Comparative analysis between aerial parts and roots (Astragali Radix) of astragalus membranaceus by NMR-based metabolomics

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ABSTRACT

Aerial parts of Astragalus membranaceus (APAM), which are derived from the overground part of Astragalus membranaceus consist of stems and leaves. The aim of this study was to provide a scientific basis for the comprehensive utilization of APAM. Nuclear magnetic resonance (NMR) was used to compare the chemical compositions of APAM and Astragali Radix (AR). The pharmacological effects of APAR and AR in cyclophosphamide (Cy)-induced mice were studied using 1H NMR-based metabolomics. The results showed that APAM and AR were different from each other in primary and secondary metabolites, and they could recover the amounts of white blood cell, monocytes, lymphocyte and neutrophils content, IL-2 and IL-6; there was no significant difference in APAM and AR in terms of immune functions. The mechanisms of APAM and AR on leucopenia were probably related to energy metabolism, amino acids metabolism, and gut microbiota-related metabolism.

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1. Introduction

Astragali Radix (AR), also known as Huangqi in China, is a commonly used herbal drug in Traditional Chinese Medicine and is important for the treatment of fatigue, diarrhea, lack of appetite, uterine bleeding, edema due to deficiency of Qi, abscesses, anemia, and

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wasting-thirst (Kuo, Tsai, Loke, Wu, & Chiou, 2009; Li et al., 2003; Shon, Kim, & Nam, 2002; Zhao, Ma, Zhu, Yu, & Weng, 2011). The main source of AR in China is the dried root of *Astragalus membranaceus* (Fisch.) Bge. or *Astragalus membranaceus* var. *mongholicus* (Bge.) Hsiao.

Aerial parts of *Astragalus membranaceus* (APAM) are derived from the overground part of *Astragalus membranaceus*, which consists of stems and leaves. Previous reports showed that APAM had many biological effects including immune stimulation and immune regulation such as increasing the valences of anti-NDV immune body, and immune organ index, and playing an important role in cardiovascular system disease (Lin et al., 2000). The chemical compositions of APAM were saponin, flavonoids, polysaccharide, and other components (Li, Gao, et al., 2014; Zhang, 2007; Zhang, 2010). However, previous studies were focused on the chemical components of APAM. There are few reports about its pharmacological activity, or the differences between APAM and AR. In China, the traditional use of AR is the root of *A. membranaceus* (Fisch.) Bge. or *A. membranaceus* var. *mongholicus* (Bge.) Hsiao, and the APAM was usually discarded after the harvest of roots. In order to utilize the wasted resources of APAM, chemical and biological comparison between AR and APAM was needed.

As a part of systems biology, metabolomics which was a newly developed strategy firstly mentioned by Nicholson and co-workers has been demonstrated as a feasible and powerful tool for chemical analysis of herbal medicines and the identification of biomarkers. In recent years, metabolomics has been widely used in many fields, such as drug discovery, disease process, toxicological evaluation (Laaksonen, 2016; Niu, Li, Du, & Qin, 2016; Patel & Ahmed, 2015), processing mechanism investigation, and species differentiation (Ernst, Silva, Silva, Vencio, & Lopes, 2014). Of the various profiling techniques in metabolomics, nuclear magnetic resonance (NMR) can provide nonselective and nondestructive information of samples and emerge as a preferred platform with minimal sample preparation.

In this work, NMR-based fingerprinting was used to compare the chemical compositions of APAM and AR. The biological effects of APAM and AR were also compared by using a Cy-induced leucopenia mice model coupled with 1H NMR-based metabolomics.

2. Materials and methods

2.1. Plant material

APAM and AR samples were collected from the Hunyuan County in Shanxi Province in September 2015 from the same planting area (the age of 5), and were authenticated by Prof. Yun-E Bai, School of Pharmaceutical Science of Shanxi Medical University. The voucher specimens were deposited in the herbarium of the School of Pharmaceutical Science of Shanxi Medical University until use.

