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Metabolic and biochemical changes in streptozotocin induced obese-diabetic rats treated with *Phyllanthus niruri* extract

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ABSTRACT

Herbal medicine has been proven to be an effective therapy offering a variety of benefits, such as moderate reduction in hypoglycemia, in the treatment and prevention of obesity and diabetes. *Phyllanthus niruri* has been used as a treatment for diabetes mellitus. Herein, the induction of type 2 diabetes in Sprague–Dawley rats was achieved by a low dose of streptozotocin (STZ) (25 mg/kg bw). Here, we evaluated the *in vivo* antidiabetic properties of two concentrations (250 and 500 mg/kg bw) of *P. niruri via* metabolomics approach. The administration of 500 mg/kg bw of *P. niruri* extract caused the metabolic disorders of obese diabetic rats to be improved towards the normal state. The extract also clearly decreased the serum glucose level and improved the lipid profile in obese diabetic rats. The results of this study may contribute towards better understanding the molecular mechanism of this medicinal plant in managing diabetes mellitus.

1. Introduction

Diabetes mellitus (DM) is a chronic disease in which the pancreas does not produce enough insulin (type 1) or the body is unable to use the insulin effectively (type 2). Worldwide, 9% of adults aged 18 years and above were living with diabetes in 2014. In addition, DM caused 1.5 million deaths in 2013, with 80% of them occurring in low- and middle-income countries [1]. Type 2 DM is the most common type, which affects 90% of people with diabetes worldwide [1]. This disease is also mostly diagnosed in adults. Type 2 DM is functional disorder in the proper use of insulin and can be caused by the partial destruction of the insulin-producing β-cells of the pancreas [2]. These receptor cells become less sensitive to insulin and as a result, sugar accumulates in the blood. Type 2 DM is often related to obesity, physical inactivity and genetics. The simulation of diabetes in animal models has been used in many studies investigating this disease and its complications [2–5]. To induce diabetes, streptozotocin (STZ) is typically administered to specifically destroy pancreatic β-cells [6]. The STZ-induced diabetic rat model may represent a useful platform to study the effects, complications and mechanism of type 2 DM using advanced approaches.

Metabolomics has been previously applied to study various diseases, including type 2 DM [4,7,8]. The combination of analytical tools with multivariate data analysis (MVA) in metabolomics is useful for the simultaneous detection of various metabolites, and the changes in these metabolites can be used to reflect the normality or abnormality of biochemical pathways [9,10]. Recently, nuclear magnetic resonance (NMR) has been successfully applied to investigate the complications associated with diabetes in several research studies because of its simplicity, reproducibility and rapidity [9,11]. The analysis of metabolite variations in biofluids using NMR techniques has also been well established [9,11]. The potential use of NMR to identify biomarkers in biological specimens could improve our ability to propose specific metabolic pathways and study the complications and effects associated with diabetes and its treatment with natural drugs.

The benefits and values of consuming plants as functional or nutraceutical foods on human health are subject of prominent research in the food science and technology field. The bioactive

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composition of *Phyllanthus niruri* has been widely characterized to identify the chemical species responsible for its health benefits. Knowledge about the traditional uses of *P. niruri* may facilitate the application of this plant as a derived functional food product. Thus, the effect of this plant in the prevention and/or treatment of DM and its metabolic disorders can be tested. Phenolics and lignans are the bioactive constituents of *P. niruri*, and several studies have emphasized the contributions of these metabolites to this plant's bioactivity and functionality [12,13]. In addition, it has been previously reported that natural phenolics and lignans possess potential α-glucosidase inhibitory activities and display antidiabetic capacities [14]. Previously, *P. niruri* was reported to exhibit potent α-glucosidase inhibitory activity compared to other *Phyllanthus* species [13]. However, data reflecting the *in vivo* antidiabetic activity and mechanism of *P. niruri* in managing DM complications remain scarce. Thus, the aim of the present study was to identify the metabolic disorders in obese diabetic rats and evaluate the functionality and benefits of *P. niruri* extract on ameliorating the complications associated with type 2 DM using a ^{1}H NMR-based metabolomics approach. The individual metabolic changes resulting from the diabetic challenge were also studied using a targeted metabolomics approach. The variations in the excreted amounts of identified metabolites were examined by comparing diabetic rats with controls and investigating the correlations between detected metabolites in each group. An assimilated assessment of the variations in the quantities and synergistic effects of different metabolites may reveal the details of the pathways leading to complications associated with induced diabetes. The findings were also supported by detailed biochemistry data. This metabolic approach was implemented to fill the knowledge gap, concerning the possible effects of *P. niruri* extract on the urine composition of obese diabetic rats and the associated changes.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals and reagents used in this study include analytical-grade ethanol, potassium dihydrogen phosphate (KH_2PO_4), deuterated methanol-*d4* (CH_3OH-d_4), sodium deuterium oxide (NaOD), deuterium oxide (D_2O), and trimethylsilyl propionic acid-d_4 sodium salt (TSP), which were supplied by Merck (Darmstadt, Germany). Streptozotocin (STZ), carboxymethyl cellulose (CMC), sodium azide, and metformin were purchased from Sigma–Aldrich (St. Louis, MO, USA). The animal chow and high-fat diet ingredients were obtained from Gold Coin (Kuala Lumpur, Malaysia).

2.2. Characterisation of P. niruri extract

To process the *P. niruri*, the aerial part was first cleaned and stored overnight at −80 °C prior to freeze-drying to a constant weight. The dried sample was ground and sieved to small-sized powder. For extraction, the dried powder was mixed with 80% ethanol in a ratio of 1/25 v/w and sonicated for 1 h at room temperature. Then, the mixture was filtered twice through Whatman No.1 filter paper to remove the debris before concentrating the crude extract using a rotary evaporator and freeze-drying to remove excess water. The secondary and primary metabolites were identified in *P. niruri* extract using NMR and LC–MS and quantified by HPLC in our previous work [15,16].

2.3. Animal disease model

The diabetic rat model was obtained according to the established protocol [17]. For the preliminary study, acute toxicity studies and oral glucose tolerance tests (OGTTs) were performed (results not shown). For the animal experiments, male Sprague–Dawley (SD) rats were purchased from Chenur Supplier (Selangor, Malaysia) with weights ranging from 150 and 200 g and were housed in the animal housing facility of the Faculty of Medicine and Health Sciences, UPM. The procedures for the use and handling of the rats were approved by the Animal Care and Use Committee of the mentioned faculty (UPM/FPSK/PADS/BR-UUH/00493). The rats were kept comfortably in appropriate cages. The environmental conditions of temperature, relative humidity and light cycle were standard for normal and relaxed living. The rats had free access to food and water, except during overnight fasting for urine collection. At the beginning of the experiment, the rats were acclimatized for 1 week. All experiments were performed during the day. The high-fat diet was prepared three times per week in the processing laboratory of the Faculty of Food Science and Technology.

