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^{1}H-NMR Metabonomics Study of the Therapeutic Mechanism of Total alkaloids and Ajmalicine from *Rauvolfia verticillata* in Spontaneously Hypertensive Rats

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Abstract: The total alkaloids extracted from *Rauvolfia verticillata* have been developed into drugs-Verticil to treat hypertension. It has a slow and sustained reduction in blood pressure, for the treatment of early and mild hypertension. Ajmalicine, an indole alkaloid from total alkaloids, has been shown to not only reduce blood pressure but also expand blood vessels. However, the mechanism of the antihypertensive effect of total alkaloids and ajmalicine has not been fully characterized. In this research, we chose to study a hypertensive rodent model, spontaneously hypertensive rats. Spontaneously hypertensive rats are a useful model for the study of essential hypertension which is a complex, polygenic, and multifactorial disorder. Studies suggest the usefulness of an ^{1}H-NMR-based metabonomic approach using spontaneously hypertensive rats for the study of hypertension. The spontaneously hypertensive rats were given total alkaloids and ajmalicine by oral gavage from 10 to 16 weeks of age. Plasma samples were analyzed using ^{1}H-NMR spectroscopy and metabolic information was extracted from the NMR data by principal components analysis as well as partial least square-discrimination analysis in order to develop biomarkers associated with hypertension. The metabolic pathways of small molecule metabolites in plasma were investigated to explain the content changes. The results obtained showed that ajmalicine only has weak effect for hypertension. Total alkaloids exert its antihypertensive effect by reducing the level of tyrosine and increasing the level of choline and leucine. The use of total alkaloids plus ajmalicine showed its antihypertensive effect more strong than total alkaloids.

Key words: Total alkaloids; Ajmalicine; ^{1}H-NMR; Metabonomics; Spontaneously Hypertensive Rats

Introduction

Essential hypertension, also known as hypertension, can result in damage to the heart, brain, kidney and other organs, causing stroke, coronary heart disease, heart failure, and renal failure. Because of its high morbidity and mortality, hypertension poses a serious threat to human health and quality of life [1]. Spontaneously hypertensive rats (SHR) are internationally recognized as the best animal model of human hypertension pathogenesis [2] and there have been a number of studies published on SHR [3-4]. Blood is the most commonly used biological fluid in metabolomics studies, carrying a lot of information about small molecule metabolites, and more fully reflects the actual state of the body. Currently, drug treatments for hypertension are fast, clear and effective, but they also have shortcomings including significant side effects, and rapid changes in blood pressure. Therefore, it is very important to look for drugs that play a role through multi-channel and multi-target regulation.

The total alkaloids extracted from *Rauvolfia verticillata* (Lour)Baill. have been developed into drugs-Verticil to treat hypertension. It has a slow and sustained reduction in blood pressure, for the treatment of early and mild hypertension. Ajmalicine, an indole alkaloid, is used as an antihypertensive and sedative agent, and can also expand blood vessels. When rabbits were injected with 0.25 mg·kg^{-1} ajmalicine intravenously, the small blood vessels of the leptomeningeal expand and   brain perfusion is increased; when the dose was increased to 1 mg·kg^{-1}, it produced hypotension in dogs and the blood flow in the femoral, carotid, coronary arteries was increased. It is also has antibacterial effects [5]. The total alkaloids and ajmalicine were extracted from *Rauwolfia* (Yuanyang, Yunnan, China) and the medicinal material was identified as Apocynaceae RauvolfiaLinn. [*Rauvolfia verticillata* (Lour)Baill.] by Jingming Jia (Professor, Shenyang Pharmaceutical University, China). *Rauwolfia* from Yuanyang Yunnan.

Metabonomics is regarded as an important research method, which can provide detailed information about biological systems at different levels [6-8]. It has also found widespread application in the study of a variety of diseases and drug actions [9-10]. Metabolomic studies are a rapidly emerging area of "-omics" research and are defined as the comprehensive and quantitative analysis of all metabolites [11-13]. The discovery of specific biomarkers from traditional Chinese medicines is playing an important role in monitoring disease status, predicting disease progression and clarifying of mechanisms of action [14]. It also offers an alternative method for monitoring the biochemical changes associated with the generation and development of the effects of exogenous and endogenous factors. [1]H Nuclear magnetic resonance (NMR) spectroscopy is widely used in metabonomics research because of its high resolution, sample preservation and its ability to analyze intact biological systems [15-17]. The characteristic profiles generated by ^{1}H NMR spectroscopy can be used to provide metabolic information, either visually and/or with suitable chemometric analysis such as principal components analysis (PCA). Rather than examining just one or two specific metabolites, the study is a multi-targeted analysis of low molecular weight, endogenous metabolites [18]. Although there have been many metabonomic studies on the actions of traditional Chinese medicines [19–23], no such studies on the total alkaloids and ajmalicine have been reported to date.

The focus of this research was on the plasma metabolites of five groups of rats: a control group, a hypertension model group, a hypertension model group treated with ajmalicine, a hypertension model group treated with the total alkaloids and a hypertension model group treated with the total alkaloids and ajmalicine. Here, the applicability of NMR-based metabonomics in assessing the effects of the total alkaloids and ajmalicine on hypertension in rats was evaluated to identify potential biomarkers and reveal the mechanisms of action of the total alkaloids and ajmalicine.

Materials and methods

Chemicals and Materials

Rauvolfia was obtained from Yuanyang Yunnan, China. An animal non-invasive blood pressure meter (Softron BP-98A) was purchased from Ruanlong Technology Co., LTD. in Beijing, China. A Medlab v5.0 biological signal acquisition and processing system was obtained from Mei Yi Technology Co., LTD. (Nanjing, China). Deuteroxide and sodium 3-trimethylsilyl-propionate (TSP) were purchased from Merck Drugs & Biotechnology in Germany. Dipotassium phosphate was purchased from Xilong Chemical Co., LTD. in Guangdong, China. Sodium hydroxide was purchased from Yuwang Chemical Co., LTD. in Shandong, China. Distilled water was obtained using a Milli-Q Reagent Water System.

