow, while the untreated diabetic, metformin-treated diabetic and MDE-treated diabetic groups were fed a high-fat diet (HFD). The HFD contained 53.7% basal chow, 20% saccharose, 15% lard, 10% egg yolk powder, 1.1% cholesterol and 0.2% sodium cholate. The diets were given continuously throughout the study.

At the beginning of the 6th week, rats fed a HFD received streptozotocin (STZ) (30 mg/kg, i.p., dissolved in 0.1 mol/L cold citrate buffer, pH 4.5) to induce diabetes. Three days after STZ injection, rats with a fasting blood glucose (FBG) level >11.1 mmol/L as measured by a glucometer were confirmed as T2DM. Metformin (250 mg/kg) or MDE (600 mg/kg) was administered intragastrically for 5 weeks following the induction of diabetes. The untreated diabetic and control groups received saline.

Biochemical analysis and histological examination

At the end of the study (week 11), rats were fasted for 12 h, following which FBG levels were measured. Rats were administered 2 g/kg glucose intragastrically, and their blood glucose levels were subsequently measured 30, 60 and 120 min later.

After the oral glucose tolerance test (OGTT), blood samples from each rat were collected via the abdominal aorta. Serum samples were separated and stored at -80°C until analysis. Serum levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea nitrogen (BUN) and serum creatinine (Scr) were measured using an AU640 automatic analyser (Olympus, Tokyo, Japan). The superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) activities were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum insulin levels were determined using an ELISA kit.

The pancreas was fixed in 10% formalin for 24 h, embedded in paraffin, and serially sectioned. The sections were subsequently stained with haematoxylin and eosin (H&E) for histological examination.

Metabolomic analysis

Serum samples from control, untreated diabetic group and MDE-treated group were evaluated via UHPLC/QTOF-MS. A 100 μL aliquot of each serum sample was added to 300 μL methanol (containing 5 $\mu\text{g}/\text{mL}$ 2-chloro-L-phenylalanine as an internal standard) and vortex mixed for 2 min. The mixture was then centrifuged at 13,000 rpm, 4 °C for 10 min, and the supernatant (200 μL) was transferred to a sample vial for testing. The internal quality control (QC) was prepared by mixing an equal amount of each sample to further monitor the system stability.

LC-MS analysis was performed using an Agilent 1290 Infinity UHPLC system coupled to an Agilent 6545 UHD and Accurate-Mass Quadrupole Time-of-Flight mass spectrometer (Agilent, Palo Alto, CA, USA). An XSelect HSS T3 column (2.5 μm , 100 \times 2.1 mm, Waters, Milford, MA, USA) was applied at 25 °C and a flow rate of 0.35 mL/min. The optimal mobile phase was composed of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The gradient elution of the serum samples was as follows: 0–2 min, 5% B; 2–10 min, 5–95% B; 10–15 min, 95% B; 15–20 min, 95–5% B.

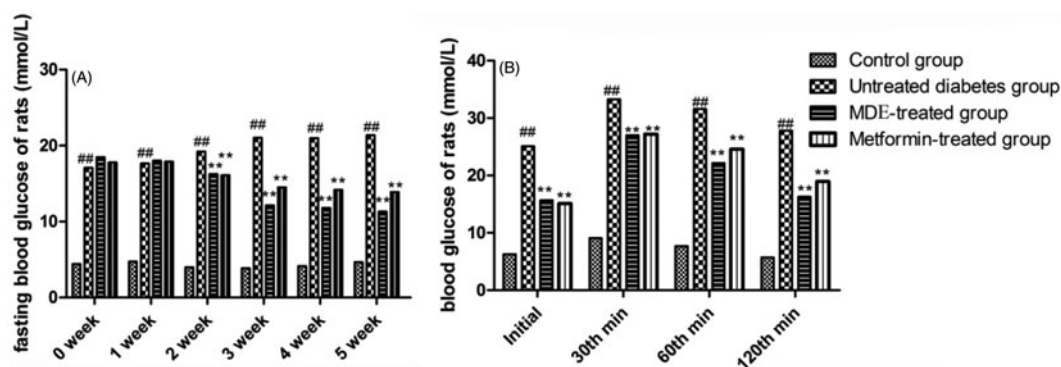


Figure 1. Determination of fasting blood glucose (A) and oral glucose tolerance (B) among all groups. Values are presented as mean \pm SD, $n=6$. ** $p < 0.01$ compared with the untreated diabetic group; * $p < 0.05$ compared with the untreated diabetic group. ## $p < 0.01$ compared with the control group; # $p < 0.05$ compared with the control group.

The mass spectrometer was operated using an electrospray ionization (ESI) source in positive and negative mode, with a capillary voltage of 4 kV in positive mode and 3.5 kV in negative mode. The dry gas flow was 10 L/min and the gas temperature was 325 °C. The nebulizer pressure was set at 20 psi. The fragmentor voltage was set at 120 V and the skimmer voltage at 45 V. The scanning mass range was 100–1000 Da. MS^E was applied for the MS/MS analysis with a low collision energy of 5 eV and a high collision energy of 20–30 eV.