2.2. Solvents and chemicals

Methanol and ethyl acetate (Analytical grade) were bought from Beijing Chemical works (Beijing, China). Cy, purchased from Jiangsu sdsensor Pharmaceutical Co., Ltd (Batch No. 15092125), was dissolved in sterilized physiological saline (8.0 mg/ml) and stored at 4°C before use.
Sodium 3-trimethylsilyl [2, 2, 3, 3-d₄] propionate (TSP) was obtained from Cambridge Isotope Laboratories Inc (Andover, MA, USA). D₂O was bought from Norell (Landisville, NJ, USA), and NaOD was purchased from Armar (Dottingen, Switzerland). Analytical grade K₂HPO₄·3H₂O and NaH₂PO₄·2H₂O were obtained from Beijing Chemical Works (Beijing, China). Interleukin-2 (IL-2) and Interleukin-6 (IL-6) Enzyme-Linked Immunoassay (ELISA) kits were supplied by R&D (USA).

2.3. APAM and AR aqueous crude extract preparation for chemical analysis and biological evaluation

According to the traditional use, the crudes of APAM and AR were pulverized into a powder. Each powdered sample (100 g) was weighed accurately and extracted with water by refluxing twice (1000 mL and 2 h each). The mixture was filtered, concentrated under reduced pressure, and subsequently freeze-dried.

2.4. NMR analysis of APAM and AR

For NMR analysis, two different sample preparation procedures were used for the comprehensive chemical characterization of APAM and AR aqueous crude extract. In the first procedure (M1), the freeze-dried powder (about 50 mg) was dissolved in KH₂PO₄ buffer in D₂O (adjusted to pH 6.0 by 1N NaOD) containing 0.05% TSP. After centrifuging for 15 min at 13,000 rpm, the supernatant (600 µL) was transferred into a 5-mm NMR tube for NMR analysis. In the other procedure (M2), 10 g of freeze-dried powder was suspended in 100 mL of water and partitioned by an equal volume of ethyl acetate. The combined extracts were evaporated to dryness, and 650 µL of deuterated methanol solution was added. The supernatant (600 µL) was transferred into 5-mm NMR tubes for ¹H NMR analysis after centrifuging for 15 min at 13,000 rpm.

All spectral data (¹H NMR and 2D NMR) were obtained at 25°C on a Bruker Avance 600-NMR spectrometer (600.13 MHz proton frequency, Bruker, Germany). NMR parameters were studied as previously described (Kim, Choi, & Verpoorte, 2010; Li, Zhang, Du, & Qin, 2015).

2.5. Animal model, drug administration, and sample collection

Kunming mice (18–20 g) were purchased from Experimental Animal Center of Academy of Military Medical Sciences, the Chinese People’s Liberation Army (license number (Military) SCXK2012-0004). All animals were kept in animal room under a constant temperature of 20–24°C with a relative humidity of 65 ± 10% and a 12 h dark–light cycle with free access to food and water. All animals were treated with humane care throughout the experiment under the above conditions for one week acclimation period.

The animal experiment was conducted as previously described with minor modifications (Wang, Tong, Li, Cao, & Su, 2012). After one week’s environmental adaptation, the mice were randomly divided into four groups, the drugs were orally administered to mice once per day for 10 consecutive days, and a total volume of 0.2 mL per 10 g of body weight was administered to the mice once a day. Cy was intraperitoneally (i.p.) injected on days 4–6. Group I (Control group) received physiological saline, group II
(Model group) received Cy (80 mg/kg), group III (APAM group) received APAM crude extracts (10 g/kg/day) and Cy (80 mg/kg), group IV (AR group) received AR crude extracts (10 g/kg/day) and Cy (80 mg/kg). Mice were put to death after blood was collected from the ophthalmic venous plexus on the 11th day. One part of blood was collected by retro-orbital bleed into heparin, which was used to determine the white blood cell (WBC), monocytes (MO), lymphocyte (LY), and neutrophils (NE) by HEMAVET950FS automatic animal blood analyzer. The amounts of IL-2 and IL-6 in serum were analyzed by the mice IL-2 and IL-6 ELISA kits according to the instructions. The other part of the blood was centrifuged at 3500 rpm for 15 min, and then was stored at −80°C for metabolomic analysis. Mice femurs were collected and studied as previously described (Qu, Wang, et al., 2016).

2.6. Preparation of serum samples for NMR measurement

The serum samples were thawed before analysis and were prepared as follows: 100 μL serum was added to 450 μL D$_2$O, and then the mixture was centrifuged at 13,000 rpm for 10 min in 4°C. The supernatants (500 μL) were transferred into 5-mm NMR tubes for $^1$H NMR analysis (Zhao, Huang, et al., 2011).

2.7. NMR measurements for serum

$^1$H NMR spectral data were acquired on a Bruker 600-MHz AVANCE III NMR spectrometer (Bruker, Germany) operating at 600.13 MHz. NMR parameters were studied as previously described (Zhao, Huang, et al., 2011).