2.4. Experimental induction of diabetes in rats

After 1 week of adaptive feeding, the animals were randomly divided into the control and experimental groups as follows:

1. Normal diet (ND) rats gavaged with 0.03% CMC;
2. Obese (OB) rats gavaged with 0.03% CMC;
3. Obese + Streptozotocin (25 mg/kg body mass) (OBSTZ) rats gavaged with 0.03% CMC;
4. OBSTZ rats + 150-mg/kg metformin (OBSTZ-MET);
5. OBSTZ rats + 250-mg/kg *P. niruri* extract (OBSTZ-LD); and
6. OBSTZ rats + 500-mg/kg *P. niruri* extract (OBSTZ-HD)

The normal diet (ND) group rats were given the regular diet, and the experimental group rats were fed a high-fat diet consisting of 441 kcal of fat, 128 kcal of carbohydrate, and 7 kcal of protein. After 12 weeks of high-fat diet feeding and an overnight fasting, the rats were intravenously injected with ice-cold STZ. The STZ dosage was 25 mg/kg body mass for the obese rats. Three days after STZ injection, blood was collected from the tail tip, and the fasting blood glucose (FBG) level was determined. The rats with plasma glucose levels of 13 mmol/L or above were considered diabetic and selected for further pharmacological studies [18]. The animals were fed their respective diets until the end of the study.

2.5. Treatments

The animals were treated one week after being injected with STZ. Diabetic and normal rats were divided into groups of 12 rats each. After overnight fasting, the fasting blood glucose level (0 h) was measured. Subsequently, the rats were gavaged with CMC, as a negative control, and metformin and both doses of *P. niruri* extract (all dissolved in 0.03% CMC). Tubes containing 0.1% sodium azide were utilized for urine collection to avoid bacterial contamination. The total volume of urine excreted by each rat in 24 h was measured. The collected urine samples were stored at −80 °C prior to further analysis.

2.6. ^{1}H NMR analysis and preparation of urine

Urine samples were collected from the rats at week 12 (significant obesity induction; initial, I), week 13 (diabetes induction; baseline, B) and week 17 (after the treatment period; final, F). The thawed urine samples were centrifuged at 5000 rpm for 5 min to separate any debris. Then, 0.4 mL of the urine was mixed with 0.2 mL of KH_2PO_4 buffer in D_2O (pH 7.4) containing 0.1% TSP in a centrifuge tube and vortexed. The solution was centrifuged again at

Table 1
The content of metabolites in two tested doses of *P. niruri* extract.

Dose (mg/kg Bw)	Metabolites content (μg)							
	Ellagic acid	Catechin	Quercetin	Quercetin rhamnoside	Chlorogenic acid	Epicatechin	Phyllanthin	Hypophyllanthin
250	11.67 ± 0.94	0.41 ± 0.02	0.43 ± 0.05	0.15 ± 0.03	11.08 ± 0.36	43 ± 5.74	11.5 ± 0.54	5.79 ± 0.50
500	23.34 ± 1.89	0.82 ± 0.04	0.87 ± 0.11	0.30 ± 0.06	22.16 ± 0.72	86 ± 11.48	23 ± 1.07	11.58 ± 1.00

13000 rpm for 5 min, and then, 5.5 mL of the supernatant was transferred to an NMR tube and subjected to ^{1}H NMR analysis. The NMR spectra were obtained at 500 MHz (Varian Inc., California, USA). The preset parameters used to collect the ^{1}H NMR spectra consisted of 64 scans with an acquisition time of 3.53 min. The preparation procedure was performed as previously described [19]. In addition, two-dimensional (2D) J-resolved NMR was used to facilitate assigning the overlapped signals of some metabolites. The spectra were binned into small frequency windows and reduced into a dataset. The freely available Chenomx NMR Suite (v. 6.2, Alberta, Canada), the Human Metabolome Database (HMDB), and published data were adopted for the identification of the metabolites. The PCA loading plot showed the typical metabolites for each group of rats, which allowed the novel biomarkers for diabetic rats and the mode of action resulting from the consumption of *P. niruri* to be identified.

2.7. Blood collection and biochemical analysis of serum

The collection of serum samples for biochemical analyses was performed according to the timeline described for urine collection (initial, baseline, and final). The blood collected at the initial and baseline time points was obtained using a retro-orbital sampling technique. The rat was subjected to general anesthesia with ether, followed by the soft penetration of the orbital sinus of the eye using a special capillary tube. For the final collection, the blood sample was withdrawn by cardiac puncture. The red-topped serum-collection tubes were used without the addition of an anticoagulant. After collection, the blood was centrifuged at 6000 *g* for 10 min at 4 °C, and the resulting supernatant (serum) was immediately placed in a sterile (plain) tube. The serum samples were stored at −80 °C prior to analysis. The biochemical parameters of the rat serum were determined as described previously [17], including glucose, triglycerides, and cholesterol, which were examined using commercial kits from Spinreact (Gerona, Spain).

2.8. Statistical analysis

The NMR dataset was similarly binned to 365 points with a bin size of 0.04 ppm to minimize the impact of the variation in ionic concentration. The data were aligned to the TSP signal. The regions containing the urea (δ 5.55–5.95) and water (δ 4.60–4.85) signals were removed. In addition, the region from 4.50 to 5.14 ppm corresponding to an overlapped area of sugars was also removed to eliminate its previously reported impact as DM biomarker. The ASCII data of the NMR spectra of the urine samples were used as an independent variable (x) in multivariate data analysis. The Pareto scaling method was chosen to scale the data to give all the metabolite signals the same importance and reduce the effects of noise [20]. The NMR data were subsequently subjected to PCA using an unsupervised statistical method in Simca-P software 13 (Umeå, Sweden) to determine the variation among the samples. Significant differences among the individual metabolites were identified using *P* values obtained in the InStat V2.02 statistical package (GraphPad Software, San Diego, CA, USA). The heat map for correlation analysis, the hierarchical clustering analysis (HCA) and the variable importance in the projection (VIP) among the relative levels of significant metabolites were determined using MetaboAnalyst 2.5, which is freely available online metabolomics analysis software (http://www.metaboanalyst.ca). Furthermore, the correlations among all the 24 metabolites were also calculated using the Pearson test. The predictive abilities of the regression models were evaluated calculating their regression coefficients (R^2).

