DOI: 10.15806/j.issn.2311-8571.2015.0015 World J Tradit Chin Med 2015; 1(3): 9–20

Metabolomics Analysis of Health Functions of Physalis Pubescens L. using by Ultra-performance Liquid Chromatography/Electrospray Ionization Quadruple Time-of-Flight Mass Spectrometry

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ABSTRACT

Herbal medicines may benefit from metabolomics studies, and applying metabolomics may provide answers about which herbal interventions may be effective for individuals, which metabolic processes are triggered, and the subsequent chemical pathways of activity. *Physalis pubescens* L (PPL) is an herbal fruit for one year living plant and has been developed into healthy function's food. However, the mechanisms of health functions are still unclear. To comprehensively and holistically assess its anti-fatigue and antioxidant effects, a novel integrative metabolomics approach was applied. In this study, we present metabolomics analysis applying ultra performance liquid chromatography coupled to quadrupole with time-of-flight mass spectrometry (UPLC-Q/TOF-MS) to determine metabolite alterations after oral administration PPL to rats. Fifteen metabolites in urine were identified as potential biomarkers. Pattern analysis of the UPLC-Q/TOF-MS data disclosed that PPL could relieve fatigue rats by ameliorating the disturbance in amino acids metabolism and energy metabolism, alleviating the oxidative stress from reactive oxygen species and the inflammatory damage, and recovering the destructed regulation. Based on these results, we demonstrated that *PPL* is a promising source of natural anti-fatigue and antioxidants material for use in functional foods and medicines.

Key words: Metabolomics, *Physalis pubescens* L, antioxidant, anti-fatigue, metabolic pathway, biomarkers

INTRODUCTION

Physalis pubescens L (PPL, **Figure 1A**) is an herbaceous fruit for one year living plant. The plant mainly grown in the edge of the forest and meadow and cultivate in the northeast of China. It was sweet and sour taste and contains several vitamins and amino acids. In view of rich in nutrients, edible convenient, PPL could be developed into healthy care function's food. In recent years, the search for natural antioxidants originated from plants that were used to substitute synthetic antioxidants has been a hot topic[1]. Natural antioxidants not only can be used for cure diseases, but also for food fortification, as a functional food or dietary supplement[2]. PPL as a common fruit, is a kind of natural antioxidants which represents a potentially source with a wide range of edible. The exhaustive swimming and climbing pole test have been used as experimental exercise models to evaluate anti-fatigue of food[3]. However, there is no report on the effects of PPL on resistant sport fatigue. The literature data has rarely demonstrated that the PPL fruit (**Figure 1B**) possess *in vivo* antioxidant activity. Thus, experiments for predicating the possible use of PPL as natural antioxidant sources in an in vivo model are required.

Metabolomics, an omic science in systems biology, is the comprehensive profiling of metabolic changes occurring in living systems[4]. The field of metabolomics continues to grow rapidly in the past ten years and has been shown to be a powerful technology in complex phenotypes prediction and interpretation in variety biological systems[5]. It is the scientific technology to study metabolic pathways and measure unique biochemical molecules generated in a living system[6]. It may be a powerful tool to generate a metabolic fingerprint in body fluids at a given time point to compare metabolic physiological changes[7]. Metabolic profiling of urine is positively absorbing because urine collection is non-traumatic, and urine contains metabolic charateristics of many biochemical pathways[8]. The non-targeted approach of urine could provide new in-take biomarkers, contributing to the development of the food metabolome[9]. Urine metabolomics studies serve in two different but closely related modes: as a way to identify metabolites as potential biomarkers distinguishing the food metabolome from normal and as a metabolic fingerprinting tool separating groups based on metabolic patterns change[10]. By applying non-targeted urine metabolomics through UPLC-Q/TOF-MS, we aimed to study metabolic biomarkers of PPL versus normal controls. The main aims of this work were to evaluate the *in vivo* antioxidant activity and anti-fatigue and anti-hypoxia capacities of the fruit from PPL with a view to its potential

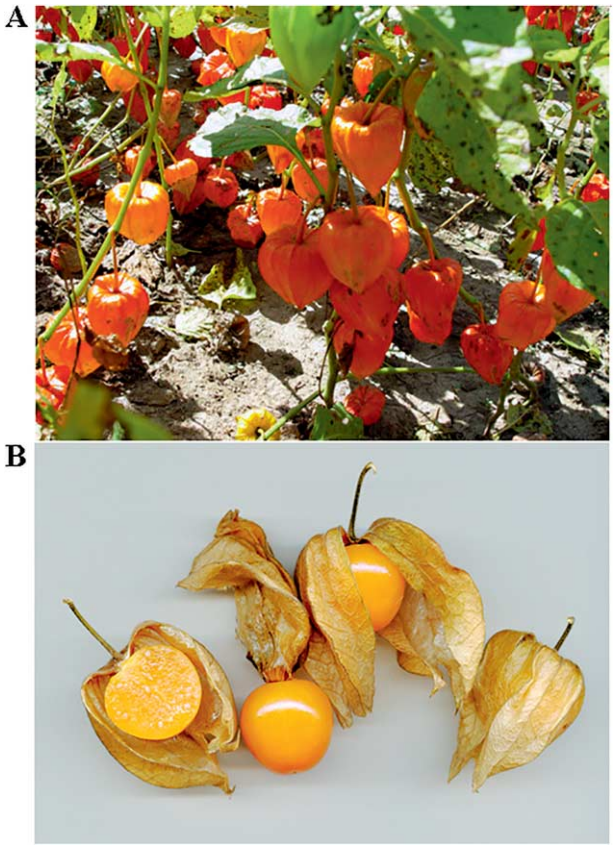

Figure 1. *Physalis pubescens* L (A) and its fruit (B).

use in functional foods and medicines, the urinary metabolomic was determined by UPLC-Q/TOF-MS. To the best of our knowledge, all these are reported for the first time.

MATERIALS AND METHODS

1. Chemicals and reagents

Methanol and ACN (HPLC grade) was purchased from Merck (Darmstadt, Germany). Leucine enkephalin was purchased from Sigma-Aldrich (MO, USA). Other reagents and chemicals were of analytical grade. Deionized water was purified on a Milli-Q system (Millipore, Bedford, USA). The assay kits for blood lactic acid (BLA), urea nitrogen (BUN), hepatic glycogen (HG), superoxide dismutase (SOD), lutathione peroxidase (GSH-PX) and malondialdehyde (MDA) were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals and reagents used in this study were of analytical grade and made in China.