Raw data were converted to the common (mz.data) format by the Agilent Masshunter Qualitative Analysis B.08.00 software (Agilent Technologies, USA). The converted data were imported into the XCMS software (<http://metlin.scripps.edu/xcms/>) for calculation of the normalized peak intensity, exact mass and retention time. Subsequently, these data were enrolled in the final dataset for multivariate statistical and pattern recognition analysis. These data were also introduced to SIMCA-P (V14.1. Umetrics, Sweden) for orthogonal partial least squares discriminant analysis (OPLS-DA) and principal components analysis (PCA). The SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the normalized integral values to determine significant differences between metabolic changes. The variables with a variable importance in project (VIP) >1 in the OPLS-DA model and $p < 0.05$ in the t -test are considered potential biomarkers. Potential biomarkers were interpreted using the Human Metabolome Database (<http://www.hmdb.ca>), LIPIDMAPS (<http://www.lipidmaps.org>) database and METLIN (<https://metlin.scripps.edu/>) database. Metabolic Pathway Analysis (MetPA) was also performed (<http://www.metaboanalyst.ca/>) to identify the affected metabolic pathways and facilitate further biological interpretation.

Statistical analysis

All data are presented as the mean \pm standard deviation (SD). The differences among FBG, OGTT, TG, TC, HDL-C, LDL-C, SOD, MDA, CAT, GSH-Px, ALT, AST, BUN, Scr and serum insulin were assessed using Fisher's Exact test as indicated, with $p < 0.05$ being considered statistically significant.

Results

Antidiabetic effects of MDE

T2DM rats showed a significant increase in FBG (Figure 1). Following MDE treatment for 5 weeks, the FBG levels in rats in the MDE-treated group were markedly lower than those in the

untreated diabetic group. The hypoglycaemic effect of MDE was similar to that of metformin (250 mg/kg) (Figure 1(A)). The oral glucose tolerance test showed that the MDE-treated group had a significantly lower blood glucose concentration at 30, 60 and 120 min in comparison with the untreated diabetic group (Figure 1(B)). The TC, TG, HDL and LDL levels were markedly changed in the untreated diabetic group as compared with those in the control group, all of which showed dyslipidemia in T2DM rats. MDE treatment elicited a reduction in TC, TG and LDL levels and an increment in HDL levels as compared with those in the untreated diabetic group (Table 1). There were significant differences in oxidative stress between the untreated diabetic and the control groups. A rising trend in the MDA activity and a decreasing trend in GSH-Px, SOD and CAT activities were found in the untreated diabetic group. MDE treatment significantly improved the activities of the antioxidative enzymes, GSH-Px, SOD and CAT as compared with those in untreated diabetic rats, and the concentration of MDA was lower than that in untreated diabetic rats (Table 1). The untreated diabetic rats showed an increase in serum AST, ALT and BUN levels, and a decrease in Scr levels. Treatment with MDE reversed all these parameters (Table 1). In addition, the serum insulin levels in the MDE-treated group were significantly decreased as compared with those in the untreated diabetic group (Table 1). These results suggest that MDE can alleviate insulin resistance and liver and kidney dysfunction in T2DM rats.

Histological examination of the pancreas

The pancreatic ducts were markedly dilated in the untreated diabetic group, with significant infiltration of inflammatory cells in the intralobular and periductal areas (Figure 2(A)), and the pancreas was atrophic and fibrotic (Figure 2(B)). Rats in the MDE-treated group exhibited a remarkable reduction in pathological damage as compared with that in untreated diabetic rats (Figure 2(C)). Similarly, metformin alleviated pancreatic damage in T2DM rats (Figure 2(D)).

Metabolomic analysis of MDE

Metabolomic profiling of serum samples was performed by UHPLC/QTOF-MS. A total of 2596 features were obtained in the positive mode and 1978 in the negative mode. The stability of the analytical method is crucial for obtaining valid metabolomic data. The analytical stability of the system throughout the experiment was evaluated by analysing QC samples. The PCA

Table 1. Determination of insulin, TC, TG, LDL – C, HDL – C, SOD, MDA, GSH – PX, CAT, ALT, AST, BUN and Scr levels in serum among all groups.

	Control group	Untreated diabetic group	MDE-treated group	Metformin-treated group
Insulin (μ U/L)	12.18 \pm 1.8	23.85 \pm 2.41 ^{##}	15.12 \pm 1.34 ^{**}	17.43 \pm 1.62 ^{**}
TC (mmol/L)	1.45 \pm 0.12	9.54 \pm 0.87 ^{##}	2.95 \pm 0.31 ^{**}	6.31 \pm 1.1 ^{**}
TG (mmol/L)	0.67 \pm 0.1	5.21 \pm 0.25 ^{##}	1.35 \pm 0.15 ^{**}	3.83 \pm 0.21 [*]
LDL – C (mmol/L)	1.47 \pm 0.25	2.05 \pm 0.22 [#]	1.73 \pm 0.28 [*]	1.69 \pm 0.15 [*]
HDL – C (mmol/L)	0.68 \pm 0.11	0.37 \pm 0.02 ^{##}	0.58 \pm 0.04 ^{**}	0.44 \pm 0.05 [*]
SOD (U/mL)	120.68 \pm 11.54	46.21 \pm 3.87 ^{##}	79.67 \pm 6.33 ^{**}	51.39 \pm 5.49
MDA (nmol/mL)	3.12 \pm 0.13	10.11 \pm 1.54 ^{##}	6.56 \pm 0.59 ^{**}	7.86 \pm 0.79 [*]
GSH – PX (U/mL)	777.23 \pm 25.14	565.86 \pm 21.28 ^{##}	645.93 \pm 31.74 ^{**}	605.91 \pm 29.21
CAT (U/gHb)	91.68 \pm 9.98	47.9 \pm 3.78 ^{##}	65.01 \pm 4.99 ^{**}	72.32 \pm 4.23 ^{**}
ALT (U/L)	40.22 \pm 3.87	114.17 \pm 15.37 ^{##}	73.38 \pm 10.45 ^{**}	63.65 \pm 9.75 ^{**}
AST (U/L)	112.56 \pm 18.61	160.5 \pm 18.3 [#]	131.54 \pm 11.39 [*]	121.52 \pm 14.27 [*]
BUN (mmol/L)	6.69 \pm 0.81	11.18 \pm 1.87 ^{##}	8.21 \pm 1.03 [*]	10.41 \pm 2.17
Scr (μ mol/L)	36.75 \pm 4.22	28.12 \pm 2.17 [#]	30.4 \pm 2.98 [*]	32.19 \pm 1.83 [*]