3. Results

3.1. Preliminary ^{1}H NMR spectroscopic data of P. niruri extract

The extract of *P. niruri* contained various bioactive metabolites, including epicatechin, ellagic acid, catechin, quercetin, quercetin rhamnoside, chlorogenic acid, phyllanthin, and hypophyllanthin. The amounts of the metabolites that had been previously quantified by HPLC and calculated for the two tested doses (250 and 500 mg/kg bw) are presented in Table 1. Other secondary and primary metabolites in *P. niruri* extract were identified by NMR and LC–MS in our previous work [15,16]. According to a visual inspection of the overlay NMR spectra of the urine samples of the treated groups, some signals were slightly shifted despite the use of a buffer to avoid the effect of pH. The spectra of urine samples and *P. niruri* extract were compared to determine whether they shared the same metabolite signals and thus directly identify some metabolites as present in the urine samples. However, no clearly analogous resonances were found.

3.2. Identification of metabolites in urine samples

The characteristic ^{1}H NMR signals and their multiplicities were determined with the help of 2D NMR and Chenomx library and revealed the identities of 23 metabolites in the urine of normal, obese and obese-diabetic rats. The metabolites identified included glucose, pyruvate, lactate, citrate, fumarate, succinate, 2-oxoglutarate, β-hydroxybutyrate, acetoacetate, acetone, acetate, dimethylglycine, malonate, alanine, hippurate, creatinine, dimethylamine, trimethylamine, allantoin, formate, taurine, betaine, and creatine. For the obese-diabetic group, the respective signals for taurine and creatine were difficult to assign because of their extensive overlap with the glucose signals. Other identified metabolites were similar for both normal and obese diabetic rats. Metabolites such as citrate, fumarate, succinate, 2-oxoglutarate, formate, acetate, malonate, hippurate and creatinine contributed to differentiating the obese from the normal groups. Additionally, differences in acetone, taurine, alanine, dimethylamine, allantoin, glucose, pyruvate, lactate, β-hydroxybutyrate, acetoacetate, creatinine and trimethylamine were observed between these two groups. Comparing the normal and obese-diabetic rats revealed that glucose, choline, taurine and creatine were the major biomarkers in the obese-diabetic group. Other metabolites assigned in ND rats were low in the obese-diabetic group, except for acetone. Between obese and obese-diabetic rats, significant increases in glucose and decreases in pyruvate, acetoacetate and acetate were noted.

3.3. Metabolite analysis of urine samples and biomarkers of treated and non-treated obese-diabetic rats

A supervised projection to latent structures-discriminant analysis (PLS-DA) was subsequently applied to identify the significant

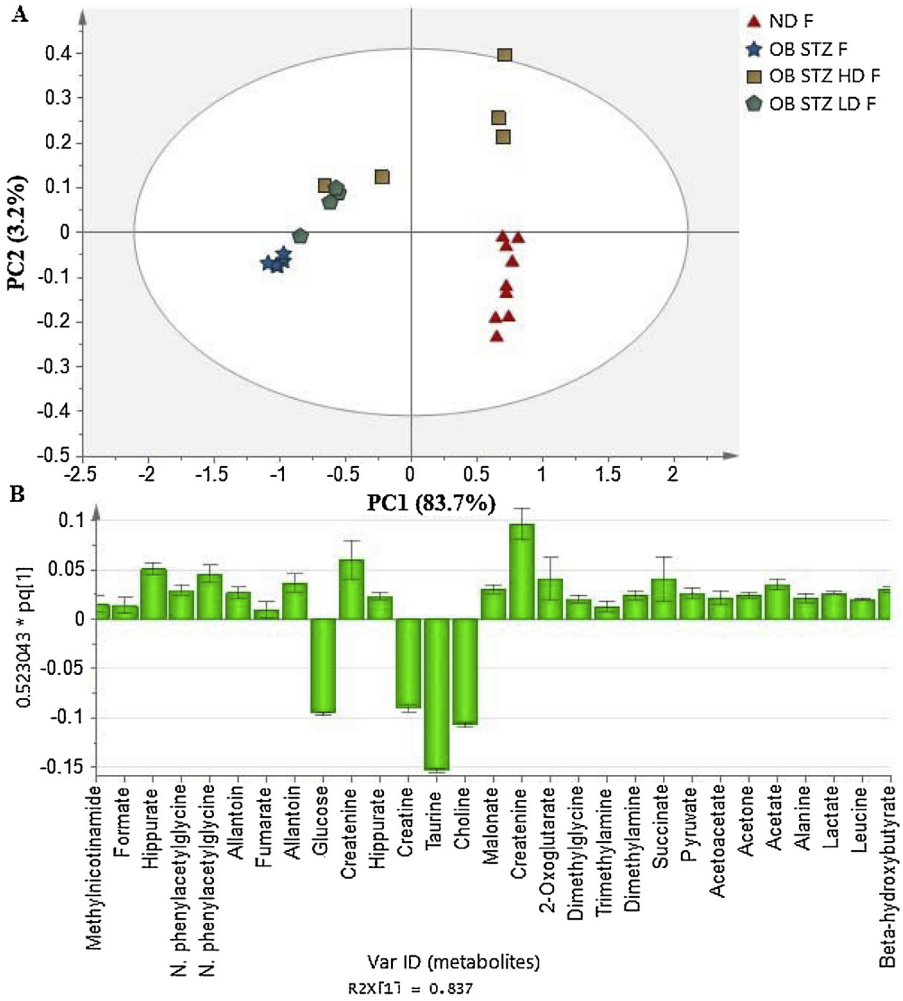

Fig. 1. The PLS-DA score (A) and loading column (B) plots of the effects of low and high doses of *P. niruri* extract on the obese diabetic rats after 4 weeks of treatment. ND: normal diet rats. OB STZ: obese diabetic rats. HD: 500 mg/kg of *P. niruri* extract. LD: 250 mg/kg of *P. niruri* extract. F: final (after 4 weeks of treatment).

metabolites associated with the high-fat diet and treatment with both metformin and *P. niruri* (Fig. 1). The score plot of the urine samples after treatment is shown in Fig. 1A. Both PC 1 and 2 described a total variance of 86.9% with high class discriminant R2Y and Q2 values of 92.3 and 87.8, respectively, indicating that the suggested metabolites were significantly affected by the treatment. The OBSTZ group is clustered separately on the left side of the score plot. It exhibited two different distinct clusters corresponding to the two diabetic groups (treated versus untreated). However, a clear separation occurred for OBSTZ treated with *P. niruri*, especially for HD, after the treatment period. The clusters were separated because of the variability of the diabetic biomarkers, especially glucose, choline, taurine and creatine in the untreated groups. Treatment with *P. niruri* helped to reduce the sugar level compared to that of the untreated group. Furthermore, the treatment with HD-*P. niruri* regulated and/or reduced the blood glucose to normal levels, as confirmed by the proximity of the HD-*P. niruri*-treated to the ND control group.