2. Plant material and preparation

The plant material, PPL fruit were purchase from Harbin farmers market. All crude drugs were authenticated by Prof. Xijun Wang, department of pharmacognosy of Heilongjiang University of Chinese Medicine. Fresh PPL fruit bodies were squeezed into juice, then filter the seed. Seeds were vacuum-dried in 65 °C and ground into powder. Fine powder was suspended in the fruit juice, as low dose oral solution. In rotary evaporation instrument reduced pressure concentration, as PPL middle dose oral solution. Concentrate on dose in the twice as high dose oral solution.

3. Animal handing and sampling

Male KM mice (20 ± 2 g body weight) and Wistar-derived rats (200 ± 20 g body weight) in clean grade were provided by the Laboratory Animal Center of Heilongjiang University of Chinese Medicine (Harbin, China). Rats were housed individually in metabolism cages. Animals were housed in controlled environmental conditions (temperature, 24 ± 2 °C; relative humidity, 55 ± 5%), and under a 12 h light/12 h dark cycle conditions, free access to standard diet and water. The experimental animals were housed under the above conditions for 1 week acclimatization and fasted for 12 h with free access to water, prior to the experiments, rats were put in the metabolism cages during the urine collection periods specified below. All the mice and rats were randomly divided into 4 groups as follows: control group (distilled water), low-dose group (8.35 mL/kg PPL, LG), middle-dose group (16.7 mL/kg PPL, MG), high-dose group (33.4 mL/kg PPL, HG). Sample was administered i.g. once a day for 30 days. Urine samples were collected predose (− 24 to 0 h) and 30th d. Urine samples were centrifuged at 13000 rpm (4 °C) for 15 min to remove any solid debris. The urine supernatants were frozen at −80 °C prior to LC-MS analysis. After all urine samples were collected, 5 urine samples were randomly selected from the urine collected on the last day of each treatment group and mixed together as the ''quality control'' (QC) sample. All experiments were performed in accordance with the approved animal protocols and guidelines established by Medicine Ethics Review Committee for animal experiments of Heilongjiang University of Chinese Medicine (HUCM-2014768).

4. Anti-fatigue test

To determine anti-fatigue activity, the mice were placed individually in a square swimming pool. The details of this apparatus were an acrylic plastic pool (70 × 70 × 60 cm) filled 35 cm deep with water maintained at 25 ± 5 °C. 10 mice were randomly selected from each dose group after 30 min at the late of administration in mice, a weight equivalent to 5% of body weight was attached to the root of mouse tail. Mice were judged to be fatigued when they failed to rise to the water surface to breathe within 8 s period as the index of swimming capacity, recorded from the beginning of the time in the pool to exhaustion. Pole-climbing test were performed to assess motor function. Use organic glass rod as climbing rod rack. The mice were allowed to seize on the top of the rod. The time were record from climbing pole until muscle fatigue and fell, three times of continuous, the sum of the time take into account.

5. Determination of biochemical variables for anti-fatigue activity

5.1 Determination of blood lactic acid (BLA)

Blood samples were obtained from eyeball of mice. Ten mice were randomly selected from each group until 30 days. The mice swam for 60 minutes without weight after orally

administered 30 minutes. Blood was obtained by eyeball of mice after 30 minutes rest. The serum was prepared by centrifugation at 4000 rpm for 15 min at 4 °C after coagulation at room temperature 10-20 minutes. The supernatants were removed, stored at − 20 °C and thawed before analysis. The BLA activities in serum were determined using commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

5.2 Determination of blood urea nitrogen (BUN)

Blood samples were obtained from eyeball of mouse. Ten mice were randomly selected from each group until 30 days. The mouse swam for 90 minutes without weight after orally administered 30 minutes. Blood was obtained by eyeball of mouse immediately. The serum was isolated to measure BUN. The BUN activities in serum were determined using commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

5.3 Determination of hepatic glycogen (HG)

Ten mouse were randomly selected from each group and sacrificed by breaking the neck after oral determination. Liver samples were collected rapidly and rinsed with physiological saline solution to remove the blood or content, blotted on filter paper, and weighed 100 mg, and then stored at − 80 °C until analysis. The HG activities in serum were determined using commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

6. Anti-hypoxia Assay

Hypoxia tolerance test under ordinary pressure was taken in mouse. 10 mice were randomly selected from each dose group after 30min at the late of administration in mouse. Put mouse in 250 ml ground glass bottles which have good pressurizing capability. Each bottle only put one mouse, in order to cause anoxic environment, gauze bag with 15 g of sodium lime be loaded into the bottle to absorb moisture and CO_2. Immediately hermetic seal tightly with vaseline after the mouse was put into the bottle, the time was recorded from seal to systemic convulsion of mouse. Immediately remove the mouse, when it began to systemic spasm symptoms. Chemical hypoxia-resistance assay was taken by sodium nitrite poisoning experiment. 10 mice were randomly selected from each dose group. After 30 min at the late of administration in mouse, animals were taken intraperitoneal injection of sodium nitrite by 250 mg/kg. Record the time from injection until the mouse died as index.

7. Antioxidant enzyme activities

The whole blood samples were collected under heparin treatment from the caudal vein 30 minutes later after the last oral administration, and the samples were used for measuring the activity of lutathione peroxidase (GSH-PX). The serum samples were collected after anesthetized by sodium pentobarbital from the inferior vena cava. After leaving to rest for 30 minutes, the blood serum was prepared by centrifugation at 4000 rpm and 4 °C for 10 min. The superoxide dismutase (SOD), Malondialdehyde (MDA) activities in serum and GSH-PX activity were determined using the respective commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

8. Metabolic profiling

8.1 Chromatography and MS conditions

Chromatographic analysis was performed in a Waters Acquity^{TM} Ultra Performance LC system (Waters, Milford, USA) and equipped with quaternary pump, vacuum degasser, autosampler, diode-array detector. The system was controlled with MassLynx (V4.1). An aliquot of 4 μL of sample solution was injected into an ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) held at 45 °C and the flow rate was 0.4 mL/min. The mobile phase consisted of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid with gradient elution (linear gradient from 99% B to 89% B in 2.5 min, followed by linear gradient from 89% B to 15% B between 2.5 and 4.5 min, and linear gradient from 15% B to 26% B between 4.5 and 7 min, from 26% B to 43% between 7 and 7.5 min, from 43% B to 99% B between 7.5 and 10 min, from 99% B to 1% B between 10 and 11 min, from 1% B to 99% B between 11 and 11.5 min, finally followed by a hold at 99% B until 13 min.