Values are presented as mean \pm SD, $n = 6$.

^{**} $p < 0.01$ compared with the untreated diabetic group.

^{*} $p < 0.05$ compared with the untreated diabetic group.

^{##} $p < 0.01$ compared with the control group.

[#] $p < 0.05$ compared with the control group.

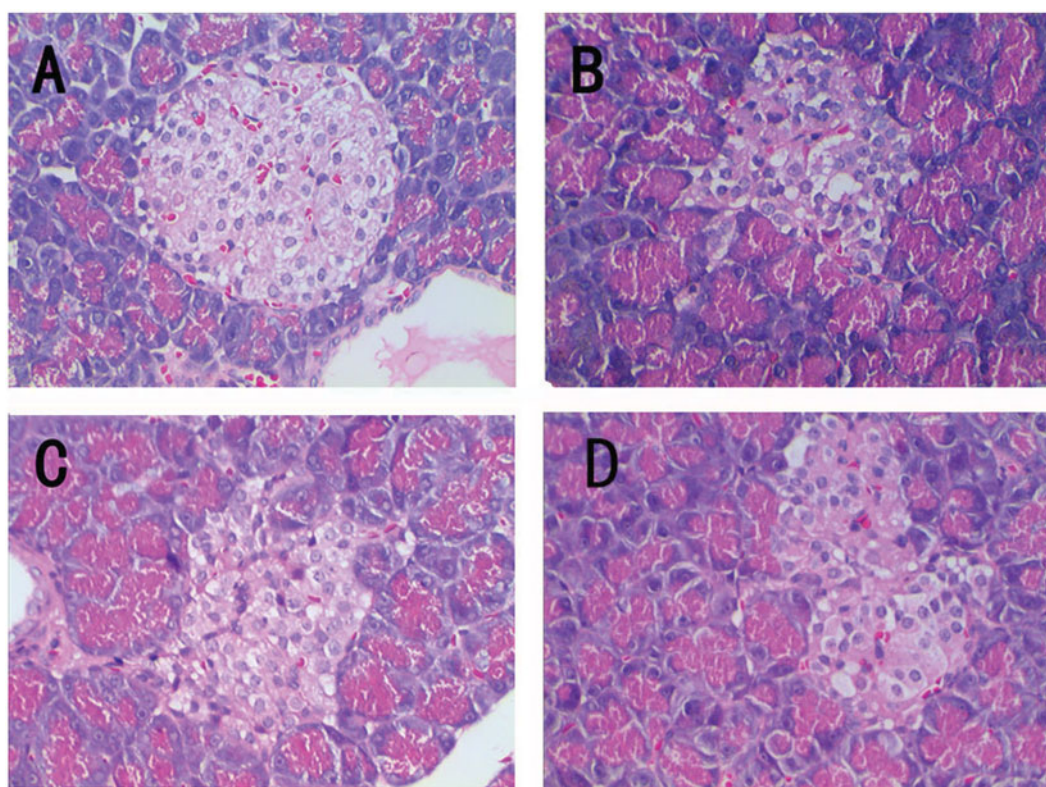


Figure 2. Effects of MDE on histopathological changes of pancreas in T2DM rat ($\times 40$, H&E staining). (A) control group, (B) untreated diabetic group, (C) MDE-treated group, (D) metformin-treated group.

results (Figure 3) show that the QC samples (green circle) were tightly clustered together, indicating good stability of the UHPLC/QTOF-MS system, which was sufficient for metabolomic analysis.

PCA is an unsupervised method of multivariate analysis, with a score plot as the output result. In the PCA score plots (Figure 3), the 3 groups were well-separated. The coefficient of determination in the positive ion mode was 0.67, and that in the negative ion mode was 0.65, indicating significant inter-group differences following treatment. These results suggest that 5 weeks of treatment with MDE altered the metabolic state of T2DM rats.