The trajectory of HD-*P. niruri* extract treatment displayed a clear shift to the left side of the score plot, where the datasets for the obese-diabetic group after treatment with *P. niruri* extract were clustered near the initial group before STZ injection (Fig. 2A). The same behavior was observed for the groups treated with HD-*P. niruri* and metformin (Fig. 2B). The metformin was used as a standard control drug for comparison with the extract and ensure that the obese-diabetic were met (Type 2 diabetes). Examining the loading plot for treatment with *P. niruri* extract revealed that the left cluster can be discriminated from the right cluster by its high glucose, choline, taurine and creatine levels. Based on the loading plot (Fig. 2C), the discriminating factors here are the high concentrations of formate and fumarate in the final treated group.

For the heatmap analysis, based on the results of the PLS-DA and VIP plots, the major metabolites contributing to the chemical classification of the animal model were determined to be primary and secondary metabolites, including taurine, choline, glucose, and alanine, along with other chemical compounds. Therefore, visual HCA was performed to simultaneously identify the metabolic discrepancies between the treated and non-treated rats. Clustering analysis is built on the concept of similarities. One way to mathematically define the similarity between two objects is based on the Euclidean distance, as adopted in this study. The data were Pareto scaled prior to being subjected to HCA with a Euclidean and clustering algorithm using the Ward method [21]. Using these data, the metabolomics pattern of the key metabolites was expressed as squares in a heatmap. These squares represent the metabolites, and their contents are indicated by colors based on a normalized scale from −3 (low) to 3 (high). The HCA result is visually displayed on the heatmap (Fig. 3A). The diabetic group exhibited higher levels of taurine, choline, glucose, and creatine and a lower level of formate alanine, lactate, in accordance with the above results. The treated groups had higher levels of other metabolites. These analyses were consistent with the separation of the animal model into

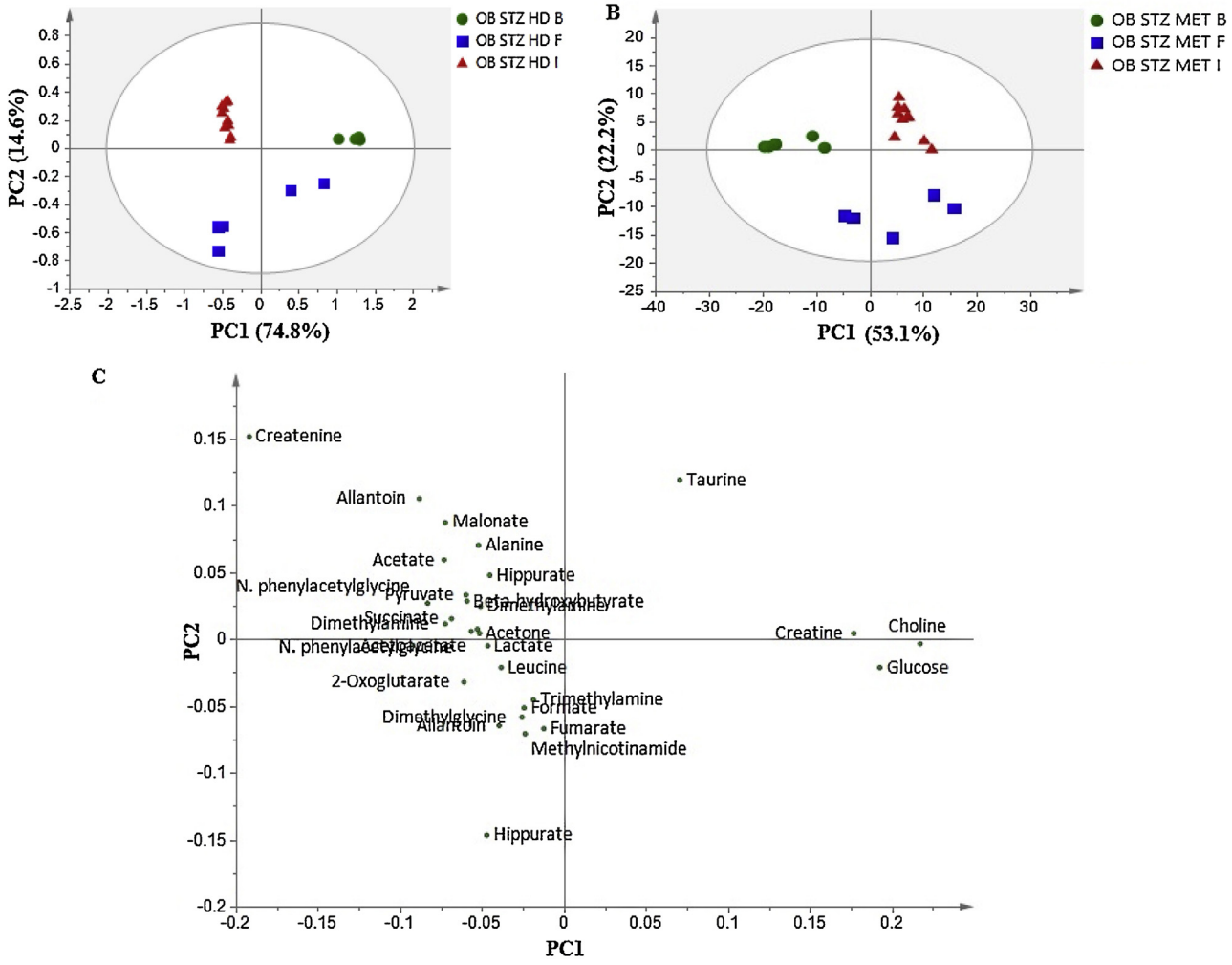

Fig. 2. The PCA score showing the trajectories of *P. niruri* treatment with HD (A) and metformin (B) and the loading plot of *P. niruri* treatment with HD (C). I: initial, before injecting STZ. B: baseline after injecting STZ. F: final after 4 weeks of treatment. OB STZ: obese diabetic rats. HD: 500 mg/kg of *P. niruri* extract. MET: metformin.

different groups based on the PLS-DA. To identify the most significant contributing variables, the VIP analysis of PLS-DA model was considered (Fig. 3B). A total of 15 metabolites met the criteria of being significant metabolites in the separation because their VIP values exceeded 0.5. The relative quantification of these individual metabolites was then achieved through normalization to the internal reference (TSP). The changes in the metabolite levels were quantitatively assessed between the treated and diabetic rats. The box plots of significant individual metabolites are presented in Fig. 4. The glucose, choline, taurine and creatine levels in the rats treated with *P. niruri* extract exhibited improvements compared to the diabetic rats.