The $^1$H-$^1$H correlated spectroscopy (COSY) spectrum consisted of 25 scans with the following parameters: spectral widths of 6602.1 Hz in both dimensions, and relaxation delay (RD) of 1.5 s. The heteronuclear single quantum coherence (HSQC) spectrum consisted of 110 scans with the following parameters: spectral widths of 6602.1 Hz in the $^1$H dimension and 36 219.4 Hz in the $^{13}$C dimension, and RD of 1.5 s.

2.8. NMR data processing

The $^1$H NMR spectra were processed using the MestReNova software (version 8.0.1, Mestrelab Research, Santiago de Compostella, Spain). All spectra of the serum were manually phased and baseline-corrected and referenced to that of creatine at 3.04 ppm. The spectra were integrated into regions with the bucket width of 0.01 ppm intervals across the region δ 0.30–8.00 ppm. The regions containing the resonance from residual water (δ 4.60–5.20) were excluded. Normalization to a total sum of all integrals was conducted prior to analysis.

2.9. Multivariate data analysis

Multivariate data analysis was performed with Simca-P 13.0 software (Umetrics, Sweden). Principal component analysis was first performed on mean-centered data to identify outliers and to obtain the overview of data distributions. Partial least-squares discriminant analysis (PLS-DA), with $R^2$X representing the explained variations and $Q^2$ for the
model predictabilities, applies PLS to discriminate between groups of samples that are
defined as separate response variables. The validity of the model was performed with
SIMCA-P software by permutation tests (200 permutations), a seven-fold cross-validation
method, and CV-ANOVA method.

Relative amounts of metabolites were evaluated based on the integrated regions
(buckets) from the least overlapping NMR signals of metabolites. Results of WBC, MO,
LY, NE, DNA content, IL-2, IL-6, and the peak areas of the differential metabolites
were further compared with t-test using SPSS16.0 software, and differences were con-
sidered to be statistically significant with a p-value threshold set at .05.

3. Results

3.1. Chemical analysis of APAM and AR

3.1.1. Chemical analysis of APAM and AR by NMR

In this study, two methods were used for the NMR analysis of APAM and AR aqueous
 crude extracts. The signals were assigned based on comparisons with the chemical
shifts of standard compounds, NMR databases such as Biological Magnetic Resonance
Data Bank (BMRB) (http://www.bmrb.wisc.edu) as well as reported literature data (Li,
He, Sun, Qin, & Du, 2014; Li, Li, et al., 2015; Liu, Nyberg, Jäger, & Staerk, 2017; Zhao,
Huang, et al., 2011). In addition, 2D NMR spectra including 1H-1H COSY and HSQC
were also used in the metabolite identification (Figure S1, S2).

The 1H NMR spectra in the M1 fractions can be divided into four regions (In
Figure 1(a,c)). In the amino acids region, some amino acids including leucine, isoleuc-
cine, valine, threonine, alanine, arginine, proline, glutamine, GABA, aspartic acid,
asparagine, glycine, and phenylalanine were assigned clearly; in the organic acids
region, acetic acid and succinic acid were also identified; in the sugar region, xylose,
glucose, galactose, sucrose, and raffinose were identified; in the aromatic region,
uridine, fumaric acid, tyrosine, adenine, formic acid, trigonelline, and adenosine
were evident. In addition, some nitrogen compounds, such as choline and betaine,
were also detected.