Mass spectrometry detection was performed using a Waters Acquity^{TM} UPLC system equipped with an ESI ion source operating in both positive and negative ion modes. The full-scan data were acquired from 50 to 1000 Da with a 0.40 s scan time and a 0.1 s inter scan delay over a 13 min run time, using a capillary voltage of 2800 V, desolvation temperature of 300 °C, sample cone voltage of 30 V for positive ion mode and 35 V for negative ion mode, extraction cone voltage of 3.5 V for positive ion mode and 3.0 V for negative ion mode, source temperature of 110 °C, cone gas flow of 50 L/h and desolvation gas flow of 600 L/h. Data were centroided and mass was corrected during acquisition using an external reference (Lock-Spray^{TM}) consisting of a 0.2 ng/mL solution of leucine enkephalin infused at a flow rate of 100 μL/min via a lockspray interface, generating a reference ion for positive ion mode ($[M+H]^+$ = 556.2771) and negative ion mode ($[M-H]^-$ = 554.2615) to ensure accuracy during the MS analysis. All data collected were acquired using MassLynx^{TM} (V4.1) software in centroid format. The metabolite identifications were confirmed by comparing the retention time under the same chromatographic conditions and by matching the fragmentation pattern of the parent ion from the biological sample to that of the standard metabolite using tandem mass spectrometry (UPLC–QTOF-MS/MS). To optimize the conditions of UPLC-Q/TOF-MS, the QC sample was used, as it contained the most information of whole urine samples.

8.2 Multivariate data analysis and data processing

After UPLC-QTOF-MS analyses, the raw data were imported into the MassLynx software for peak detection and alignment. For data analysis, The individual ion intensities were normalized with respect to the total ion count (TIC) to generate a

database that covered retention time, mass data (m/z) , and the normalized peak area. Multivariate data matrices were generated from the procession by EZinfo software (Waters Corporation) for the loading plots of principal component analysis (PCA), orthogonal projection to latent structures discriminate analysis (OPLS-DA) analyses. From the OPLS analysis, the magnitude of the parameter obtained correlates with the group discriminating power of a variable. A list of ions showing considerable group discriminating power was generated from the loadings S-plot and variable importance plot (VIP) for metabolic pathway analysis.

8.3 Biomarkers identification

PCA and OPLS-DA were applied for identify various metabolites as being responsible for the separation, and were therefore viewed as potential biomarkers. To help identify the metabolite molecular mass, exact molecular mass data from redundant m/z peaks were first used. Metabolite peaks were assigned by MS/MS analysis and matched with available biochemical databases. Used the MassFragment™ application manager (Waters Corp., Milford, USA) to facilitate the MS/MS fragment ion analysis process by way of chemically intelligent peak-matching algorithms, and then, combined with searching available biochemical databases for possible candidates of the ions, the potential markers were confirmed.

8.4 Construction of metabolic pathway

In order to better understand the construction and interaction among various metabolites, Ingenuity Pathway Analysis (IPA) of potential biomarkers were performed. MetPA (http://metpa.metabolomics.ca.), is a free web tool based on database sources including the KEGG and HMDB database to identify the most relevant pathways analysis and make it visually.

9. Statistical analysis

Experimental data were expressed as mean ± standard deviation (SD). The statistical analysis was performed using the SPSS software (Version 20 for windows, IBM, Chicago, IL). Differences between groups were tested for statistical significance, followed by least significant difference (LSD) test and independent differences sample *t*-test. The result of $P < 0.05$ or $P < 0.01$ was considered statistically significant.

RESULTS

1. Anti-fatigue effect analysis

The anti-fatigue behavioral assay of the PPL fraction was measured as the swimming endurance capacity of mouse using a swimming pool for measuring maximal swimming time. The endurance of mouse administered control, LG, MG and HG groups were 11.75 ± 3.25, 14.75 ± 6.25, 59.83 ± 9.51 and 61.42 ± 10.53 min, respectively (**Figure 2A**). MG and HG groups of PPL administration were significantly different ($P < 0.05$) compared with the control group. The total pole-climbing test scores were 6.13 ± 1.98, 6.73 ± 1.51, 12.96 ± 3.19, 19.85 ± 5.11min for control, LG, MG and HG group, respectively (**Figure 2B**). M and H groups of PPL administration were significantly different ($P < 0.01$) compared with the control group.

2. Effect of PPL on BLA, BUN, and HG levels with exercise in mouse

BLA levels in the control, LG, MG and HG groups were 4.77 ± 1.05, 4.53 ± 0.75, 3.02 ± 0.67, 2.42 ± 0.96 mmol/L, respectively (**Figure 2C**), and were significantly lower, by 37% ($P < 0.01$) and 49% ($P < 0.01$), with MG and HG group, respectively, than control treatment. BUN levels in the control, LG, MG and HG groups were 6.78 ± 1.26, 6.22 ± 0.57, 5.15 ± 0.41, 4.47 ± 0.48 mmol/L, respectively (**Figure 2D**), and were significantly lower, by 24% ($P < 0.01$) and 34% ($P < 0.01$), with MG and HG group, respectively, than control treatment. HG levels in the control, L, M and H groups were 3.22 ± 0.67, 3.71 ± 0.69, 4.79 ± 0.82, 4.94 ± 0.64 mg/g, respectively (**Figure 2E**), and were significantly higher, by 1.49- ($P < 0.01$) and 1.53-flod ($P < 0.01$), with MG and HG group, respectively, than control treatment. Trend analysis revealed that PPL treatment had a significant dose-dependent effect on decreasing blood lactic acid content and blood urea nitrogen content and increasing hepatic glycogen levels ($P < 0.01$ for MG and HG group, compared with control group).

3. Anti-hypoxia behavioral assay

The results of hypoxia tolerance test under ordinary pressure and chemical hypoxia-resistance effect were shown in **Figure 2F,G**. As the data represent, high-dose group of PPL could obviously prolonged the survival time of hypoxic mouse ($P < 0.01$) and MG, HG could remarkably prolonged the survival time of mouse subjected to sodium nitrite poisoning ($P < 0.05$). To conclude, PPL could significantly postpone living times while mouse was in normal or chemical hypoxia.

4. Effect of PPL on GSH-PX, SOD and MDA levels

The GSH-Px activities in blood of four groups rats after experiment are represented in **Figure 2H**. The GSH-Px activities of MG and HG were increased significantly compared with control group (CG) ($P < 0.01$) and was not significant in LG ($P > 0.05$). The SOD activities in serum of four groups rats after experiment are shown in **Figure 2I**. Compared with CG, an significant increase were observed for SOD activities of three PPL administration groups (respectively high-dose, middle-dose and low-dose groups) ($P < 0.01$ or $P < 0.05$). The levels of MDA in serum of rats are shown in **Figure 2J**. A significantly decreased level of MDA content of was observed in MG and HG compared with CG ($P < 0.01$) was not significant in LG ($P > 0.05$).