3.4. Correlations among metabolites according to Pearson's correlation analysis

The urine metabolites derived using MVA were subjected to correlation analysis. The 15 most significantly ($P < 0.05$) changed urinary metabolites were quantified after PLS-DA and PCA analyses. The correlation among the metabolites was displayed by correlating their quantities separately for each group of rats using Pearson's correlation (Fig. 5). The correlation coefficients ranged from 0.1 to −0.1, and a value of 0 indicates no correlation. Clearly, glucose, taurine, choline, and creatine were highly correlated with each other. However, the variations in the diabetic and treated rats in the overall correlation plot were negative for these 4 metabolites relative to the other metabolites. Overall, the high positive correlation was displayed for most of metabolites rather the former 4 ones with one another. For instance, the positive correlations between succinate and 2-oxoglutarate and between beta-hydroxybutyrate with alanine, the pyruvates and other species were very strong, as indicated by the dark red color in the plot ($R > 0.9$). Fumarate and acetoacetate showed the lowest correlations with most of the metabolites, possibly because they are isolated from other metabolites, as indicated by the light-orange stripes in the correlation network by light orange stripes ($R < 0.1$).

3.5. Biochemical analysis

The levels of fasting serum triglycerides, cholesterol, HDL, LDL and glucose were analyzed and compared between the control groups and the treated ones (Fig. 6). The OB STZ groups treated with the two doses of *P. niruri* and metformin exhibited reductions in the tested parameters, except for HDL prior to treatment, and tended to return to normal levels relative to the OB control group. To investigate the impact of *P. niruri* on the lipid profile, the levels of blood glucose, creatinine, triglyceride, LDL and HDL were measured as shown in Fig. 6. The levels of blood glucose, creatinine, triglyceride, and LDL were remarkably high in OBSTZ relative to those in the control OB group, except for that of HDL. However for the groups treated with metformin and the two doses of *P. niruri*, these parameters' levels were improved by the treatment. The explana-

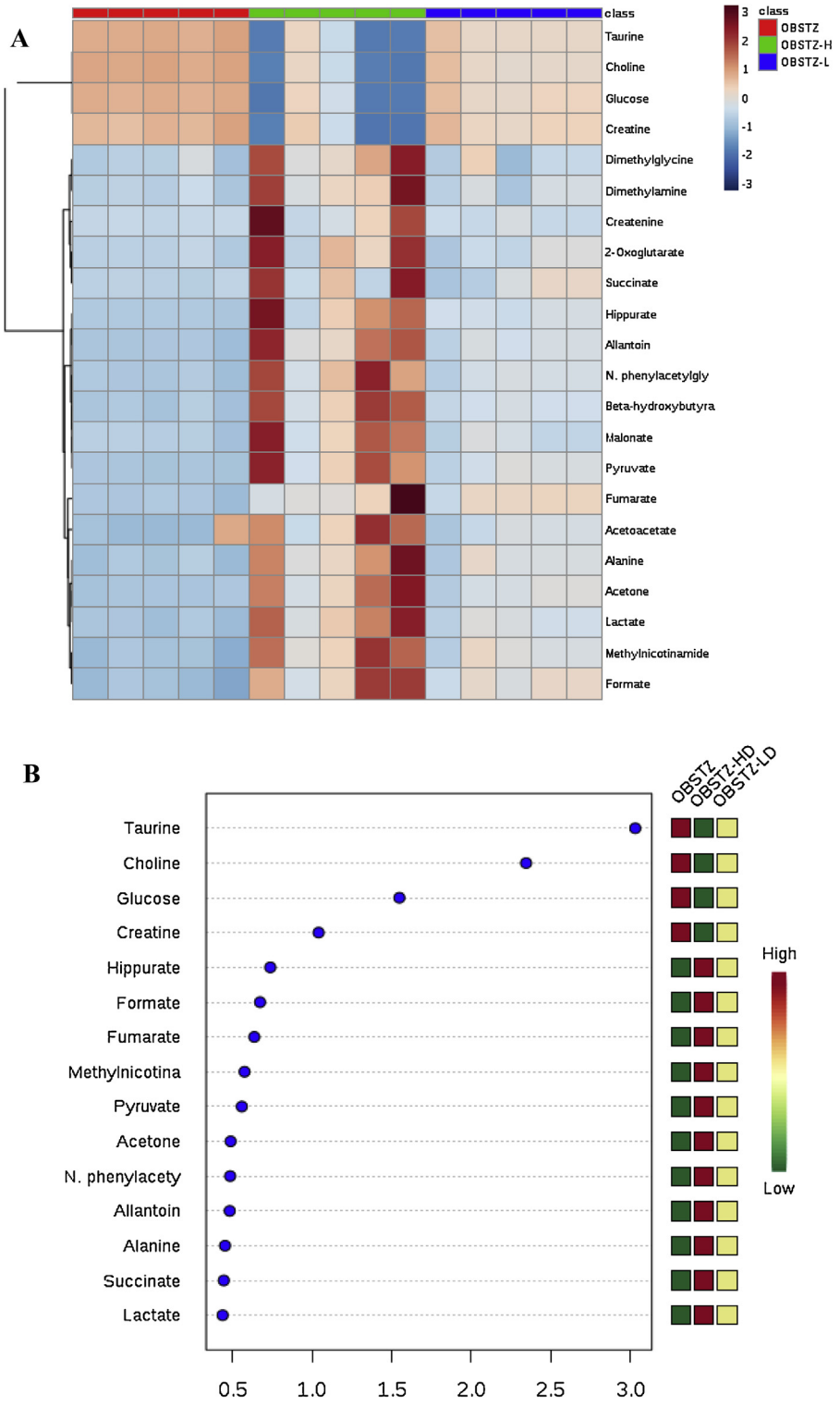

Fig. 3. (A) The heatmap of the effects of low and high doses of *P. niruri* extract on the treated obese diabetic rats after 4 weeks of treatment. (B) The VIP values derived from PLS-DA. OB STZ: obese diabetic rats. HD: 500 mg/kg of *P. niruri* extract. LD: 250 mg/kg of *P. niruri* extract.

tion for this observation is that *P. niruri* may prevent hyperlipidemia by decreasing lipid accumulation. The blood glucose levels of the OBSTZ groups after injection with STZ were higher than those of the OB control group and increased further in the non-treated groups during the 4 weeks of the treatment period. The blood glucose levels of *P. niruri*-treated diabetic rats with both LD and HD and metformin were significantly different from those of the OBSTZ control group. After the 4-week treatment period with both the LD and HD of *P. niruri*, significant reductions were observed.