In addition to the large amount of primary metabolites, the signals of secondary
metabolites were also detected. Both in APAM and AR of M2 fraction (Figure 1(b,d)),
saponins such as astragaloside I, astragaloside II, astragaloside III, astragaloside IV,
and flavonoids such as calycosin, calycosin-7-O-D-glucoside, 1, 2-dihydroxy-3, 4
-dimethoxyisoflavan-7-O-β-D-glucoside, formononetin, and 9, 10-dimethoxypterocarpan-3-O-β-D-glucoside were detected. Visual inspection of the fraction showed that
threonine, adenosine, and adenine were only detected in AR and tyrosine was only
present in APAM. Meanwhile, the contents of saponins and flavonoids in APAM and
AR were obviously different, which were present at higher concentrations in AR than
in APAM. The chemical shifts and coupling constants of all the identified metabolites
were summarized in Table S1. Adenosine was present with signals at 6.07 (d, J =
7.44 Hz, H-1′), 8.24 (s, H-2), 8.35 (s, H-8). Sucrose was detected by the signals at 5.42
(d, J = 3.90 Hz, H-1), 3.56 (dd, J = 3.78, 6.18 Hz, H-2), 4.05 (t, J = 8.56 Hz, H-4′), 4.22
(d, J = 8.76 Hz, H-3′), 3.68 (s, H-1′), 3.82 (m, H-6,6′), the assignment can be verified
by 2D NMR spectra(1H-1H COSY and HSQC NMR). δ 6.85 (d, J = 8.04 Hz, H-5′), δ
6.93 (dd, $J = 3.24$, 6.42 Hz, H-6'), $\delta$ 7.04 (brs, H-2'), $\delta$ 8.04 (d, $J = 9.0$ Hz, H-5), $\delta$ 8.11 (s, H-2) were assigned to be calycosin, a major isoflavone in APAM and AR. Formononetin, another isoflavonoid, was at $\delta$ 7.04 (d, $J = 9.0$ Hz, H-3',5'), $\delta$ 7.45 (d, $J = 9.0$ Hz, H-2',6'), $\delta$ 8.07 (d, $J = 8.76$ Hz, H-5), $\delta$ 8.18 (s, H-2).

In the picture, different numbers represent different metabolites, and all the identified metabolites were summarized in Table S1.

Figure 1. Typical $^1$H NMR spectra of primary and secondary metabolites. A: $^1$H NMR spectra of aerial parts of APAM of primary metabolites; B: $^1$H NMR spectra of APAM of secondary metabolites; C: $^1$H NMR spectra of aerial parts of AR of primary metabolites; D: $^1$H NMR spectra of AR of secondary metabolites.
3.1.2. The differences of the contents of the major components between APAM and AR

The relative contents of primary and secondary metabolites were determined by the peak areas of the least overlapping signals of metabolites, and TSP and deuterated methanol were used as internal standard, respectively (Table S3). The result showed that the relative contents of primary metabolites such as leucine, isoleucine, valine, alanine, acetic acid, GABA, succinic acid, aspartic acid, asparagine, choline, betaine, α-galactose, fumaric acid, tyrosine, formic acid, trigonelline, as well as secondary metabolites such as astragaloside I, astragaloside IV, formononetin, 1,2-dihydroxy-3,4-Dimethoxyisoflavan-7-O-β-D-glucoside were higher in APAM.

3.2. Biological evaluation of APAM and AR in Vivo
3.2.1. Blood parameters

As shown in Figure 2, a significant decrease of WBC, NE, LY, and MO levels in the model group was detected on the 11th day by comparison with the control group ($p < .01$), the levels of WBC, NE, LY, and MO can be reversed significantly to control levels in APAM and AR groups, and there was no significant difference in APAM and AR groups.

![Figure 2](image_url)

**Figure 2.** Scatter figures of periphery blood parameters in control, model, aerial parts of APAM and AR groups. Compared with the control group,* $p < .05$, **$p < .01$; compared with the model group, ¨ $p < .05$, ## $p < .01$. 
3.2.2. DNA content of bone marrow cells
As shown in Figure 3, compared with the control group, it indicated that Cy treatment caused the decrease of DNA content of bone marrow cells in the model group ($p < .01$). In this study, APAM- and AR-treated groups prevented the reduction of DNA content of bone marrow cells ($p < .05$), suggesting that APAM and AR exerted bone marrow protective effects against Cy-induced bone marrow injury.

3.2.3. The amounts of IL-2 and IL-6
As shown in Figure 4, the levels of IL-2 and IL-6 in the model group were reduced after the Cy treatment, as compared with the control group ($p < .01$). The APAM and AR treatment could all recover the amounts of IL-2 and IL-6 as compared with the model group ($p < .01$), whereas the amounts of IL-2 and IL-6 in the APAR and AR groups were still lower than in the control group.

Figure 3. Scatter figure of DNA content of bone marrow cells in control, model, aerial parts of APAM and AR groups. Compared with the control group, *$p < .05$, **$p < .01$; compared with the model group, #*$p < .05$, ##$p < .01$.