In comparison with the unsupervised PCA model, the supervised OPLS-DA model can distinguish differences among groups and improve the validity and analytical ability, since it focuses

on the actual class discriminating variation. The OPLS-DA serum score plot and S-plots are shown in Figure 4. Figure 4(A1,A2) shows that the control and untreated diabetic groups are separated and distributed in different quadrants, indicating that the T2DM model was established successfully. In the positive ion mode, R^2Y and Q^2Y were 0.99 and 0.94, and in the negative ion mode were 0.99 and 0.93, respectively. According to Figure 4(A3,A4), the analysis resulted in good statistical performance for the untreated diabetic group and MDE-treated groups. In the positive ion mode, R^2Y and Q^2Y were 0.99 and 0.86, and in the negative ion mode were 0.99 and 0.81, respectively. These results suggest that MDE displayed pharmacological activity. From Figure 4(B1–B4), the S-plot exhibits most of the variables in the dataset, in which the ions furthest away from the origin

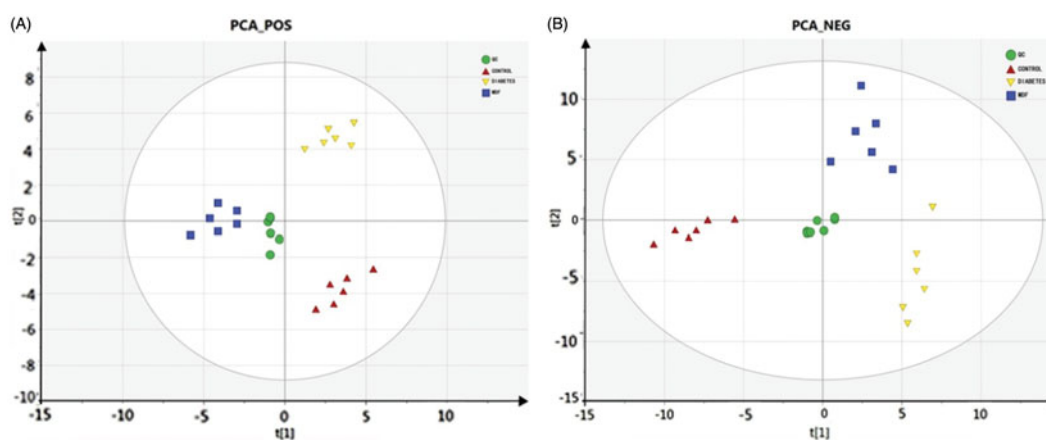


Figure 3. The PCA score plots of control group, untreated diabetic group and MDE-treated group. (A) Positive ion mode, (B) negative ion mode. ●, QC samples; ▲, control group; ▼, untreated diabetic groups; ■, MDE-treated group.

contribute significantly to the clustering of the two groups and may be regarded as potential biomarkers. The VIP was calculated to identify metabolites contributing significantly to the separation of the untreated diabetic and MDE-treated groups. According to the criteria of $VIP > 1.0$ and $p < 0.01$ in t -test between groups, the metabolites with significant changes were screened out. The accurate masses of the metabolites were matched to online databases including HMDB, METIN and KEGG. The mass tolerance between the measured m/z values and the exact mass was defined within 10 ppm. MS/MS fragmentation patterns also provided the necessary structural information about the biomarkers. MS/MS spectra of the endogenous substances were collected and matched with the reference spectra in the above databases, and a total of 17 endogenous metabolites were identified and characterized as potential biomarkers (Table 2). To illustrate the identification process, one endogenous metabolite (m/z : 180.0657, Rt: 4.79) is detailed as an example. Based on this information and its MS/MS fragmentations (Figure 5), fragment ions were obtained at m/z 105.0342 and 77.0375, which are likely the fragment ions of $-C_3H_5NO_3$ and $-C_2H_5NO_2$, respectively; therefore, this potential biomarker is considered to be hippuric acid.

These identified metabolites were taurine, nicotinuric acid, cholic acid, phosphohydroxypyruvic acid, hippuric acid, arachidonic acid, tyrosine, phenylalanine, glucuronide, PGE2, carnitine, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Changes in these metabolites among the control, untreated diabetic and MDE-treated groups are shown in Figure 6.

Metabolic pathway analysis

The identified biomarkers responsible for the therapeutic effects of MDE play an important role in specific metabolic pathways; therefore, it is important to dissect the pathways influenced by MDE through network analysis. MetPA delivers the analysis results in an intuitive manner; pathway impact plots (Figure 7) were built to visualize the impact of altered metabolic pathways. Pathways with an influence value greater than 0.1 were selected as the major metabolic pathways. Accordingly, the major metabolic pathways in serum samples involved glycerophospholipid, fatty acid, arachidonic acid, nicotinate, taurine and amino acid metabolism, primary bile acid and amino acid biosynthesis, and pentose and glucuronate interconversion.

Discussion

Melastoma dodecandrum has been used to treat diseases in China for at least 500 years, and usually accomplishes its overall therapeutic effect via a multitarget approach (Li 2006); however, few modern pharmacological studies have focussed on the anti-diabetic mechanisms of *M. dodecandrum*. In the present study, emerging metabolomic methods were employed, with the use of sophisticated instruments and multivariate data analysis to investigate the therapeutic mechanism of MDE in T2DM rats.

Following treatment for 5 weeks, MDE positively regulated hyperglycaemia, insulin resistance, dyslipidemia, oxidative stress, and liver and kidney dysfunction in T2DM rats. Furthermore, abnormal insulin secretion and islet cell dysfunction were relieved. Previous metabolomics and clinical biochemistry studies have demonstrated that T2DM may lead to a series of complex responses in multiple metabolic pathways, such as energy metabolism, carbohydrate metabolism, lipid metabolism and amino acid metabolism (Marta et al. 2016). In the present study, different potential biomarkers were discovered in serum samples, including taurine, nicotinuric acid, cholic acid, phosphohydroxypyruvic acid, hippuric acid, arachidonic acid, amino acids, glucuronide, PGE2, carnitine and phospholipids. These biomarkers were not isolated and usually reflected changes in metabolic pathways. Statistical analysis shows that multiple signalling pathways were affected by MDE treatment, including lipid, amino acid, arachidonic acid, taurine, nicotinate metabolism, glucose interconversions and primary bile acid biosynthesis.