5. Metabolomics Study

5.1 LC-MS analysis of metabolic profiling

Global metabolic profiling in both positive and negative ion modes were analyzed by LC-MS, and 3205 ions including ESI+ and ESI- ions were obtained. Using the optimal LC-MS condition described above, the comparison of the four base peak ion (BPI) chromatograms obtained from the four urine

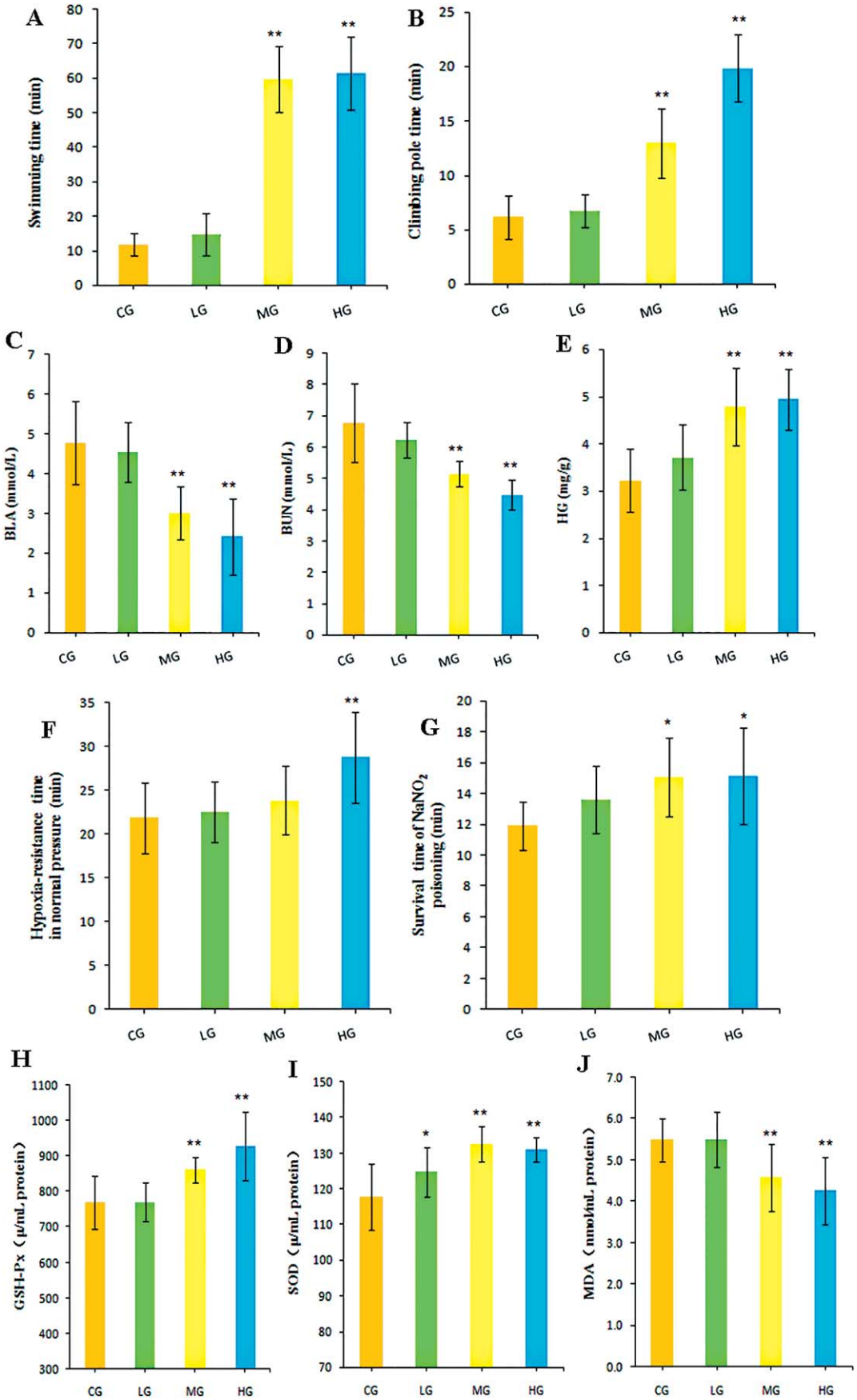

Figure 2. Antioxidant, anti-fatigue and anti-hypoxia effects of *Physalis pubescens* L. Effect of PPL on the mice swimming time (A), Effect of PPL on the climbing pole time in mice (B); Effects of PPL on BLA (C), BUN (D) and HG (E) levels in mice; Effect of PPL on the hypoxia tolerance test under ordinary pressure (F), Effect of PPL in vivo on sodium nitrite poisoning (G); Effect of PPL in vivo on the SOD (H), GSH-Px activities (I) and MDA content (J) in blood. Results are expressed as a mean ± SD (n = 10). $*P < 0.05$, $**P < 0.01$ compared with the control group (CG). HG: High-dose group; MG: Middle-dose group; LG: Low-dose group.

samples. The PPL group and the control group generated distinct metabolic phenotypes were showed in unsupervised PCA of the metabolic signature. The urinary metabolome of control and the PPL group rats were found to segregate well after one month (Figure 3). And then, using PLS-DA to maximize the difference of metabolic profiles between control and PPL groups and the detection of metabolites were facilitated consistently present in the biological samples. Using Pareto-scaling technique, data were standardized. To obtain better discrimination between the control and PPL groups, the supervised OPLS-DA was applied. OPLS-DA can not only improve biomarker discovery efforts, but also separate samples into two blocks nicely. Score plots from OPLS-DA showed obvious separation between the PPL group and control group in both positive (**Figure 4A**) and negative ion modes (**Figure 5A**), which suggests that the biochemical perturbation of urinary prominently occurs in PPL group. From the corresponding loading plots, the ions furthest away from the origin contribute significantly to be responsible for the separation between control and PPL groups and may be therefore regarded as the differentiating metabolites for PPL groups (Figure 4B, 5B). Combining the results of S-plots (Figure 4C, 5C) and VIP-plots (Figure 4D, 5D) from the OPLS analysis, the precise molecular mass, retention time, and MS/MS data were provided by the UPLC-MS analysis platform for the structural identification of biomarkers.

5.2 Biomarker characterization

According to their MS/MS product ion analysis and molecular ion masses, and then compared with database resources or authentic standards, structure identification was performed. To evaluate the accuracy of possible formulas, some parameters, such as double bond equivalent (DBE), i-fit value (the isotopic pattern of the selected ion), and deviation from calculated mass (mDa or ppm) were used. Using the Mass-Fragment™ application manager based on Q-TOF, and the potential element composition, the fractional abundance of isotopes, degree of unsaturation of compounds were also obtained. We searched for the presumed molecular formula in the Human Metabolome Database, ChemSpider and other databases to identify the possible chemical compositions, and the potential structures of the ions were determined by screening the MS/MS data. According to VIP value (>2), all the detected ions were arranged. Finally, 15 marker metabolites contributing to the complete separation of PPL from control groups were identified, six of them are related to its health care function, including glycine, L-phenylalanine, pantothenic acid, tryptophan, hippuric acid, melatonin, showing the best combined classification performance (Table S1).