Figure 4. Scatter figures of IL-2 and IL-6 in control, model, aerial parts of APAM and AR groups. Compared with the control group, *$p < .05$, **$p < .01$; compared with the model group, #*$p < .05$, ##$p < .01$. 
3.2.4. Metabolomic study

3.2.4.1. Metabolite assignment. Figure 5 showed the typical $^1$H NMR spectra of serum from the control group. Analysis of NMR spectra data involved assigning specific metabolites which were assigned basing on the chemical shifts of standard compounds from the literature data (Jiang, Huang, Wang, & Tang, 2012; Shin et al., 2011; Xu et al., 2017; Zhang et al., 2011), HMDB and BMRB. In addition, 2D NMR spectra including $^1$H-$^1$H COSY and HSQC were also used in the metabolite identification (Figure S3). Twenty-six metabolites were detected in mice serum, and it was found that the samples mainly contained organic acids, amino acids, glycolysis, and tricarboxylic acid cycle (TCA) intermediates, choline metabolites, organic bases, purines, and the other metabolites. Detailed assignments of all identified compounds have been listed in Table S2. In order to obtain more detailed metabolomic changes, we further performed a multivariate analysis of these data.

Figure 5. Representative $^1$H NMR spectra of serum from the control group. In the picture, different numbers also represent different metabolites, and all the identified metabolites have been summarized in Table S2.

Figure 6. PLS-DA score plot (a), PLS-DA permutation test (b) of mice serum between the control and model groups.
3.2.4.2. Multivariate statistical analysis of NMR data. In this study, a PLS-DA method was established and employed to identify biomarkers. As shown in Figure 6(a), there is a classification between the control and model groups, but the R²Y and Q²Y were 0.153 and 0.191, respectively, which indicated that the model had bad prediction characteristics. The permutation test (Figure 6(b)) showed that the PLS-DA model built for control and model groups was invalid. Thus, the differences of control and model groups in metabolome could not be observed by multivariate analysis.

Table 1 shows the relative integral levels of metabolites in serum samples from the control and model groups. The result showed that mice in the model group had obviously lower levels of lipids, succinate, trimethylamine oxide (TMAO), and tyrosine and higher levels of leucine, glutamate, creatine, choline, glycine, and phenylalanine.

Table 1. Comparison of the integral levels of metabolites in the control and model mice in serum.

| δ¹H | Metabolites          | Control            | Model            |
|-----|----------------------|--------------------|-----------------|
| 0.87| Lipids               | 1.0313 ± 0.1131    | 0.8435 ± 0.2452* |
| 0.94| Isoleucine           | 0.3136 ± 0.04531   | 0.3306 ± 0.03122 |
| 0.96| Leucine              | 0.4178 ± 0.07178   | 0.5030 ± 0.07634* |
| 1.05| Valine               | 0.1505 ± 0.02829   | 0.1494 ± 0.03882 |
| 1.20| 3-HB                 | 0.6314 ± 0.3401    | 0.4176 ± 0.1935  |
| 1.33| Lactate              | 6.9809 ± 1.1260    | 7.3691 ± 1.5380  |
| 1.48| Alanine              | 0.3858 ± 0.07790   | 0.4006 ± 0.04831 |
| 1.72| Lysine               | 0.3019 ± 0.02568   | 0.1999 ± 0.02838 |
| 2.07| Glutamate            | 0.1836 ± 0.03188   | 0.2177 ± 0.03737* |
| 2.14| OAG                  | 0.3543 ± 0.02774   | 0.3704 ± 0.07483 |
| 2.38| Pyruvate             | 0.2867 ± 0.03479   | 0.2826 ± 0.04230 |
| 2.41| Succinate            | 0.2200 ± 0.04192   | 0.1463 ± 0.06676* |
| 2.44| Glutamine            | 0.2931 ± 0.03677   | 0.2624 ± 0.05895 |
| 2.53| Citrate              | 0.07044 ± 0.02752  | 0.0699 ± 0.02611 |
| 2.93| Dimethylglycine      | 0.05150 ± 0.008695 | 0.05754 ± 0.01914 |
| 3.04| Creatine             | 0.2411 ± 0.05539   | 0.2943 ± 0.05027* |
| 3.21| Choline              | 0.6011 ± 0.09753   | 0.7973 ± 0.2062* |
| 3.26| TMAO                 | 0.6431 ± 0.1110    | 0.5747 ± 0.05935* |
| 3.27| Betaine              | 0.7246 ± 0.1034    | 0.6995 ± 0.05587 |
| 3.36| Scyllo-inositol      | 0.08079 ± 0.02942  | 0.07755 ± 0.01928 |
| 3.56| Glycine              | 0.6482 ± 0.07403   | 0.7771 ± 0.1118* |
| 3.66| Glyceride            | 0.5824 ± 0.3039    | 0.5496 ± 0.1526  |
| 5.24| Glucose              | 0.8593 ± 0.2121    | 0.8480 ± 0.1251  |
| 6.91| Tyrosine             | 0.03156 ± 0.006873 | 0.01736 ± 0.01208* |
| 7.32| Phenylalanine        | 0.02872 ± 0.005624 | 0.03850 ± 0.00712* |
| 7.74| Histidine            | 0.02578 ± 0.008569 | 0.01887 ± 0.01335 |