Among the 17 potential biomarkers involved in the therapeutic effect of MDE in T2DM rats, 8 metabolites were associated with lipid metabolism, including PE(18:4/P-18:0), PE(22:6/22:6), PI(16:0/18:0), LysoPC(20:4), LysoPC(18:0), LysoPC(P-18:1) and palmitoyl-L-carnitine. Abnormalities in lipid and fatty acid metabolism cause dyslipidemia, which is one of the main risk factors for T2DM and its complications (Parker et al. 2019). Numerous studies have suggested that phospholipids play a vital role in glycolipid metabolism and in the development of certain metabolic diseases such as diabetes, obesity and arteriosclerosis (Donnell et al. 2019). High levels of blood phosphatidylcholine (PC) and phosphatidylethanolamine (PE) metabolites are recognized as potential biomarkers for decompensation of insulin resistance in diabetes (Geng et al. 2013), since they are signalling molecules that mediate the loss of insulin sensitivity and dysfunction in the pancreas (Prentice et al. 2019). Concentrations of PE and PC in Zucker diabetic

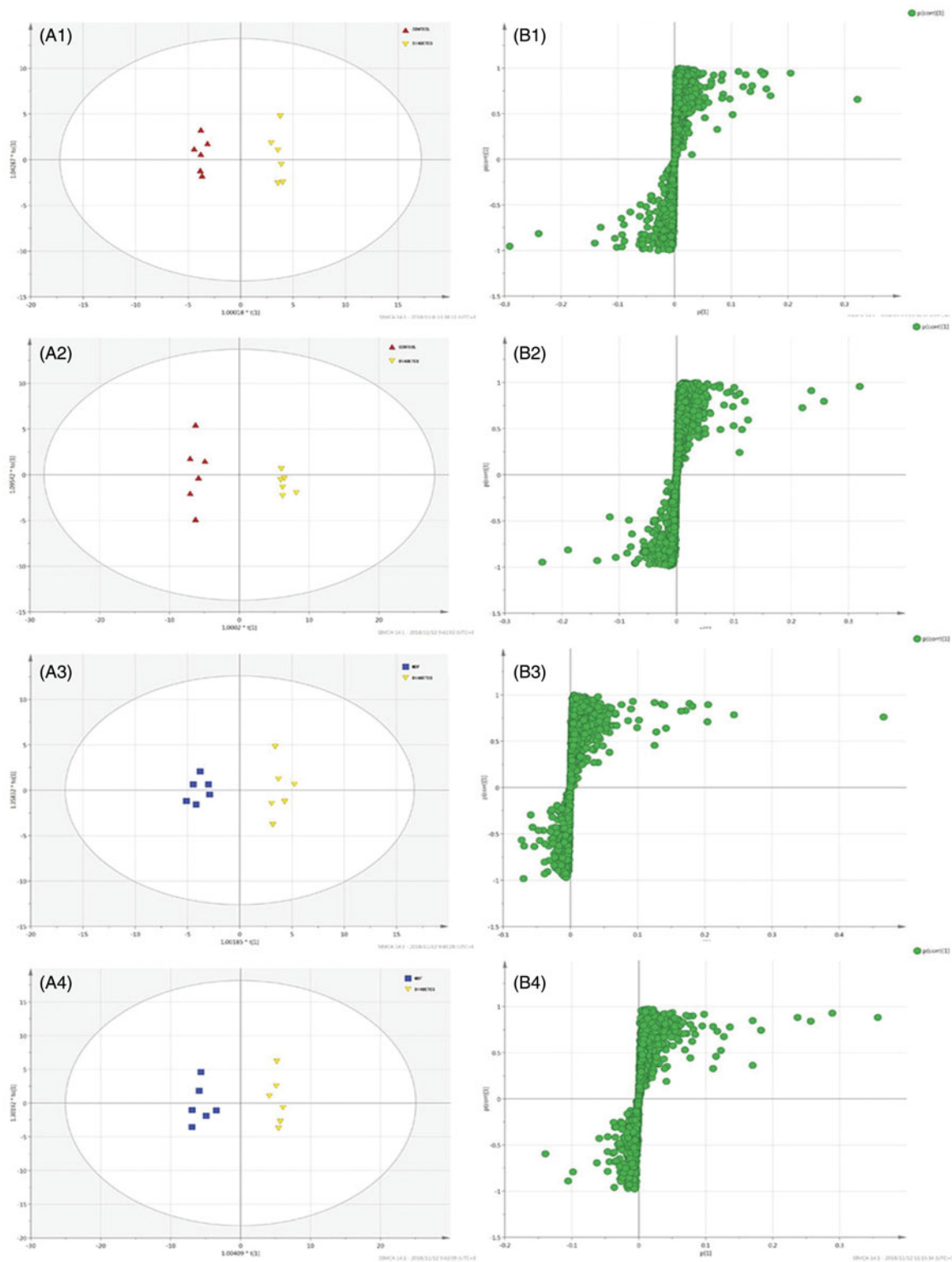


Figure 4. OPLS-DA scores plots (A) and S-plot (B) of untreated diabetic group versus control group (table 1, 2) and untreated diabetic group versus MDE-treated group (table 3, 4) in positive and negative ion mode. The label of 1 and 3 were obtained in positive ion mode, with the label of 2 and 4 in negative ion mode. ▲, control group; ▼, untreated diabetic groups; ■, MDE-treated group.