Maintenance of animals

Male rats (Wistar, 10weeks old, body weight: 180–220 g, blood pressure: 100-120 mmHg, qualified number: SCXK (LIAO) 2012-0001) were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (China). Male Spontaneously Hypertensive Rats (10 weeks, body weight: 180–220 g, blood pressure: 155-170 mmHg, qualified number: SCXK (JING) 2012-0001) were purchased from Vital River Laboratory Animal Technology Co., LTD. (Beijing, China).The rats were maintained under standard laboratory conditions (temperature: 20-25°C; relative humidity: 55±10%; and a 12 h light/12 h dark cycle) with food and water freely available.

Ethics statement

The experimental protocol was approved by the Medical Ethics Committee of Shenyang

Pharmaceutical University, and all the procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals.

Blood pressure measurement

In this study, blood pressure was measured by the caudal artery volumetric method. The tail of each rat was put into a heater, and the local temperature reached 38.5-39.5 °C after about 10 - 15 min, and then the rats were placed under a homemade cloth cover, lying on a mattress, and the blood pressure was measured while the animals were calm. Pulse and pressure signals were imported into a computer via AD interfaces, and pressure and pulse curve were directly recorded using a Medlab v5.0 biological signal acquisition and processing system. Pulse curves were recorded using a high sensitivity pulse transducer, from the first wave disappeared to the appearance of next wave, which is the systolic pressure. The blood pressure was measured every day at a fixed time and, according to the sensitivity of the systolic and diastolic blood pressure, the systolic blood pressure was chosen as an indicator in this experiment (The results were expressed in mmHg, 1mmHg = 0.133Kpa).

Extraction of total alkaloids

Dried roots of *Rauvolfia* were crushed and accurately weighed (10.0 g), then refluxed with ethanol. The extract was evaporated under reduced pressure to a constant weight, weighing, calculating extract yield. The extract were dispersed in 20 ml water and extracted with chloroform (20 ml, three times). The extracted solution were combined and evaporated, the total alkaloids were obtianed.

Extraction of ajmalicine

Rauwolfia powder (about 10 g), was mixed with 100 ml 0.1% hydrochloric acid ethanol solution. Then, the solution was heated at 70°C and refluxed 3 times, each for 30 minutes. The extract solutions were combined and the pH adjusted 6 using ammonia (free alkaloids generally are not soluble or insoluble in water, but they are soluble in alcohol, ether, chloroform, acetone, benzene and other organic solvents. Salts that react with alkaloids and acid are soluble in water or aqueous ethanol.). Ethanol was removed under reduced pressure, and the residue was dissolved in water. Hydrochloric acid was added to adjust the pH to 3 and then the material was filtered, and the insoluble material was washed with water until there was no reaction for alkaloids and acid. The acidic solutions were combined and the pH was adjusted to 9-10 by using ammonia, to obtain a yellow precipitate. This product was identified as ajmalicine. The structure was confirmed by ^{1}H-NMR and ^{13}C-NMR. The purity of ajmalicine was determined by HPLC and found to be 99%, using the peak area normalization method. The structure of ajmalicine, its ^{1}H-NMR spectrum, and its ^{13}C -NMR spectrum are shown in Fig 1, Fig 2 and Fig 3, respectively.

Drug treatment

After the rats were fed a standard diet for one week, the SHR were randomly divided into four groups: a SHR group, a ajmalicine treated group, a total alkaloids treated group, a total alkaloids and ajmalicine treated group (n = 8 rats/group) and the control group were 8 rats also.The rats in the total alkaloids-treated group were given at a dose of 20 mg·Kg^{-1}·d^{-1}, ajmalicine-treated group were given at a dose of 120 mg·Kg^{-1}·d^{-1}, total alkaloids and ajmalicine -treated group were given total alkaloids at a dose of 20 mg·Kg^{-1}·d^{-1} and ajmalicine at a dose of 120 mg·Kg^{-1}·d^{-1} (self-extracted in laboratory). The animals in the model and control groups were given the same volume of water as the treated groups, and all of the administrations were performed by oral gavage.

Plasma sample collection

Blood samples (3.5ml) were collected from each rat at weeks 0, 1, 2, 4 and 6 in the five groups. The blood samples were placed in 5 ml heparinized EP tubes, and centrifuged at 4,000 × g for 10 minutes, then the supernatant was collected in EP tubes and stored at -70 °C.

^{1}H-NMR spectroscopy

Plasma (350μl) was transferred to a 5 ml EP tube, followed by the addition of NaH_2PO_4-Na_2HPO_4 (300 μl, 0.2 M, pH7.4) buffer solution and the mixture was vortexed for 60 seconds, then centrifuged for 10 minutes at 12,000 × g. The supernatant (550μl) obtained was mixed with 150 μl TSP Deuterium oxide solution (1.5 mg·ml^{-1}) and then vortexed for 60 seconds and transferred to a 5-mm o.d. NMR tube. The analysis was conducted using a Brucker AV 600 MHz superconducting Fourier transform nuclear magnetic resonance spectrometer. ^{1}H-NMR spectra were recorded at a temperature of 298.2 K, and the water peak of water was restrained using the presaturation method and the Carr-Purcell-Meiboom-Gill (CPMG) was adopted. Sixty-four free-induction decays were collected into 64000 data points with a spectral width of 12019 Hz and the pre-saturation delay was 2.0 s. Using the free induction decay signal obtained by Fourier transform , the one dimensional NMR spectrum was obtained.

Data-reduction of ^{1}H-NMR spectra and pattern recognition

In this study, each sample was represented by an NMR graph. All the samples collected were analyzed using the validated method, and all NMR spectra were baseline corrected and phased. TSP (δ0.0 ppm) was used as a reference for the NMR spectra. Using MestReNova 5.3.1 software (Mestrelab Research, USA), the ^{1}H-NMR spectra were automatically reduced to TXT files. The data from each spectrum (δ 0–10 ppm) were automatically reduced to 250 integrated segments of equal width (0.04 ppm). The regions of 4.60–5.20 ppm were excluded, because this was the resonance of residual water. The TXT files were imported into Microsoft Excel for labeling, and then imported into SIMCA-P 11.5 for principal component analysis. Prior to the analyses, the values of all of the variables were centered and scaled according to literature reports [3, 24-25] and combined with our laboratory's own standard library, using Chenomx NMR suite software, to identify the NMR spectra of the metabolites.