The serum cholesterol and triacylglycerol levels of all OBSTZ groups after the STZ injection increased significantly compared to those of the OB control group. However, during the *P. niruri* treatment, the levels declined continuously, and prior to the end of the 4-week treatment period, significant reductions were observed in

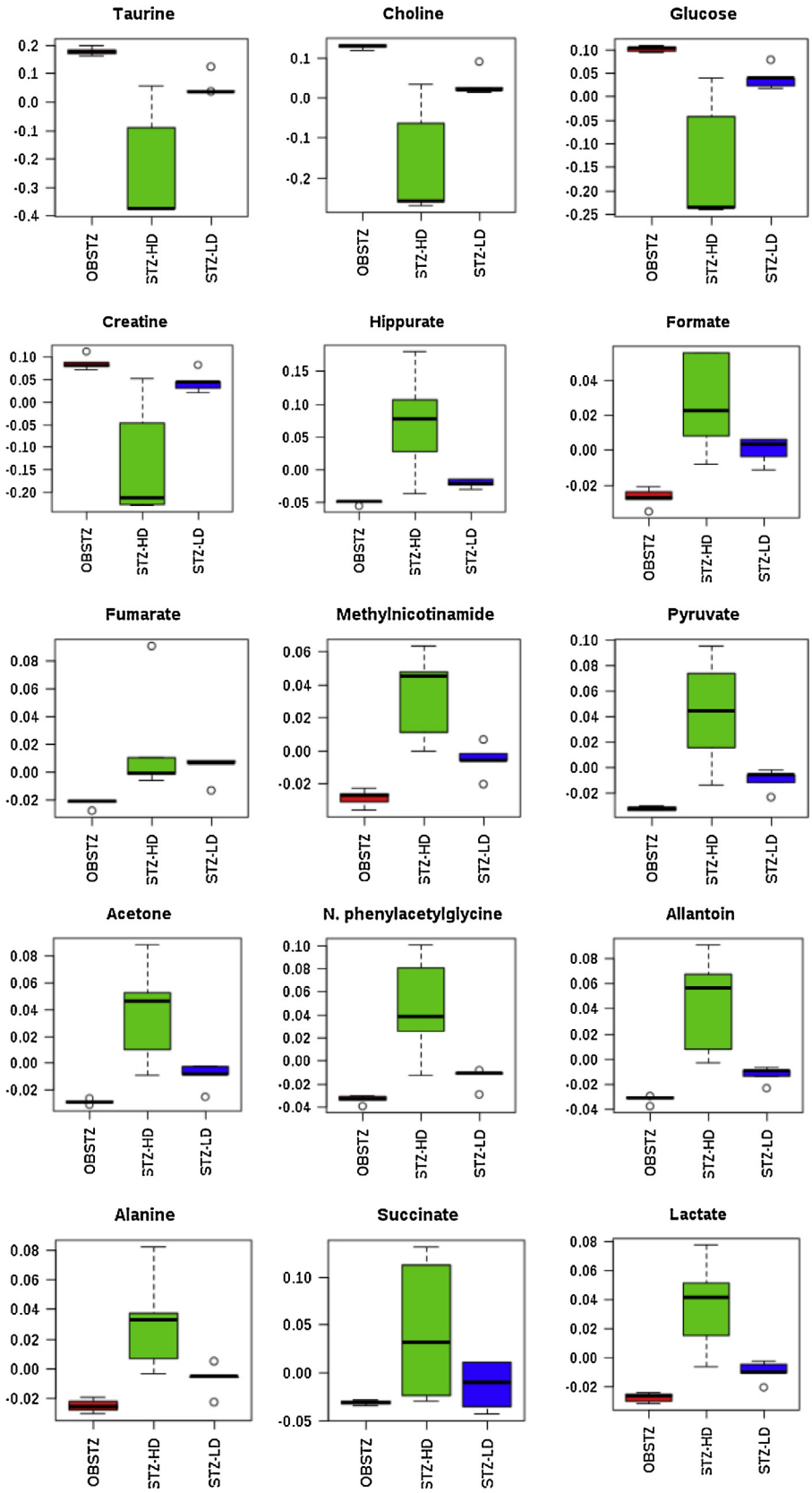

Fig. 4. The box plots of the relative quantities of the most significant metabolites in the urine samples from the treated and untreated groups.

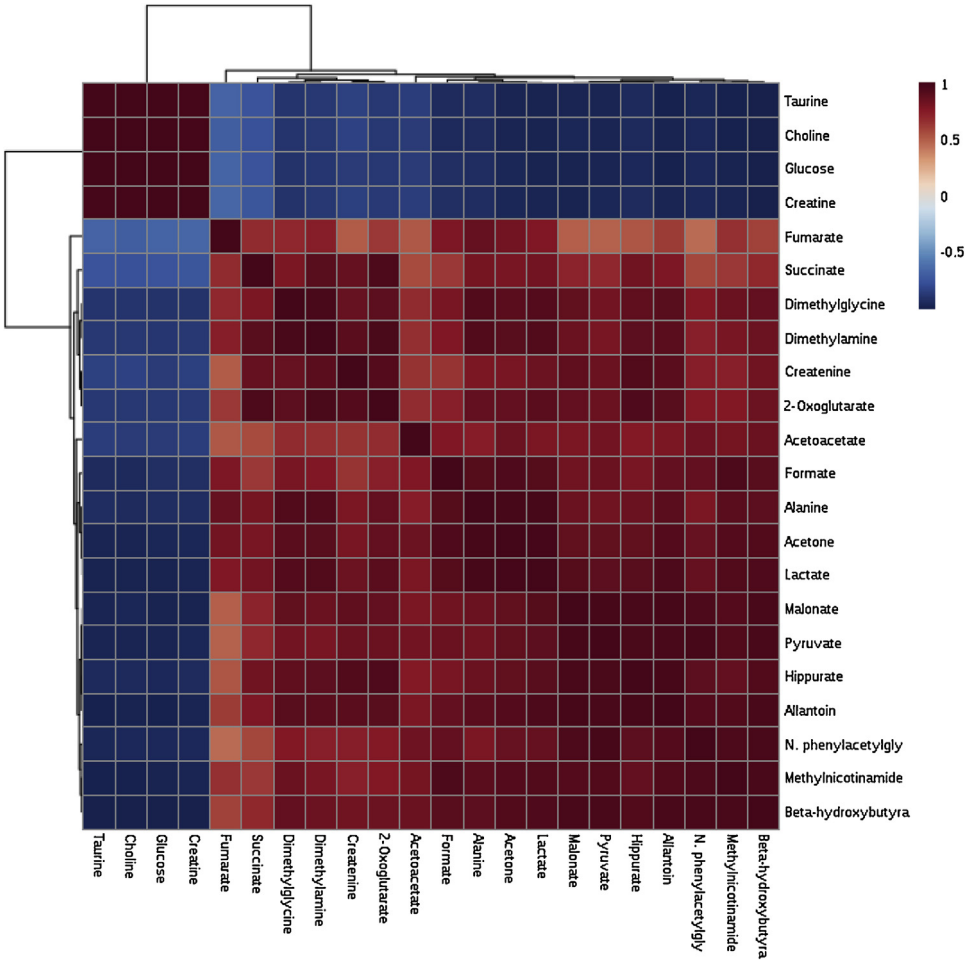

Fig. 5. The overall Pearson's correlation of the most significant metabolites in the rats' urine samples.