fatty rats have shown regular changes following the development of diabetes; phospholipid metabolism disorder aggravates development of the disease (Hsu et al. 2000). Clinical studies have also found that the plasma phospholipid concentration in diabetic patients is significantly higher than that in normal subjects (Jia et al. 2006). It is well known that L-carnitine free radical scavenging and antioxidant activities, which can reduce the

damage caused by lipid oxidation. L-Carnitine would facilitate fatty acid export from tissues in the form of acyl-carnitines, thereby alleviating lipid-induced insulin resistance. A study comparing the serum concentration of L-carnitine found that it was decreased by 15–20% in T2DM patients as compared with that in normal subjects (Bene et al. 2018). It has been reported that the combined effect of L-carnitine and phospholipids can

Table 2. Potential metabolites selected and identified between MDE – treated group and untreated diabetic group. MDE – treated group vs. untreated diabetic group

RT	m/z	Δppm	VIP	Metabolites	Formula	FC	p Value	HMDB	Trend	Pathway
1.28	124.0066	3	1.66	Taurine	C ₂ H ₇ NO ₃ S	2.6140	0.0148	00251	↑	Taurine and hypotaurine metabolism
1.85	203.0436	4	9.68	Nicotinic acid	C ₉ H ₇ NO ₃	3.3018	0.0278	03269	↑	Nicotinate and nicotinamide metabolism
2.52	182.0784	3	2.68	L-Tyrosine	C ₉ H ₉ NO ₃	1.8166	0.0490	00158	↑	Phenylalanine, tyrosine and tryptophan biosynthesis
4.26	188.0709	4	1.41	L-Phenylalanine	C ₉ H ₉ NO ₂	0.7693	0.0415	00159	↓	Phenylalanine, tyrosine and tryptophan biosynthesis
4.79	180.0657	0	2.03	Hippuric acid	C ₉ H ₉ NO ₃	0.7572	0.0429	00714	↓	Phenylalanine metabolism
5.25	184.9852	3	2.63	Phosphohydroxyppyruvic acid	C ₃ H ₅ O ₇ P	2.9595	0.0344	01024	↑	Glycine, serine and threonine metabolism
5.71	297.0982	0	1.34	2 – Phenylethanol glucuronide	C ₁₄ H ₁₈ O ₇	0.6400	0.0485	10350	↓	Pentose and glucuronate interconversions
5.92	413.2181	0	1.42	20 – Hydroxy – PGE2	C ₂₀ H ₃₂ O ₆	0.4700	0.0064	03247	↓	Arachidonic acid metabolism
7.63	407.2807	1	1.71	Cholic acid	C ₂₄ H ₄₀ O ₅	0.4547	0.0156	00619	↓	Primary bile acid biosynthesis
8.86	566.3221	1	2.23	LysoPC(20:4)	C ₂₈ H ₅₀ NO ₇ P	0.7965	0.0455	10395	↓	Glycerophospholipid metabolism
9.27	400.342	1	1.29	Palmitoyl-L-carnitine	C ₂₃ H ₄₅ NO ₄	2.7078	0.0436	00222	↑	Fatty acid metabolism
9.53	506.373	2	1.14	LysoPC(18:1)	C ₂₆ H ₅₂ NO ₄ P	0.3254	0.0140	10408	↓	Glycerophospholipid metabolism
10.05	568.361	2	7.01	LysoPC(18:0)	C ₂₆ H ₅₂ NO ₄ P	0.5984	0.0462	10384	↓	Glycerophospholipid metabolism
13.2	722.5171	4	1.65	PE(18:4/P – 18:0)	C ₄₁ H ₇₄ NO ₈ P	0.3508	0.0334	09214	↓	Glycerophospholipid metabolism
14.63	839.5649	1	4.34	PI(16:0/18:0)	C ₄₃ H ₈₃ O ₁₃ P	0.4017	0.0097	09781	↓	Glycerophospholipid metabolism
16.47	858.4975	5	1.15	PE(22:6/22:6)	C ₄₉ H ₇₄ NO ₈ P	2.6785	0.0410	09705	↑	Glycerophospholipid metabolism
17.63	303.2328	4	6.35	Arachidonic acid	C ₂₀ H ₃₂ O ₂	0.3616	0.0153	01043	↓	Arachidonic acid metabolism

↑: upregulated. ↓: downregulated.

facilitate the excretion of blood cholesterol and fatty acids to reduce TC, TG and LDL-C levels (Banas et al. 2000), which is in accordance with our findings.

Amino acids are considered the most important biological compounds, being involved in the synthesis of proteins and hormones and playing prominent metabolic and physiological roles within organisms (Neinast et al. 2019). In the present study, 4 biomarkers related to amino acid metabolism were detected, including L-tyrosine, L-phenylalanine, hippuric acid and phosphohydroxyppyruvic acid. Phenylalanine is an essential amino acid and the precursor for tyrosine, which plays an important role in promoting energy metabolism, scavenging free radicals and relieving fatigue. Dyslipidemia causes dysfunctional phenylalanine metabolism by inhibiting the conversion of phenylalanine to tyrosine, decreasing the tyrosine concentration in the blood. The metabolic signatures of tyrosine and phenylalanine strongly predict diabetes development (Magnusson et al. 2013). It has been reported that phenylalanine and tyrosine are involved in the synthesis of catecholamines, which are important brain neurotransmitters. Disorders of phenylalanine metabolism also exacerbate abnormalities in the neuromodulation of insulin secretion (Spronsen et al. 2017). Hippuric acid is produced via the metabolic conversion of phenylalanine by intestinal bacteria. An increased hippuric acid content may imply abnormal metabolism of intestinal flora in T2DM rats, but *M. dodecandrum* effectively improved this trend.