Principal Component Analysis (PCA) and Partial least squares projection to latent structure-discriminant analysis (PLS-DA) were used in this study. The former was applied to identify outliers and detect data grouping or separation trends, and it also produced an overview of the data set. The latter, supervised pattern recognition, focused on the actual class discriminating variation of data compared with the unsupervised approach, PCA [26]. This is a statistical method that allows a graphical overview of large multivariate datasets and provides good discrimination between groups. This pictorial representation of the full data in one plot simplifies the detection of sample grouping because each sample is represented by one point on the plot. The samples that appear close together on the graph are said to cluster and are similar in biochemical composition. The OPLS-DA model was validated by describing R^2 and Q^2 values. R^2 was used to explain the differences in the models and Q^2 was used to provide an estimation of the predictive capability of the models. Theoretically, the closer R^2 and Q^2 are to 1 the better the model description. Typically, R^2, and Q^2 were higher than 0.5 (50%) and the difference between the values should not be too large. It should be noted that the R^2 and Q^2 values are closely related to the main component and the value of R^2 and Q^2 will increase with the growth of the main component and, when the main component can only increase the value of R^2, the calculation should be terminated [27]. Some of

the intensities from the spectral data of the key metabolites selected according to a variable importance plot (VIP) analysis of OPLS were expressed as the means ± sd. The significance of variations between groups in the data regarding biological parameters was determined using the paired-sample t-test (SPSS19.0). P-values less than 0.05 were considered to be statistically significant.

Results

Results of blood pressure

We measured the blood pressures of control and SHR groups from 10 weeks to 20 weeks. Between 12-20 weeks, the blood pressure of the Wistar rats remained stable, and there was no significant difference in SBP from week to week (p> 0.05); SHR at 12 weeks of age had a significantly higher blood pressure than Wistar rats (p <0.05), whose SBP increased gradually over the weeks. At 14 weeks, the SBP reached a higher level. Between 14~18 weeks , the blood pressure levels remained relatively stable and the blood pressure was higher than at 20 weeks. Compared with 14 weeks, the blood pressure of SHR at 12, 13 and 20 weeks exhibited a significant difference (p <0.05), while there were no significant differences compared with 16, 18 weeks (p > 0.05).The results are shown in Table 1. The total alkaloids and ajmalicine were given to SHR for 6 weeks and the blood pressures of the five groups are summarized in Table 2.

^{1}H-NMR analysis and identification

The ^{1}H-NMR spectra of the plasma of rats in different groups are shown in Figure 4. The identification of metabolites in rat plasma are shown in Table 3.

Analysis of potential biomarkers in the plasma

Metabolic changes in plasma were identified after centralization and normalization of integral data. In Figure 5, one sample was represented by each point, and different metabolic patterns were revealed by different samples. In Figure 5A, the PCA of the ^{1}H-NMR spectra from the control group and spontaneously hypertensive rat group are shown. The samples of the two groups separated from each other, but the classification was not remarkable. Therefore, we employed OPLS-DA to discriminate between the normal and spontaneously hypertensive rat model groups, which are shown in Figure 5B. The OPLS-DA model had a high R^2Y and Q^2 value, indicating the overall goodness of fit. The good predictive capabilities of the proposed model were also shown. In Figure 5C, the results of the control, spontaneously hypertensive rat model and ajmalicine treatment groups are shown. The spontaneously hypertensive rat model and ajmalicine treatment groups can not be separated effectively. In Figure 5D, the results of the control, spontaneously hypertensive rat model and total alkaloids treatment groups are shown. In Figure 5E, the results of the control, spontaneously hypertensive rat model and total alkaloids and ajmalicine treatment groups are shown. In Figure 5D and 5E, the three groups could clearly be discriminated due to marked differences in their metabolic profiles. The total alkaloids treatment group was found between the control and spontaneously hypertensive rat model groups, suggesting that the metabolic profiles of the spontaneously hypertensive rats recovered and the plasma metabolites were restored to normal levels after drug treatment.

According to the VIP values from the pattern recognition model, potential biomarkers were selected. In the OPLS model, 55 variables displayed VIP values greater than 1. In Figure 5F, these 55 points were relatively far away from the dense cluster, suggesting that these samples made a greater contribution to the classification. Points with VIP values greater than 1 and with P values less than 0.05 were regarded as final biomarkers. Following structural identification, 8 potential

biomarkers were identified and these were listed in Table 4. The results of other metabolites in the plasma were listed in Table 5.

Discussion

The blood pressures of SHR rise with age and become significantly higher than those of Wistar rats after 5 or 6 weeks of age, and hypertension is almost established around 10 weeks of age [28]. Thus, 10-week-old SHR are still in the stage of developing hypertension and are useful as a model of the early stage of hypertension. The present experimental results showed that the SHR can be discriminated from the age-matched Wistar rats in terms of their plasma metabolite profiles. The metabolites contributing to the discrimination are of great interest in relation to the mechanisms of blood pressure regulation. It has been reported that the age and gender of groups may have an effect on the metabolic study [29-30]. The present study was conducted with rats of the same age and gender (10 weeks, male), and the age difference between the groups was not statistically significant, excluding interference from these factors.

Lactate, citrate and glycogen are involved in carbohydrate metabolism. The decrease in lactate indicates a change in glucose metabolic pathways, resulting in enhanced aerobic metabolism. The decrease in citrate levels, which is an intermediate product of the tricarboxylic acid cycle, indicates the deregulation of energy metabolism. The decrease in glycogen indicates that glycogenesis is inhibited or glycogenolysis is enhanced. Glycerol, which is transformed into dihydroxyacetone phosphate, participates glucose metabolism.