Note: Compared with the control group, *p < .05, **p < .01. 3-HB: 3-D-Hydroxybutyrate; OAG: O-acetylated glycoproteins; TMAO: trimethylamine oxide.

3.2.4.2. Multivariate statistical analysis of NMR data. In this study, a PLS-DA method was established and employed to identify biomarkers. As shown in Figure 6(a), there is a classification between the control and model groups, but the R²Y and Q²Y were 0.153 and 0.191, respectively, which indicated that the model had bad prediction characteristics. The permutation test (Figure 6(b)) showed that the PLS-DA model built for control and model groups was invalid. Thus, the differences of control and model groups in metabolome could not be observed by multivariate analysis.

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3.2.4.3. Metabolomic study of APAM and AR. Mean levels of 10 biomarkers including lipids, leucine, glutamate, succinate, creatine, choline, TMAO, glycine, tyrosine, and phenylalanine were used to evaluate therapeutic effects of APAM and AR on leukopenia mice. In Figure S4, eight biomarkers including lipids, leucine, succinate, creatine, TMAO, glycine, tyrosine, phenylalanine could be reversed by APAM and seven biomarkers including leucine, glutamate, succinate, TMAO, glycine, tyrosine, phenylalanine could be reversed by AR. Lipids and creatine could be reversed only by APAM, and glutamate could be reversed only by AR.
4. Discussion

4.1. Chemical compositions of APAM and AR

The present study revealed that primary metabolites such as threonine, adenosine, and adenine were detected only in AR, tyrosine was present only in APAM, but APAM and AR had same secondary metabolites including saponins and flavonoids. Meanwhile, the relative contents of metabolites in APAM and AR were different. Biosynthesis of threonine in plants is determined by competitive affinities of threonine synthase and cystathionine γ-synthase for a common pool of O-phosphohomoserine (Joshi, Joung, Fei, & Jander, 2010). Tyrosine is a precursor for the plant defense compound dhurrin. Recent advances in understanding the biosynthesis of tyrosine have come from the characterization of enzymes that catalyze the N-hydroxylation of the precursor amino acid to the oxime intermediate (Celenza, 2001). It may be that different enzymes were contained in APAM and AR; therefore primary metabolites were not similar to each other.

4.2. Immune functions of APAM and AR

WBC is composed of NE, LY, MO, eosinophil, and basophil granulocyte; total WBC count has been considered as a useful marker of immune changes in humans, with absolute numbers providing more reliable information than percentages (Qu, Li, Zhao, Li, & Qin, 2016; Serdar, LeBlanc, Norris, & Dickinson, 2014). IL-2 and IL-6 in mice serum are cytokines that were associated with immune functions (Jones, 2005; Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006). All the results showed that there was no significant difference in APAM and AR in terms of immune functions.

4.3. Metabolic pathway changes induced by APAM and AR

The metabolites including lipids, leucine, glutamate, succinate, creatine, TMAO, glycine, tyrosine, and phenylalanine could be regarded as potential biomarkers; the corresponding perturbed metabolic pathways may play a key role in APAR and AR for the treatment of leucopenia. The changes of those potential biomarkers were related to some metabolic pathways including energy metabolism, amino acids metabolism, and gut microbiota-related metabolism. A schematic diagram of the perturbed metabolic pathways is shown in Figure 7.

4.3.1. Energy metabolism

Leucine and succinate were all involved in energy metabolism. Leucine is an important ketogenic amino acid, which can be converted to intermediate product of 3-HB for energy production (Shi, Xiao, Wang, & Tang, 2013). Succinate which is related to energy metabolism is the major intermediate of TCA. In the study, decreased succinate and increased leucine were observed in the model group, suggesting that Cy may affect the energy supply. In APAM and AR groups, these two metabolites were all reversed, which suggested that APAM and AR had a call-back effect on regulation of the dysfunction of energy metabolism in leukopenia mice.