5.3 Metabolic Pathway Analysis

For searching out the potential pathways, we set the impact (pathway impact value calculated from pathway topology analysis) threshold to 0.10, and as potential pathways, should above this threshold. It was shown in Table S2 that the related metabolite biomarkers were mainly involved in the following pathways: phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, glycine, serine and threonine metabolism, tryptophan metabolism, primary bile acid biosynthesis, pantothenate and CoA biosynthesis, glutathione metabolism were found to be disturbed in PPL groups (**Figure 6**). Using the reference map by searching KEGG, the detailed construction was generated. As the highest score in rats, the detailed construction of phenylalanine, tyrosine and tryptophan biosynthesis pathway was shown in **Figure 7**. It is likely that PPL perturbed several amino acid pathways for combining the results in Tables S1 and S2, including phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, glycine, serine and threonine metabolism, tryptophan metabolism. Metabolic pathway enrichment analysis of the metabolites (table S2) showed that phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, glycine, serine and threonine metabolism, tryptophan metabolism is the main mechanism of action of PPL.

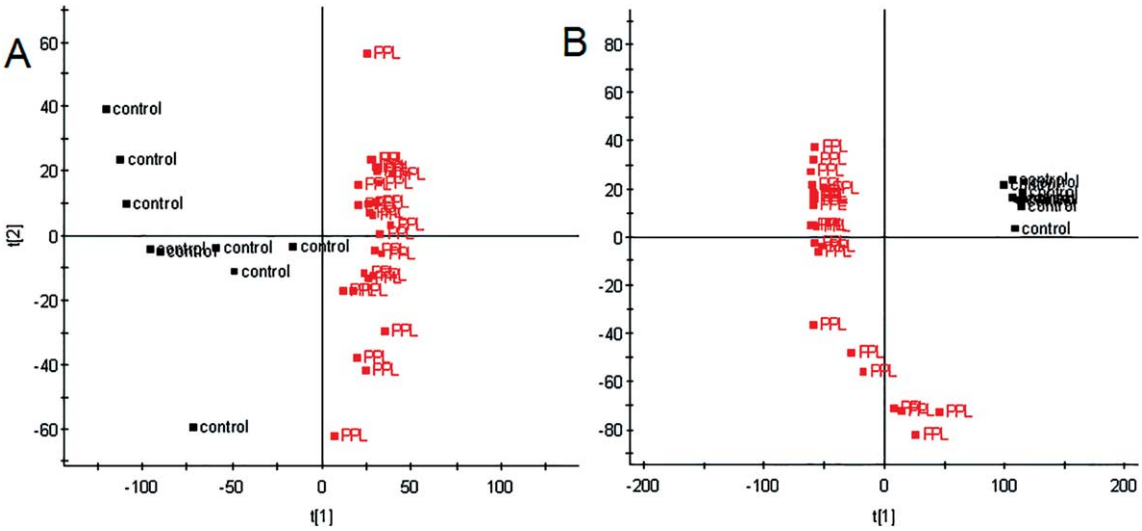

Figure 3. The PCA score plot of PPL group and controls in the ESI$^+$ (A) and ESI$^-$ (B) model.

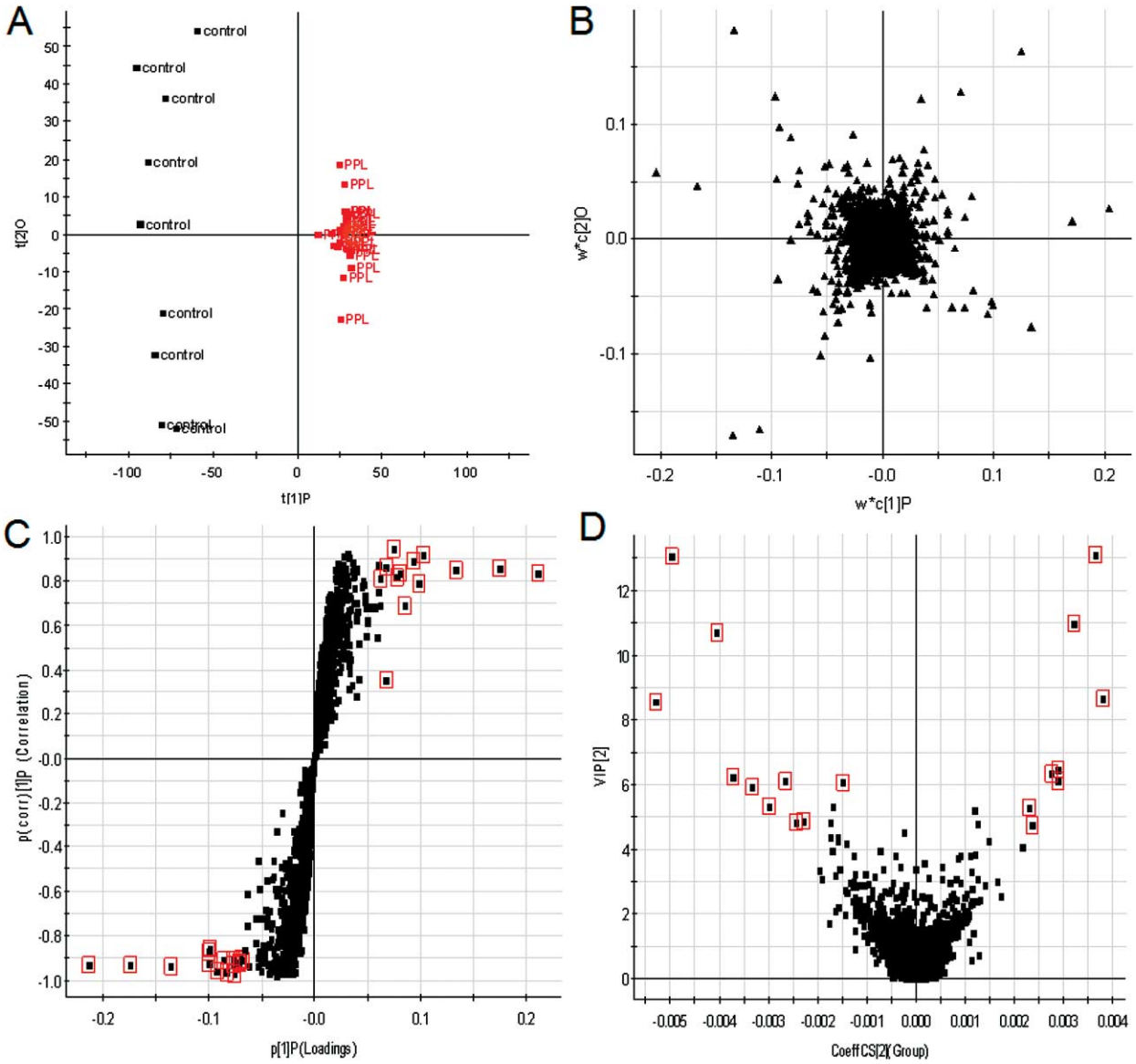

Figure 4. Metabolomic profiling of PPL in ESI^{+}mode. OPLS-DA model results for PPL group (A). Loading plot of OPLS-DA model for PPL group (B). S-score plot constructed from the supervised OPLS analysis (C). VIP-score plots constructed from the supervised OPLS analysis of urine (D).