Tyrosine, valine, glycine, leucine, taurine, methionine, and lysine are related to amino acid metabolism. The levels of tyrosine and valine increased, while glycine, leucine, taurine, methionine and lysine decreased. The results we obtained were basically the same as literature reports. Many scholars have studied the relationship between amino acid levels and high blood pressure in vivo. Guangsheng Zhao [31] compared the relationship between serum free amino acid levels and the blood pressure of eight groups. The results showed that valine, cysteine and threonine were positively related to blood pressure, while glycine, phenylalanine and leucine were negatively correlated. It was also found that serum taurine, total sulfur amino acids (methionine + taurine + cystathionine) , lysine and serine were significantly negative related to blood pressure. The mechanisms of the effects of amino acids on blood pressure have been widely reported in the literature, and mainly involve sulfur amino acids, taurine and aromatic amino acids. Sulfur-containing amino acids include methionine, cysteine and histidine and sulfur-containing amino acids can lower blood pressure and prevent hypertension and stroke. After cell metabolism, methionine is demethylated to homocysteine. Homocysteine is an independent risk factor for cardiovascular disease, and increased plasma concentrations are closely related to heart and cerebrovascular disease. In particular, it was significantly associated with damage to target organs caused by hypertension. Taurine derived from cysteine, with a variety of pharmacological activities, can reduce triglycerides in the arterial walls and inhibit non-enzymatic glycation and radical generation. It has a protective effect on cells and improves sensitivity to insulin, resulting in hypoglycemic, anti- atherosclerotic and anti- high blood pressure effects. Taurine is one of the important factors associated with reduced atherosclerosis, hypertension and concurrent body injury. Research studies have shown that taurine in serum and platelets of subjects with primary essential hypertension was significantly lower than in control groups [32]. Body tyrosine (TYR) is mainly the metabolic transformation product of phenylalanine (PHE). TYR generates dopa by TH .Then dopa decarboxylase converts dopa into dopamine (DA). TH is the rate-limiting enzyme

of catechu diaminodiphenol for synthesis. Another way to TYR is to generate hydroxyl tyrosine phenylketonuria under the catalysis of tyrosine aminotransferase, and then it is transformed into homogentisic acid. Finally, if fumarate and acetoacetate are generated, these are involved in glucose and fatty acid metabolism.

The mechanism of action of aromatic amino acids may also involve tyrosine and tryptophan. Tyrosine is a precursor of neurotransmitter catecholamine biosynthesis and catecholamine levels are increased in serum, especially dopamine and norepinephrine，leading to an increase in blood pressure. The outer periphery tryptophan circulates in blood in two forms. One form is bound, TRP is loosely combined with serum albumin. Plasma fatty acid can bind to albumin competitively while the second form is free, and not bound to albumin and can cross the blood -brain barrier. f-TRP penetrates the blood-brain barrier by means of special carriers, and   may be combined with a carrier and other neutral amino acids, especially branched-chain amino acids (BCAA), including leucine, isoleucine and valine. Therefore, the f-TRP concentration in brain depends on the f-TRP and BCAA concentrations and the concentration ratio in plasma, so the synthesis and increase in 5 - HT in the brain is closely related to the blood levels of f-TRP [33].Tryptophan is an essential amino acid, in addition to participating in protein synthesis, it is also converted to 5 - HT by oxidation decarboxylation. Studies have found that increased and continuous release levels may be one of the key factors why blood pressure is persistently elevated in hypertensive patients.

Choline, betaine and trimethylamine oxide are associated with lipid metabolism. Experimental results have shown that choline levels are reduced and the betaine, and trimethylamine oxide levels are increased in hypertensive rat plasma. Choline in the blood passes into the brain, and then reacts with acetyl coenzyme A and is converted into acetylcholine by deacetylase in vivo. Acetylcholine can cause the vasodilation of endothelial cells. Under the effect of acetylcholine, endothelial cells can release nitric oxide, prostacyclin and other vasoactive substances, producing vascular smooth muscle relaxation. The secretion of a variety of vasoactive substances, such as nitric oxide and prostacyclin, will be restricted because of the reduction in acetylcholine. The diastolic capacity of vascular smooth muscle is markedly diminished. In the early stages, functional lesions are formed, along with systemic small artery spasm. In the late stages, organ disease and arteriosclerosis are produced which leads to high blood pressure. Betaine can reduce peripheral resistance, and systolic and diastolic blood pressure. However, the effect on diastolic blood pressure is significantly greater than that on systolic blood pressure. Diastolic blood pressure mainly reflects the magnitude of the peripheral resistance. It appears that betaine acts mainly on peripheral parts. Also, it has previously been reported that TMAO in plasma is produced by the following pathway: dietary phosphatidylcholine/choline → gut flora-formed TMA → hepatic FMO-formed TMAO. TMAO can increase the stability of the protein structure in vivo, with the function of anti-ion instability and anti-water pressure. The structure of TMAO is similar to betaine, methyl donor choline and adenosylmethionine. Choline and betaine are important intermediates in lipid metabolism, which can regulate tissue osmolality [34]. The amount of choline reduced may be related to changes that are glucose-induced [35].

The discovery of biomarkers and the biological explanations described above can be used to analyze the pathogenesis of hypertension through metabolic pathways, and these results can likewise play an important role in assisting the clinical diagnosis of hypertension (Figure 6).

In summary, total alkaloids acts mainly via the pathways of lipid metabolism and protein

metabolism. After hypertension model rats were bred with total alkaloids，the levels of tyrosine, valine, glycine, leucine, taurine, methionine and lysine tended to be normal. Because of a reduction in tyrosine, norepinephrine and epinephrine levels were also reduced. The hypertension physiological basis for the outer periphery is sympathetic-adrenal medulla hyperthyroidism, the main pathological aspect of which is that norepinephrine and epinephrine levels are significantly higher in peripheral plasma. Norepinephrine and epinephrine are synthesized by tyrosine under the action of hydroxyl enzymes. Meanwhile，the content of choline increased. Thus, the content of acetylcholine is also increased. Acetylcholine is a key vasodilator of endothelial cells. Under the effect of acetylcholine, endothelial cells can release nitric oxide, prostacyclin and other vasoactive substances, allowing relaxation of vascular smooth muscle. Total alkaloids has an effect on glucose metabolism, but is only a weak effect.