Lipids and creatine were also all involved in energy metabolism. Creatine is converted into phosphocreatine by creatine kinase, and phosphocreatine is an important energy storage
A compound that stores the energy of excess ATP (Guo et al., 2014; Ma et al., 2010). Lipids are a large group of organic compounds that are esters of fatty acids and related derivatives (Zhao, Huang, et al., 2011). Increased level of creatine as well as decreased level of lipid were observed in the model group; supportive evidence for our observations has already been obtained with metabolomics research on blood deficiency syndrome animal model induced by Cy. The levels of lipids and creatine could be reversed only by APAM, which suggested that APAM could regulate the dysfunction of energy metabolism through more ways.

4.3.2. Amino acids metabolism
Glutamate, glycine, tyrosine, and phenylalanine were all involved in amino acids metabolism. Amino acids are basic units for protein synthesis in an organism and some of them play important roles in the immune system (Roth, 2007). Glutamate can be hydrolyzed by glutamine through glutamine phosphatase. Studies showed that glutamate affected the proliferation of lymphocytes, the immune response of monocytes/macrophages, and the synthesis of Hsp 70 (Exner et al., 2003). Glycine is the smallest of the amino acids and consists of a single carbon molecule attached to an amino acid and a carboxyl group. Previous studies showed that glycine had been shown to have similar inhibitory effects on several WBCs, including hepatic and alveolar macrophages, neutrophils, and lymphocytes (Roth, 2007). We observed altered amino acids profiles in the model mice, including increased levels of glutamate, glycine, and phenylalanine as well as decreased level of tyrosine. In APAM and AR groups, these metabolites were all reversed toward the normal controls, which indicate that APAM and AR had regulation effect on amino acids’ metabolism.

4.3.3. Gut microbiota-related metabolism
Trimethylamine (TMA) is produced via the action of gut microbiota on choline (Zhao, Huang, et al., 2011) and is then detoxified in the liver through flavine monooxygenase forming TMAO (Wang et al., 2011). The disturbance of gut microbiota will affect the levels of TMA and TMAO, and further affect the body’s immune function, which is in agreement with reported results (Kamada, Seo, Chen, & Núñez, 2013; Richards, Yap, McLeod, Mackay, & Mariño, 2016). The model group showed lower level of TMAO, which suggested that Cy had an effect on disturbance of gut microbes. After APAM or AR treatment, the level of TMAO showed the tendency of recovery to the normal controls, indicating that APAM and AR could improve the disturbance of gut microbes induced by Cy.
In the metabolic pathways above, the enhancing effect of APAM on energy metabolism was stronger than that of AR, but was weaker on amino acids’ metabolism and gut microbiota-related metabolism. However, the metabolic pathways with APAM and AR were the same for the treatment of leucopenia.

4.4. Correlation analysis of metabolites in APAM and AR with their pharmacological activities

Despite the fact that AR showed higher levels of saponins and flavonoids, APAM and AR exhibited a similar effect on leucopenia mice, suggesting that the other compounds also contributed to the therapeutic effects of APAM. Therefore, the bioactive compounds responsible for regulating immune functions of APAM should be further studied.

5. Conclusions

In this study, we found that APAM and AR were different from each other in regard to flavonoids, saponins, and primary metabolites. Moreover, the differences in the relative contents of metabolites were obvious. The relative content differences in the levels of these metabolites may contribute to differences in biological activity. So the differences in the contents of these metabolites in APAM and AR should also be further investigated. We investigated the pharmacological effect of APAM and AR on a Cy-induced leucopenia mice model using a metabolomic approach based on $^1$H NMR. The difference in regulating biomarkers induced by Cy in APAM and AR was relatively small. AR possesses various bioactivities, and the difference in biological effects between APAM and AR should also be investigated further by diverse animal models and multianalytical techniques.

Disclosure statement

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**Appendix**

Assignments of $^1$H NMR spectral peaks obtained from APAM and AR (Table S1), $^1$H NMR assignments of major metabolites from mice serum (Table S2), 2D NMR spectra of APAM and AR of primary metabolites (Figure S1), 2D NMR spectra of APAM and AR of secondary metabolites (Figure S2), 2D NMR spectra of mice serum (Figure S3), Histograms of potential biomarkers in the Cy-treated mice associated with APAM and AR treatment in mice serum (Figure S4). This information is available free of charge via the internet.