DISCUSSION

Many natural sources have been researched as supplements to improve health. Exercise endurance is an important variable in evaluating antifatigue treatment. The time used to swim reflects the fatigue level of animal during exercise, while the time used to climb a pole reflects the fatigue level of animal during exertion of force. In our study, the endurance of climbing pole and the exhaustive physical-exercise swim test was significantly elevated, compared the fatigue-enduring effects of three PPL groups and control on endurance in exercised and weight-loading mouse, maximal climbing pole and swimming time was increased dose-dependently with the PPL doses. In order to study the effect of PPL on anti-fatigue, the physiological and biochemical indexes were measured, including BLA, BUN and HG.

The swimming exercise can induce the blood biochemical changes as known[11]. Glycolysis is the main energy source for intense exercise in a short time, and blood lactic acid (BLA) is the glycolysis product of carbohydrate under an anaerobic condition. Therefore, BLA is an important indicator for judging the degree of fatigue[12]. To put it another way, the degree of fatigue after exercise and the condition of recovery can be represented by BLA[13,14]. The results indicated that PPL can efficiently postpone the raising of lactic acid in the blood and delay the appearance of physical fatigue. Blood urea nitrogen (BUN), the metabolism product of protein and amino acid, is a sensitive indicator to assess the bearing capability when the body undertake a physical load. Several studies have shown that the athletes after long-term exercise, BUN in the blood rises significantly[15,16]. In other words, more significantly the BUN level increases means the body is worse adapted for exercise resistance[17,18]. Thus, BUN is another index to reflect the degree of fatigue. As the results indicated, PPL may reduce the protein catabolism for energy.

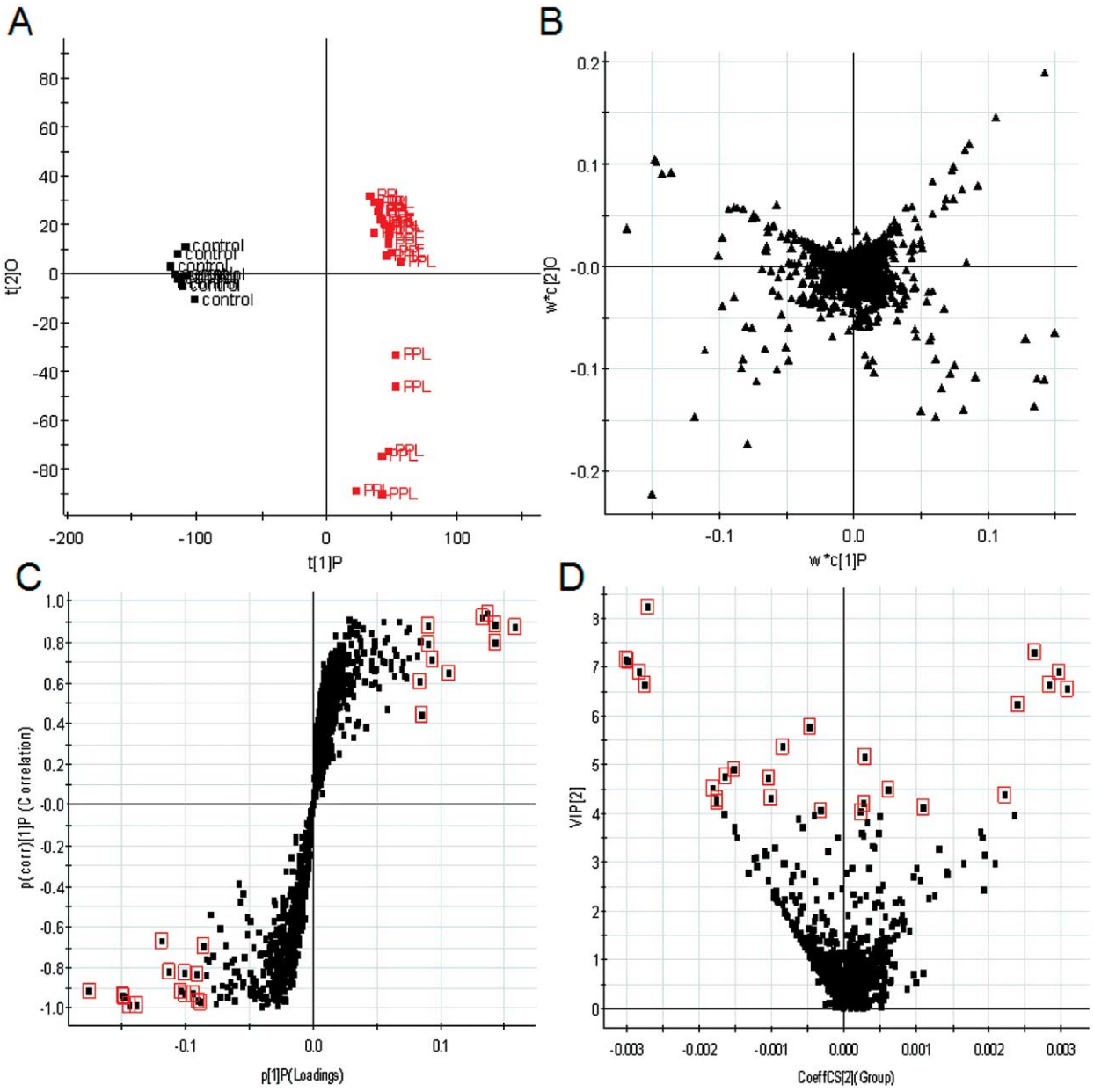

Figure 5. Metabolomic profiling of PPL in ESI⁻ mode. (A) OPLS-DA model results for PPL group. Loading plot of OPLS-DA model for PPL group (B). S-score plot constructed from the supervised OPLS analysis (C). VIP-score plots constructed from the supervised OPLS analysis of urine (D).