In this study, ^{1}H-NMR metabonomics combined with PCA and OPLS-DA were used to analyze metabolite profiles. According to the variations in endogenous metabolites, the mechanism of total alkaloids was identified. The results obtained showed that ajmalicine only has weak effect for hypertension, we can not explain its mechanism. Total alkaloids exert its antihypertensive effect by reducing the level of tyrosine and increasing the level of choline and leucine. The use of total alkaloids plus ajmalicine showed its antihypertensive effect more strong than total alkaloids. Such scientific evidence can be used for mechanism studies involving traditional Chinese medicines.

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Author Contributions

Conceived and designed the experiments: MF CZ MW. Performed the experiments: MF CZ MW MZ YW. Analyzed the data: MF CZ MW YW. Contributed reagents/materials/analysis tools: FM CZ MW MZ YW. Wrote the manuscript: FM CZ MW MZ YW.

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Figure 1. The structure of ajmalicine

Figure 2. 600 MHz ^{1}H-NMR spectrum of ajmalicine

Figure 3. 600 MHz ^{13}C-NMR spectrum of ajmalicine

Figure 4. Typical 600 MHz 1H-NMR spectrum of plasma samples. 1. LDL/VLDL 2. Leucine 3. Isoleucine 4. Valine 5. 3-Hydroxybutyrate 6. Lactate 7. Alanine 8. Lysine 9. Arginine10. N-Acetyl-L-cysteine 11. Glutamate 12. Methionine 13. Acetoacetate 14. Acetone 15.Succinate 16. Pyruvate 17. Glutamine 18. Citrate 19. Creatinine 20. Tyrosine 21. Choline 22. Betaine 23. TMAO 24. Taurine 25.Tryptophan 26. Glycine 27.Glycerol 28.á-Glucose 29. Glycogen

Figure 5. PR analysis of the 1H-NMR spectrum of plasma. (A): PCA analysis of spontaneously hypertensive rats and normal rats (R2X=0.843, Q2=0.685). (B): OPLS-DA analysis of spontaneously hypertensive rats and normal rats (R2X=0.891, R2Y=0.974, Q2=0.935). (C): OPLS-DA analysis of spontaneously hypertensive rats, normal rats and ajmalicine treated rats (R2X=0.841, R2Y=0.881, Q2=0.684). (D): OPLS-DA analysis of spontaneously hypertensive rats, normal rats and total alkaloids treated rats (R2X=0.911, R2Y=0.859, Q2=0. 755). (E): OPLS-DA analysis of spontaneously hypertensive rats, normal rats and total alkaloids and ajmalicine treated rats (R2X=0.830, R2Y=0.801, Q2=0.692). (F): Loading plot of the OPLS-DA analysis of spontaneously hypertensive rats and normal rats.

Figure 6. A summary of the related metabolic pathways for metabolites that changed significantly in the SHR group. "↑" and "↓"represent a compound that is up- and down-regulated compared with the control group.

Table 1 Blood pressures of control and SHR groups (x±s, n=8, mmHg)

week	n	Control	SHR
10(SBP)	8	167.9±9.7	116.9±4.8*
11(SBP)	8	170.6±8.6	114.6±5.5*$^\Delta$
12(SBP)	8	175.9±9.2	113.3±3.9*$^\Delta$
13(SBP)	8	180.3±10.8	114.5±5.8*$^\Delta$
14(SBP)	8	186.1±8.2	115.0±4.4*
16(SBP)	8	192.5±10.9	114.0±3.4*
18(SBP)	8	197.2±11.8	115.2±3.8*
20(SBP)	8	203.6±12.4	114.7±5.2*$^\Delta$

compared with same weeks of Wistar rats, *P<0.05;

compared with 14 weeks of SHR, ΔP<0.05.

Table 2 Blood pressures of five groups (x±s, n=8, mmHg)

group	*n*	0 week (SBP)	1 week(SBP)	2 week(SBP)	4 week(SBP)	6 week(SBP)
Control	8	116.9±4.8**	114.6±5.5**	113.3±3.9**	115.0±2.4**	114.0±3.4**
SHR	8	167.9±9.7	170.6±8.6	175.9±9.2$^{\Delta}$	186.1±8.2$^{\Delta\Delta}$	192.5±10.9$^{\Delta\Delta}$
Ajmalicine treated	8	165.5±5.7	163.4±8.2	170.2±6.9**	176.2±8.1**	184.2±6.4**$^{\Delta}$
Total alkaloids treated	8	166.2±7.9	162.6±8.6	154.1±5.7**$^{\Delta\Delta}$	147.3±5.6**$^{\Delta\Delta}$	142.5±4.9**$^{\Delta\Delta}$
Total alkaloids and Ajmalicine treated	8	165.5±5.5	159.4±7.7	152.9±3.8**$^{\Delta\Delta}$	146.3±4.3**$^{\Delta\Delta}$	141.8±5.3**$^{\Delta\Delta}$

compared with SHR，*P<0.05, **P<0.01；

compared with the group before treatment，ΔP<0.05, ΔΔP<0.01.