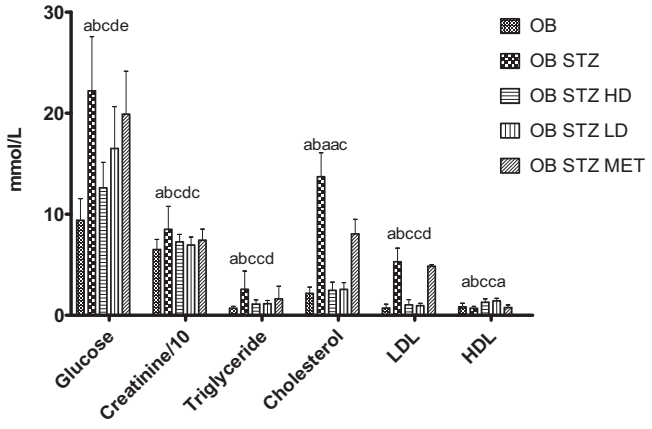

Fig. 6. The biochemical parameters of the serum of rats treated with *P. niruri* extract compared to controls after 4 weeks of treatment.
[a,b,c,e,f]The letters above the columns indicate that the results were statistically different ($P < 0.05$). OB: obese rats. OB STZ: obese diabetic rats. HD: 500 mg/kg of *P. niruri* extract. LD: 250 mg/kg of *P. niruri* extract. MET: metformin.

the LD and HD groups compared to the OB control group. The amelioration exerted by *P. niruri* in diabetic rats was evaluated in terms of blood glucose and serum creatinine. As shown in Fig. 6, the LD and HD of *P. niruri* could significantly reduce the blood glucose and serum creatinine levels. Treatment with metformin gave similar results to those obtained with both doses of *P. niruri*, which suggests that *P. niruri* can regulate the symptoms and complications of diabetes and may have a protective effect against this chronic disease.

3.6. Metabolite association and pathway analysis

To identify novel pathway abnormalities for DM pathogenesis, untargeted metabolomics is a useful technique. The insight and potential biomarkers returned by this approach can be used to extract the desired information about this disease and its complications. The MetPA coupled with METLIN and KEGG can be used for pathway analysis. This combined method revealed 3 metabolite hits in each of the 22 pathways (Fig. 7 and Supplementary Table S1). Butanoate metabolism; the citrate cycle; and glycine, serine and threonine metabolism included the most hits, with values of 0.5, 0.4 and 0.4, respectively. The synthesis and degradation of ketone bodies had the highest impact factor, followed by taurine and hypotaurine metabolism and glyoxylate and dicarboxylate metabolism, with impact factors of 0.60, 042 and 0.40, respectively. The pathway impact factor was calculated by considering the matched metabolites' importance in the network. Furthermore, a detailed pathway analysis was designed and proposed to explore the possible associations among the metabolic pathways of the significantly affected metabolites prior to treatment (Fig. 7B).

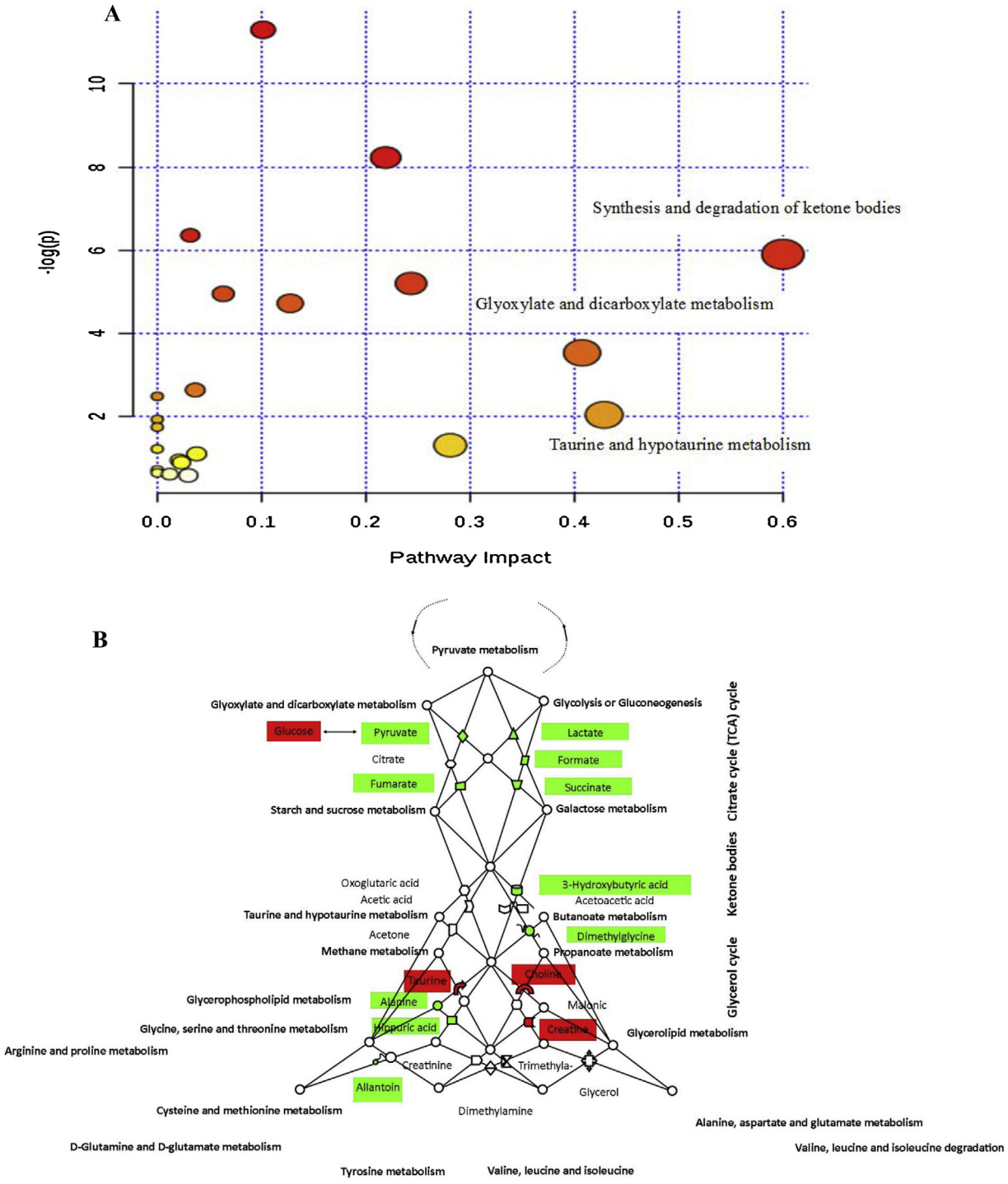

Fig. 7. (A) Summary of the pathway analysis. (B) Metabolic pathways and metabolite changes observed in the urine of obese diabetic rats treated with *P. niruri* extract compared to controls. Red indicates significantly decreased metabolites, and green indicates significantly increased ones. (For interpretation of the references to colour in this figure legend, the reader may refer to the web version of this article.)