It was known that the physical endurance capacity was decreased significantly if the energy was exhausted[19]. Energy for exercise is derived primarily from the decomposition of glycogen, after high intensity exercise muscle glycogen depletion, and then, the liver released energy forms circulating glucose[20,21]. Therefore, the glycogen content is a sensitive indicator of fatigue-related[22]. It can be indicated from the results that the rate of glycogen depletion was postponed in the PPL-treated groups, and suggested that PPL could reduce carbohydrate utilization during the exercise. Synthesis of behavioral experiments and physiological indexes confirm that PPL can dose- dependently enhanced exercise performance of the mouse, as well as increasing the HG contents, but it decreases the BLA and BUN levels. For these results, it can be confirmed that PPL had significant anti-fatigue effects on mouse and these effects were dose-dependent.

In order to evaluate hypoxia function of health food, hypoxia tolerance test under ordinary pressure is a mandatory indicator. To set the animal at normal pressure hypoxia environment, the tolerance index is measured with the living time of the mouse. Sodium nitrite make the normal bivalent iron protein into trivalent iron protein, cause loss of oxygen carrying capacity of hemoglobin and lead to tissue hypoxia and die. Evaluation of resistance to anoxia in mouse, is also measured by the survival time of sodium nitrite poisoning. The longer, the better tolerance of hypoxia. Results show that PPL could significantly postpone living times while mice are in normal or chemical hypoxia and they indicate that PPL can enhance the hypoxia tolerant capability of mouse.

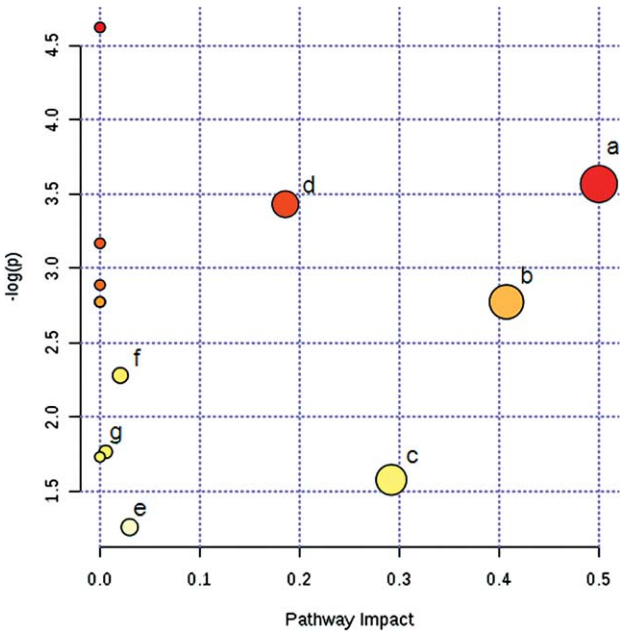

Figure 6. Summary of pathway analysis with MetaboAnalyst tool. (a) Phenylalanine, tyrosine and tryptophan biosynthesis; (b) Phenylalanine metabolism; (c) Glycine, serine and threonine metabolism; (d) Tryptophan metabolism; (e) Primary bile acid biosynthesis; (f) Pantothenate and CoA biosynthesis; (g) Glutathione metabolism

To evaluate the anti-oxidant effect of PPL in rats, we measure physiological and chemical indexes. The results show that there was an increase in the levels of the enzymes SOD and GSH-Px and decrease in the levels of the enzyme MDA. SOD catalyzes superoxide radicals and hydrogen ions to form hydrogen peroxide and molecular oxygen, therefore decreasing the propagation of free radicals, so that can be called superoxide radical scavenging factor[23,24]. GSH-Px is a widespread enzyme in the body, which can catalyze the decomposition of H_2O_2[25]. The membrane structure and function integrity of the cell can be protected by GSH-Px. Through enzymatic and non-enzymatic systems, oxygen free radicals produce in the body, which can attack the poly-unsaturated fatty acids (PUFA) in biofilm, induce and forme lipid peroxides, such as malondialdehyde (MDA). By lipid peroxide decomposition products, oxygen free radicals can cause cell damage[26]. So test the content of MDA can reflect the degree of lipid peroxidation in the body, indirectly reflect the degree of cell damage. These results suggest that PPL oral treatment caused an increase in antioxidant enzyme activity and a decrease in free radical production in rats.

Phenylalanine and tryptophan are essential amino acid, and tryptophan participates in the protein synthesis metabolism, and they can not be synthesized by the human body.