Arachidonic acid and its metabolites can exert a second messenger function in cells, both promoting and inhibiting the synthesis and secretion of insulin in islet β-cells. In diabetic patients, the protein kinase C pathway (PKC) pathway is over-activated, further stimulating phospholipase A2, which accelerates the hydrolysis of phospholipids and increases the release of arachidonic acid. However, superphysiological concentrations of arachidonic acid would result in apoptosis of islet β-cells (Papadimitriou et al. 2007). Our results show that arachidonic acid and 20-hydroxy-PGE2 levels were decreased in MDE-treated diabetic rats, suggesting that one underlying therapeutic mechanism of MDE in diabetes may be the regulation of arachidonic acid metabolism, thereby exerting protection of islet β-cells and promotion of insulin release.

Taurine plays various biological roles in the conjugation of cholesterol, antioxidation of bile acids, osmoregulation and calcium signalling. Supplementation of taurine has been reported to decrease the total weight of fat in the abdominal cavity and improve insulin sensitivity in a T2DM rat model (Franconi et al. 2006). The marked increase in taurine content in T2DM rats following treatment with MDE may be conducive to attenuation of lipid disorders and hyperglycaemia. Taurine combines with bile acid to form bile, which could influence fatty acid biosynthesis, glycolysis, gluconeogenesis and the metabolism of lipids and lipoproteins (Sato 1994). Similarly, we found that the concentration of cholic acid in MDE-treated rats was significantly decreased, indicating that the primary bile acid biosynthesis pathway was inhibited, which is also beneficial for the inhibition of cholesterol absorption (Maghsoodi et al. 2019).

Nicotinic acid is a molecule produced via the nicotinic acid metabolism pathway and is significantly altered following MDE treatment. Nicotinic acid derivatives include nicotinamide adenine dinucleotide (NAD, coenzyme I) and nicotinamide adenine dinucleotide phosphoric acid (NADP, coenzyme II), which are important coenzymes in oxidative reactions. Studies have shown that nicotinic acid regulates lipid metabolism and

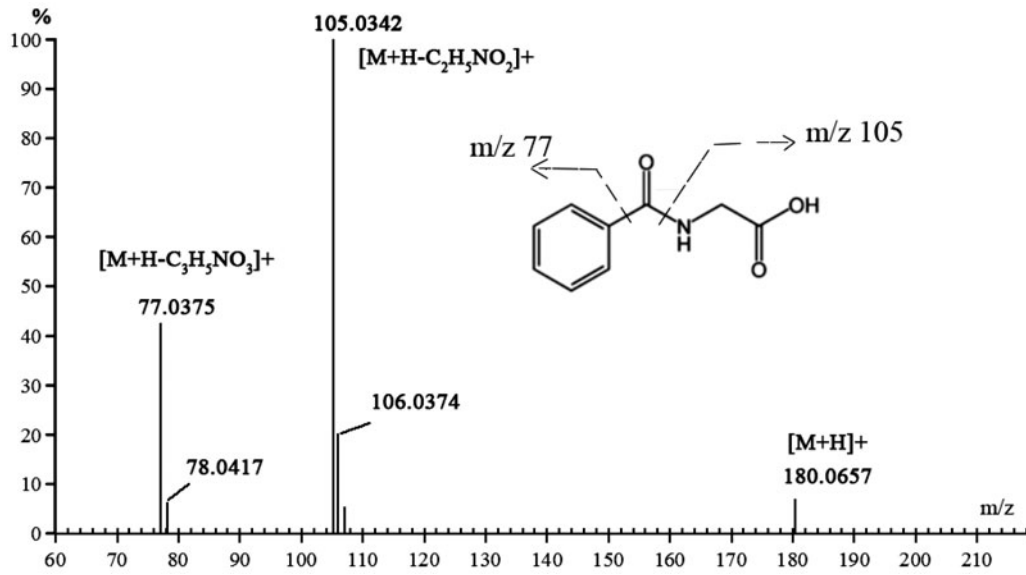


Figure 5. Product ion spectrum of biomarkers at m/z 180.0657 in positive ion mode.