4. Discussion

Obesity and diabetes are recognized to be associated with several similar causes. The disorders involved in these diseases are often related, and the main cause of type 2 DM is obesity [22,23]. In this study, obesity was induced in rats prior to evaluating the effects of diabetes and the treatments. Diabetes mellitus consists of the destabilized metabolic function of an organism characterized by disorder in the catabolism organic nutrients. Treatment with *P. niruri* extract, especially HD, was able to ameliorate the metabolic disorders of obese diabetic rats and make improvements towards the normal state after a treatment period of 4 weeks. This extract also caused the plasma glucose level to decrease and may have improved the lipid profiles and other biochemical parameters of obese diabetic rats. Furthermore, the bioactive metabolites in *P. niruri* extract (Table 1) may modulate many metabolic complications and exert direct or indirect effects in lowering the glucose level. This finding is in line with those of previous reports [24,25].

The increase in the hippurate content prior to treatment with the *P. niruri* extract observed here may be associated with the phenolics in this extract [23].

The plasma glucose levels of the diabetic rats were high after they were injected with STZ and could have resulted in some other changes in biochemical parameters and metabolites corresponding to diabetes complications and/or metabolites related to glucose metabolism. The treated and untreated groups showed distinct changes in their metabolites after treatment with *P. niruri* extract at both doses. The heatmap and statistical correlation of the 15 significantly contributing metabolites in the separation revealed the trend observed in the PLS-DA analysis and showed the biomarkers of diabetes and treatment with *P. niruri* extract. The suggested pathways and complications associated with these correlations are further discussed in detail.

4.1. Amino acid metabolism

The results showed increases in alanine and other amino acids prior to treatment with *P. niruri* extract, and the most significant difference was observed for alanine between treated and control rats (Fig. 7B). Diabetes is known to cause increases in several amino acids [26,27], which can improve insulin secretion by β-cell lines and primary islet cells through various mechanisms. Amino acids are considered to be of primary importance among the most vital biological compounds because of their functional groups, which include carboxylic acid and amine moieties. They are also involved in proteins and hormones and play prominent metabolic and physiological roles in organisms.

4.2. Lipid metabolism

The current study results revealed increases in the choline and taurine levels in OBSTZ rats, possibly because of increased lipid metabolism and the shift in energy metabolism from glucose to lipids. The levels of these species decreased treatment with *P. niruri* extract, suggesting a return to the normal state. The elevated lipid levels and their derivatives in the blood can indicate the prevalence of several chronic diseases, including diabetes. Lipids are among the metabolized energy sources used by organisms. Most of the lipids in adipose tissue and the liver or obtained by consuming food are cholesterol and fatty acid derivatives. The disruption of lipid metabolism is known to occur in diabetes, especially type 2 DM. It was also previously observed that body fat mass and lipids associated with diabetic dyslipidemia are the principal predictors of metabolic disturbances and serious medical conditions [4,5]. Indeed, diabetic dyslipidemia can be caused by the effects of the peripheral activities of insulin in muscle and adipose tissue.

4.3. Carbohydrate metabolism

The results revealed a significant increase in the glucose levels in the OBSTZ rats compared to those in the control groups and that these levels decreased was in the groups with treated *P. niruri* extract. This trend could result from the effects of *P. niruri* extract on the pathways related to carbohydrate metabolism. Carbohydrate metabolism is the most important metabolic pathway because of its role in regulating and modulating cellular energy production in organisms. Excessive intake of high-calorie and high-fat diets is usually associated with the development of obesity [8]. However, the consumption of dietary carbohydrates in the management of obesity has recently received increased attention [28]. The reduced glucose removal in adipose tissue is mostly associated with obesity, which is usually connected to the development of diabetes and its complications. In addition, it is also caused by hyperglycemia, insulin resistance, hyperinsulinemia, and hyperlipidemia [2]. The gluconeogenesis, glycolysis, pentose phosphate pathway and TCA cycle are the pathways that make up carbohydrate metabolism, which is necessary for all metabolic processes. The catabolism of glucose *via* the glycolysis pathway produces pyruvate, which requires aerobic conditions for conversion to the acetyl coenzyme A necessary for the TCA cycle. The concentration of blood glucose is a reflection of the carbohydrate metabolism in healthy and diabetic humans. Thus, the intake of carbohydrate and its sources must be controlled to manage diabetes [5].

4.4. Tricarboxylic acid (TCA)

From the results, the treatment with *P. niruri* extract increased the levels of TCA intermediates, possibly indicating an increase in glycolysis and decrease in gluconeogenesis and, thus, improvement of the TCA cycle. The TCA cycle is also recognized as the citric acid cycle and consists of chains of anabolic and catabolic biochemical pathways in the mitochondria. The regulation of TCA cycle is strongly associated with electron transport and is tightly correlated with ATP production. Furthermore, this cycle is highly important for the production of energy *via* the oxidation of acetyl-CoA to several intermediary metabolites and the aerobic oxidation of most carbohydrate as well as fatty and amino acids intermediates. However, some of the important metabolic processes occur in the mitochondria and are linked to the regulation of the TCA cycle, including gluconeogenesis, lipogenesis, cholesterol synthesis and the conversion of amino acids [29]. Therefore, the complications of type 2 diabetes may arise because of the effects of this cycle on most of the metabolic pathways in the mitochondria.

4.5. Glyoxylate and dicarboxylate metabolism

Our results revealed that the synthesis and degradation of ketone bodies represented the most affected pathway, possibly because of fatty acid oxidation. The metabolic intermediate β-hydroxybutyrate is also known as 3-hydroxybutyric acid and composes approximately 70% of the ketone bodies produced in the mitochondria of the liver. This production mainly occurs *via* the oxidation of fatty acids from adipose tissue [30]. The oxidation of ketone body metabolites proceeds *via* the TCA cycle to produce the required energy. Because of the harmful and unmanaged utilization of glucose in diabetic patients, the oxidation and decomposition of fatty acid leads to the production of ketone bodies.

5. Conclusions

Here, we attempted to apply metabolomics to evaluate the effects of two doses of *P. niruri* extract on the management of type 2 diabetes complications. Treatment with *P. niruri* extract dropped the blood glucose to near normalcy. These findings suggest that *P. niruri* extract may improve insulin sensitivity. Furthermore, *in vivo* result revealed that *P. niruri* showed a significant reduction in blood glucose of obese diabetic rats and improved other biochemical parameters. The findings of this study support the functional and traditionally reported benefits of *P. niruri* in treating diabetes.The ^{1}H NMR-based metabolomics constituted a useful tool for comparing the biofluids of obese diabetic rats with those of normal rats to identify the biomarkers for these diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.06.003.

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