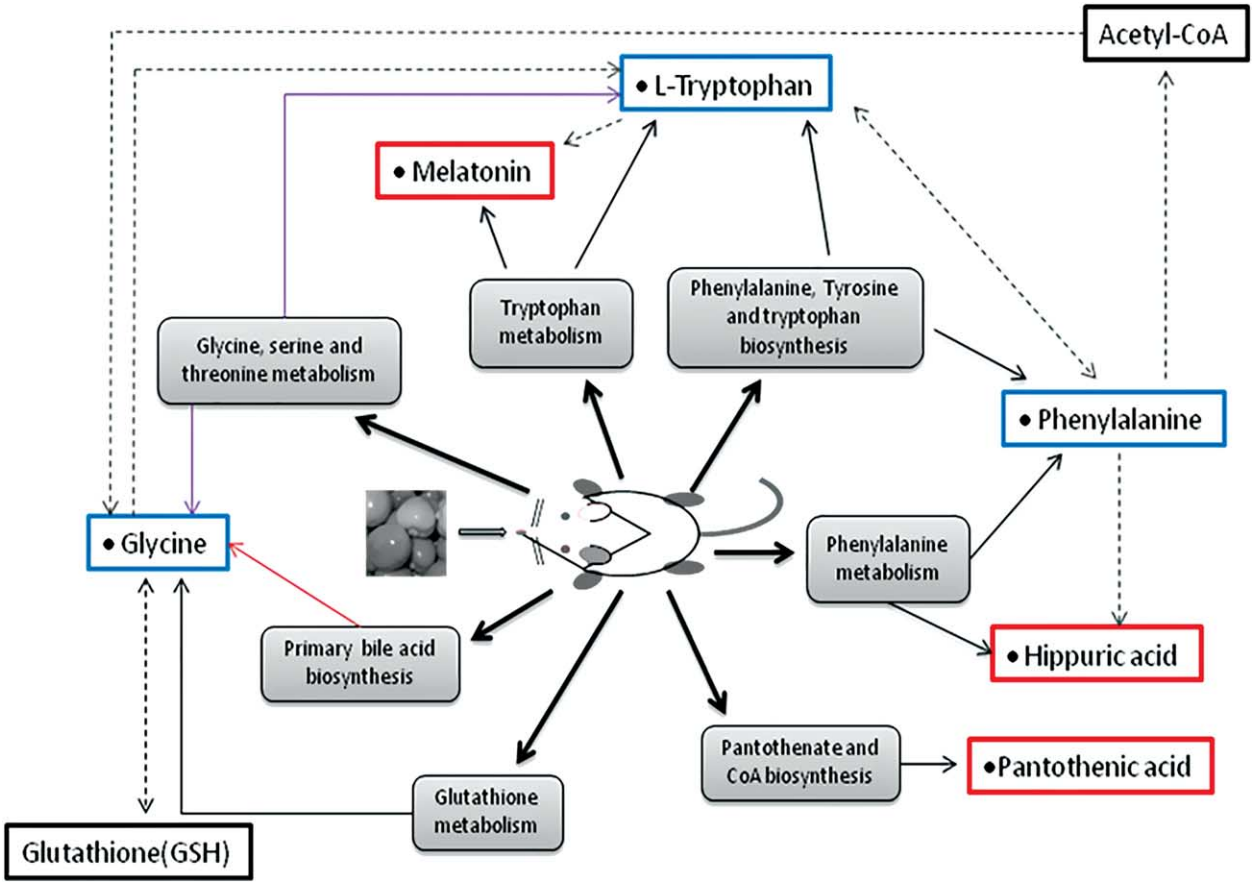

Figure 7. Construction of the altered metabolism pathways in PPL-treated rats according to the KEGG PATHWAY database. Red rectangle: up-regulated compared to controls. Blue rectangle: down-regulated compared to controls. Bold arrows: the relevant metabolic pathways. Fine arrow: the related markers. Dotted arrow: Mutual influence between the biomarkers.

Phenylalanine can help support memory retention. Tryptophan is the precursor of serotonin, serotonin is a neurotransmitter which can adjust the mood, enhance memory and protect the neurons from the damage of "excited nerve poison". Tryptophan biosynthesize serotonin at the third step, and melatonin is biosynthesized in four enzymatic steps in mammals[27]. Melatonin is not only has function as synchronizer of the biological clock, but also is a powerful free-radical scavenger and wide-spectrum antioxidant and it has been shown to be neuroprotective and inflammation in animal models[28,29]. Glutathione (GSH), as a kind of hydroxyl free radical scavenger which can decrease the level of ROS and block the tissue damage caused by free radicals, could be generated by glycine in glutathione metabolism by enzyme action. Pantothenate and CoA biosynthesis. Pantothenic acid (vitamin B5), is an essential nutrient for many animals.It is a precursor of coenzyme A (CoA), as well as be required to synthesize and metabolize proteins, carbohydrates, and fats by animal[30]. This work also confirms the feasibility of using the metabolomics to evaluate the health effects and mechanisms of PPL. Taking the potential biomarkers found in this study as possible drug targets, it revealed that PPL could regulate the metabolism function. Our results show that combination of high resolution analytical tools with pattern recognition techniques provided a new methodological cue for dissecting the underlying efficacies and mechanisms of herbal medicine, and provides scientific method on the efficacy of TCM.

CONCLUSION

In our study, a powerful approach to clearly differentiate PPL groups from matched control groups are provided with an UPLC–TOF/MS-based urine metabolomics attached with multivariate statistical methods and the potential biomarkers are identified. Results indicate that an obviously significant separation between the PPL groups and other samples revealed by OPLS-DA. Pathway analysis revealed that most of the proteins were found to play a key role in the regulation of metabolism pathways. Most of the biomarkers were amino acids and its derivatives, PPL mainly affects the amino acid metabolism. The altered metabolites demonstrated perturbations of amino acids metabolism in rats. It suggests that the urine metabolomics strategy is a powerful approach used for elucidation of the mechanisms of herbal medicine, and open new perspectives to using metabolomics platform to dissect the efficacy of herbal medicine.

ACKNOWLEDGMENTS

This work was supported by grants from the Key Program of Natural Science Foundation of State (Grant No. 81430093, 90709019, 81373930, 81173500, 81302905, 81202639), National Key Technology Research and Development Program of the Ministry of Science and Technology of China (Grant No. 2011BAI03B03, 2011BAI03B06, 2011BAI03B08), Natural Science Foundation of Heilongjiang Province of China (H2015038), Specialized Research Fund for the Doctoral Program of Higher Education (Grant No. 20122327120006), Fund Project of Heilongjiang Provincial Department of Education (Grant No. 12521498).

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Table S1. The informations of biomarker for pharmacological effects of *Physalis pubescens L*

No.	Rt(min)	Name	$[M+H]^+$	$[M-H]^-$	Formula	Trend
1	0.65	Glycine	76.0736	-	$C_2H_5NO_2$	↓
2	0.85	Ribothymidine	259.0958	-	$C_{10}H_{14}N_2O_6$	↓
3	0.97	Nα-Acetyl-L-arginine	217.1300	-	$C_8H_{16}N_4O_3$	↓
4	2.34	L-Phenylalanine	166.0910	-	$C_9H_{11}NO_2$	↓
5	2.65	Pantothenic Acid	220.1208	-	$C_9H_{17}NO_5$	↑
6	2.98	L-Homophenylalanine	180.1050	-	$C_{10}H_{13}NO_2$	↑
7	3.21	Tryptophan	205.1001	-	$C_{11}H_{12}N_2O_2$	↓
8	3.57	N-Acetyl-DL-valine	160.0998	-	$C_7H_{13}NO_3$	↓
9	3.99	Kynurenic acid	190.0531	-	$C_{10}H_7NO_3$	↑
10	4.16	Hippuric acid	180.0684	178.0553	$C_9H_9NO_3$	↑
11	4.69	Phenylacetylgl -ycine	-	192.0708	$C_{10}H_{11}NO_3$	↑
12	5.21	N_2-Succinyl-L-ornithine	-	231.0799	$C_9H_{16}N_2O_5$	↑
13	5.22	Melatonin	233.0845		$C_{13}H_{16}N_2O_2$	↑
14	6.81	5-L-Glutamyl-taurine	255.0692	-	$C_7H_{14}N_2O_6S$	↑
15	8.12	Isoleucyl-Valine	-	229.1522	$C_{11}H_{22}N_2O_3$	↑

Note: ↑, content increased; ↓, content decreased.

Table S2. Metabolic pathway enrichment analysis of the metabolites with MetaboAnalyst tool

Pathway name	Hits	Raw p	Impact	Metabolites
Phenylalanine, tyrosine and tryptophan biosynthesis	1	2.83E-02	0.5	L-Tryptophan, Phenylalanine
Phenylalanine metabolism	1	6.26E-02	0.41	Phenylalanine, Hippuric acid
Glycine, serine and threonine metabolism	1	2.07E-01	0.29	L-Tryptophan, Glycine
Tryptophan metabolism	2	3.24E-02	0.19	L-Tryptophan, Melatonin
Primary bile acid biosynthesis	1	2.84E-01	0.03	Glycine
Pantothenate and CoA biosynthesis	1	1.02E-01	0.02	Pantothenic acid
Glutathione metabolism	1	1.71E-01	0.01	Glycine