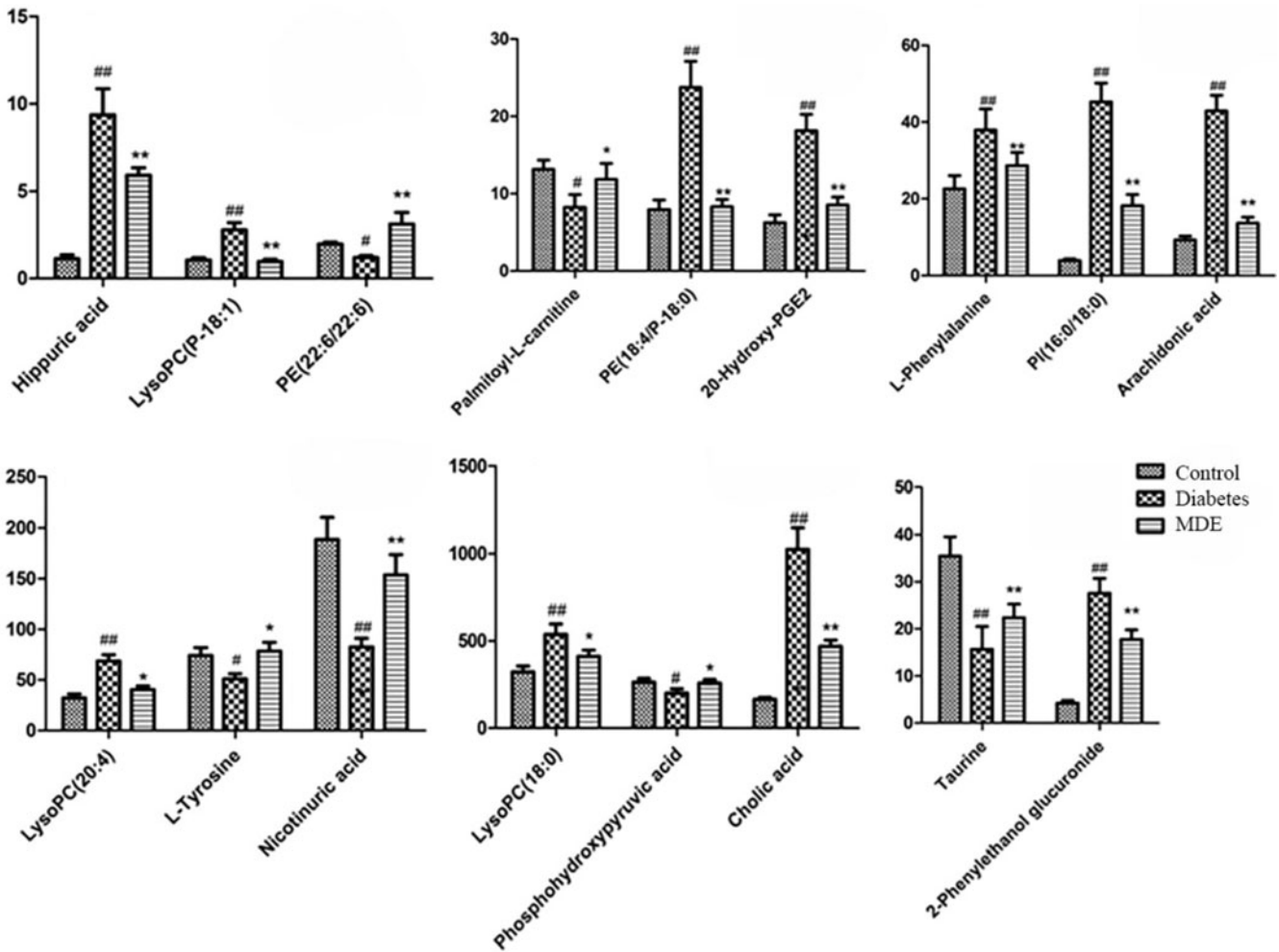


Figure 6. Relative peak area of potential biomarkers identified in serum among all groups. Values are presented as mean \pm SD, $n = 6$. ** $p < 0.01$ compared with the untreated diabetic group; * $p < 0.05$ compared with the untreated diabetic group. ## $p < 0.01$ compared with the control group; # $p < 0.05$ compared with the control group.

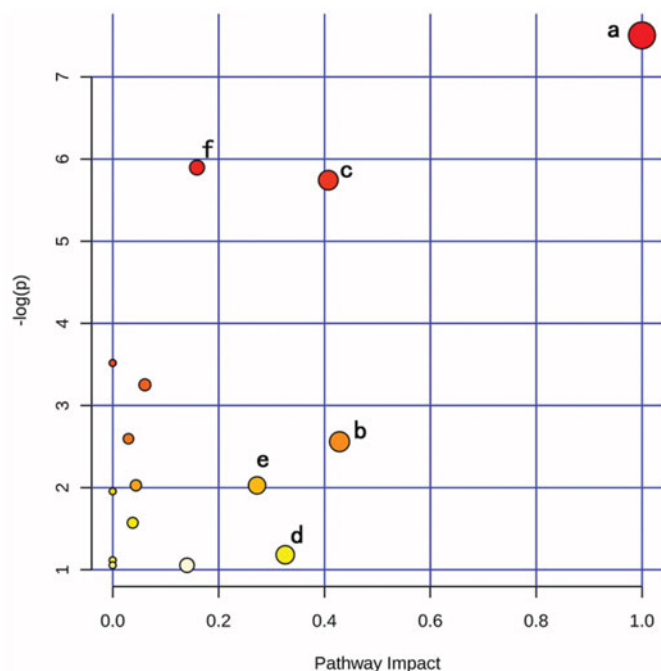


Figure 7. Summary of pathway analysis of serum of rats. (a) Phenylalanine, tyrosine and tryptophan biosynthesis; (b) glycerophospholipid metabolism; (c) arachidonic acid metabolism; (d) taurine and hypotaurine metabolism; (e) primary bile acid biosynthesis; (f) nicotinate and nicotinamide metabolism.

can reduce the synthesis of LDL-C, TC and TG (Lukasova et al. 2011).

Using holistic analysis, we found that the regulation of lipid metabolism by MDE plays an important role in its therapeutic effect. Further, study of the specific underlying mechanism of MDE with respect to lipid metabolism will provide more evidence for its effectiveness as a therapeutic agent.

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Disclosure statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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