Table 3. [1]H chemical shift assignment of the metabolites in plasma

NO.	Metabolites	δ1H(ppm)and multiplicity	Moieties
1	LDL/VLDL	0.9(m)	CH3, -(CH2)n-
2	Leucine	0.94(t)	δCH3
3	Isoleucine	0.98(d)	δCH3
4	Valine	1.06(d)	γCH3
5	3-Hydroxybutyrate	1.20(d)	CH3
6	Lactate	1.34(d),4.12(q)	βCH3,αCH
7	Alanine	1.46（d）	βCH3
8	Lysine	1.5(m)	γCH2
9	Arginine	1.73(m)	γCH2
10	N-Acetyl-L-cysteine	2.06(s)	CH3
11	Glutamate	2.08(m),2.38(m)	βCH2, γCH2
12	Methionine	2.14(s)	CH3
13	Acetoacetate	2.22(s)	CH3
14	Acetone	2.27(s)	CH3
15	Succinate	2.36(s)	CH2
16	Pyruvate	2.41(s)	CH3
17	Glutamine	2.46(m)	γCH2
18	Citrate	2.55 d, 2.68 d	Half CH2, CH2
19	Creatinine	3.06(s)	N-CH3
20	Tyrosine	3.20(ABX)	CH2
21	Choline	3.23(s)	N(CH3)3
22	Betaine	3.24(s)	N(CH3)3
23	TMAO	3.26(s)	N(CH3)3
24	Taurine	3.42(t)	CH2
25	Tryptophan	3.49(ABX)	CH2
26	Glycine	3.55(s)	CH2
27	Glycerol	3.65(ABX)	CH2
28	α-Glucose	5.24(d)	CH
29	Glycogen	5.4	CH

s=singlet; d=doublet; t=triplet; q=quartet; m=multiplet; c=complex

Table 4. Relative integrals of selected metabolites contributing to the classification

Metabolites	Control	SHR	Total alkaloids treated	SHR vs Control		Total alkaloids treated vs SHR	
				Changes	P-value	Changes	P-value
Leucine	79.1±11.4	57.6±5.6	60.3.0±11.2	↓	0.041	↑	0.03
Lactate	319.6±51.2	182.6±58.2	243.8±25.9	↓	0.034	—	0.031
Alanine	27.7+5.2	20.5+4.7	22.7+4.6	↓	0.006	—	0.293
Citrate	5.1+1.1	1.3+1.4	1.5±1.6	↓	0.001	—	0.714
Choline	306.0+23.1	220.7+36.3	281.7+26.7	↓	0.001	↑	0.001
Creatinine	16.9+5.6	51.6+8.9	45.5+15.7	↑	0.001	—	0.326
Tyrosine	18.4+6.2	29.1±7.1	23.8+4.0	↑	0.002	↓	0.001
TMAO	58.9+15.9	68.3+16.7	65.8+11.8	↑	0.003	—	0.135

Data normalized over the range 0–10.0 ppm excluding 4.60–5.20 ppm (resonance from residual water); "↑" and "↓" shows the metabolite is up- and down-regulated, "—" shows the metabolite produces no significant change; P-values determined using paired-sample t-test, P-value less than 0.05 was considered significant.

**Table 5. Relative integrals of metabolites except biomarkers in the plasma**

Metabolites	Control	SHR	Total alkaloids treated	SHR vs Control	Total alkaloids treated vs SHR
				P-value	
LDL/VLDL	205.1±42.3	212.7±32.5	186.3±34.8	0.562	0.237
Isoleucine	56.2±19	49.7±14	38.7±12	0.274	0.094
Valine	21.6±6.9	32.4±7.4	19.8±5.2	0.162	0.145
3-Hydroxybutyrate	25.1±11	30.8±12	19.8±11	0.261	0.373
Lysine	19.5±12	17.2±7.3	12.2±5.8	0.239	0.567
Arginine	32.9±13	35.2±7.8	24.1±12	0.522	0.110
N-Acetyl-L-cysteine	6.79±4.5	7.23±5.1	9.82±6.7	0.845	0.716
Glutamate	269.8±83.2	138.5±51.1	348.3±51.5	0.336	0.731
Methionine	220.2±58.6	231.0±50.5	178.6±34.7	0.788	0.347
Acetone	54.2±17	50.9±14	46.9±16	0.768	0.668
Succinate	58.8±25	63.7±42	48.9±17	0.498	0.213
Pyruvate	256.3±54.2	289.7±53.4	238.1±52.5	0.156	0.040
Glutamine	33.4±15.1	37.6±20	40.7±16	0.722	0.185
Acetoacetate	22.5±11	14.3±9.2	14.0±8.3	0.544	0.155
Betaine	12.31±2.4	8.66±3.8	21.5±4.6	0.338	0.021
Taurine	42.6±2.0	31.3±8.1	31.2±11.7	0.274	0.305
Tryptophan	29.6±6.2	24.0±13	22.4±9.5	0.345	0.815
Glycine	33.8±5.1	30.1±12	35.8±13	0.832	0.725
Glycerol	8.40±3.2	6.33±4.1	7.39±3.9	0.413	0.301
α-Glucose	36.3±1.4	37.7±10	35.3±9	0.510	0.327
Glycogen	44.8±4.0	47.4±19	41.7±21	0.725	0.539

Data normalized over the range of 0–10.0 ppm excluding 4.60–5.20 ppm (resonance from residual water); P-values determined using paired-sample t-test, P-values less than 0.05 were considered significant.
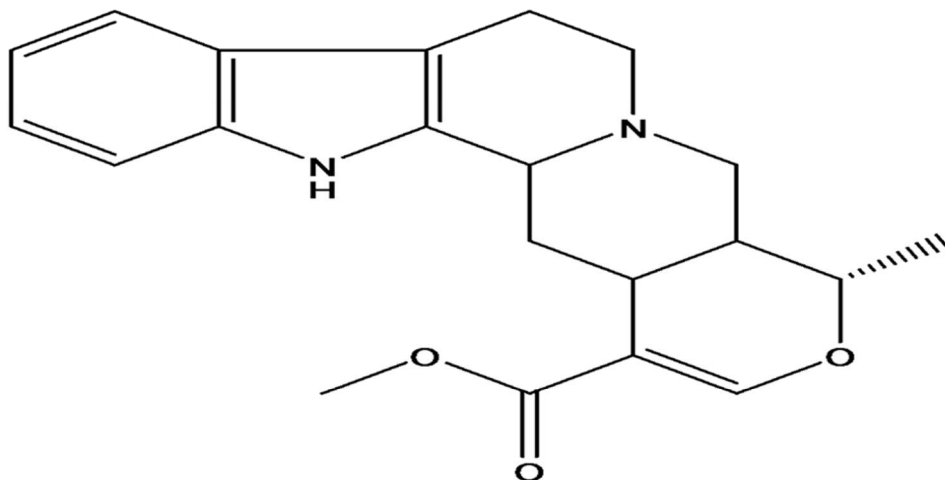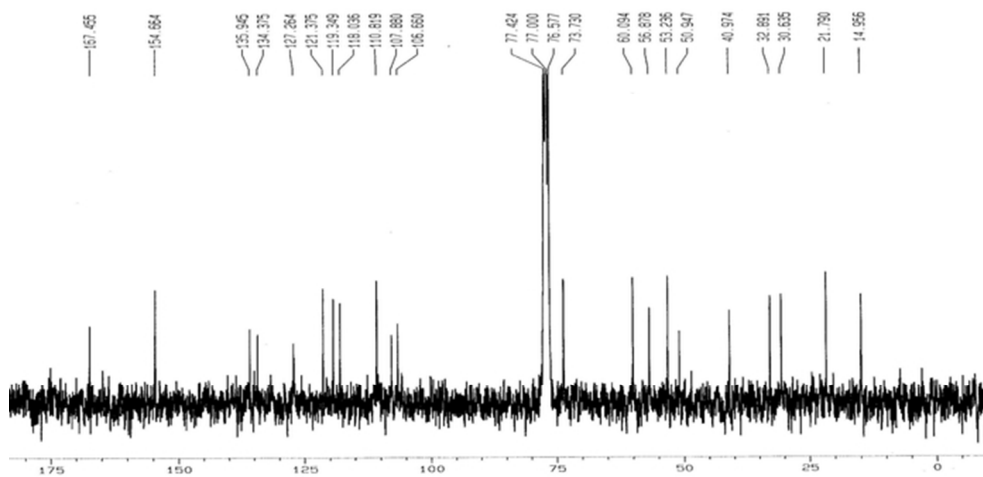
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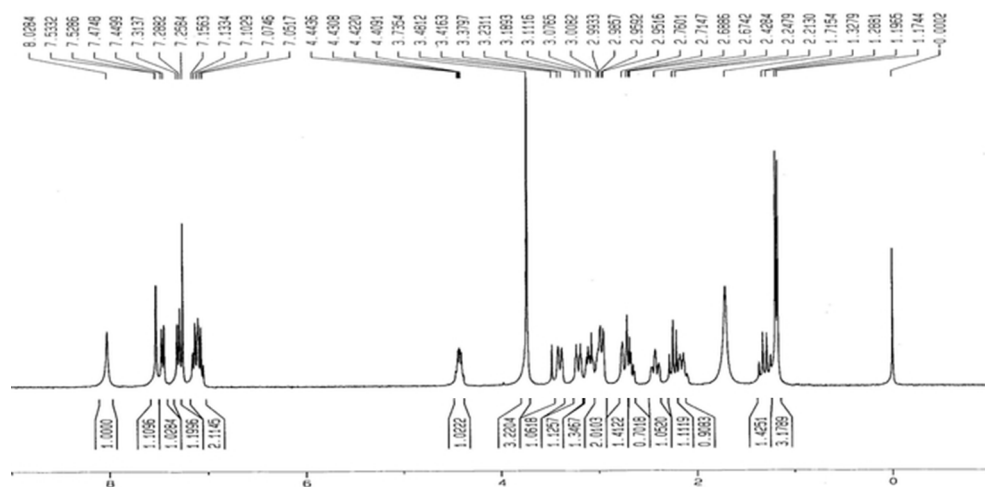
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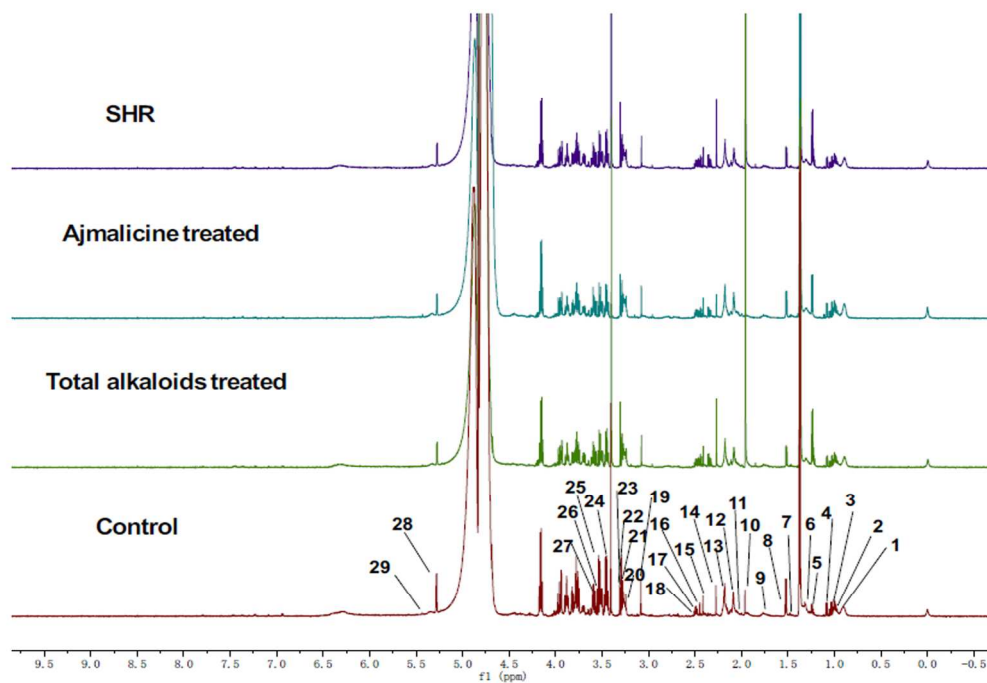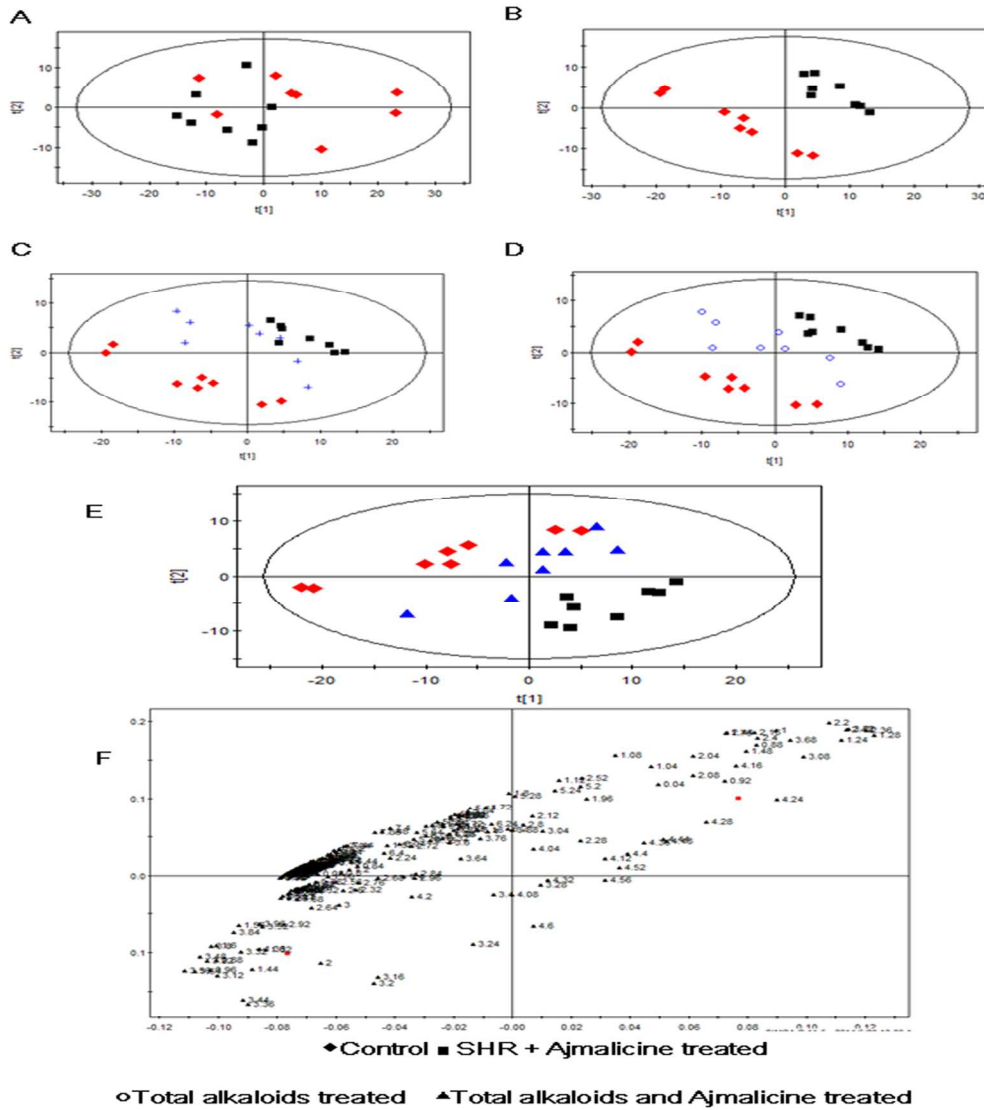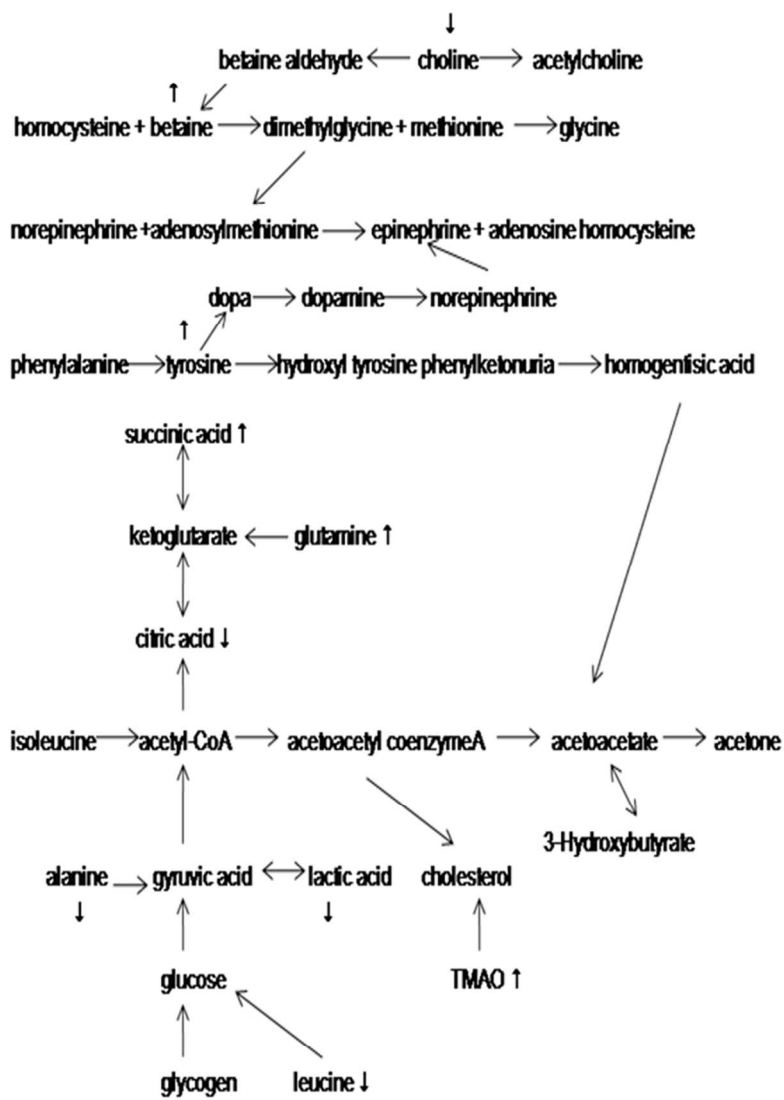46
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