Potential quality evaluation approach for the absolute growth years’ wild and transplanted Astragali Radix based on anti-heart failure efficacy

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[ABSTRACT] The quality of Astragali Radix (AR) was closely related to the growth period. However, the current commodity grades of AR were only divided by diameter but not directly related to the growth period, which leads to the contradiction between the grade standard and the quality evaluation index. Therefore, solving this problem will be the key for the quality evaluation of AR. The present study established a potential quality evaluation approach for the absolute growth years’ wild Astragali Radix (WAR) and transplanted Astragali Radix (TAR) based on the chemical components and anti-heart failure efficacy through adopting a bare-handed sections approach to rapidly identify the growth years of WAR. In this study, the absolute growth years of WAR were obtained by identifying the growth rings of 1~6 growth years root through the methods. The contents of flavonoids and saponins in 2~6 growth years’ WAR were determined by HPLC-UV-ELSD. The contents of 12 chemical components and the anti-fatigue failure effects of WAR (4-year-old) and TAR were compared on rat models of heart failure induced by doxorubicin. Meanwhile, NMR-based untargeted metabolomics studies were performed to investigate the regulative effects of WAR and TAR. The result shows that the numbers of growth rings were consistent with the actual growth periods of AR. The HPLC-UV-ELSD determination indicated that the content of total flavonoids in WAR was significantly higher than that in TAR. Pharmacodynamics analysis revealed that the effects of WAR on cardiac function parameters (EF, FS and LVIDs), contents of serum CK and BNP were superior to those of TAR. 13 metabolites of heart were identified that had a higher rate of change in WAR group than TAR. Overall, a rapid identification method for the growth years of WAR was established, and the fact that WAR were significantly better than TAR in the heart failure rats was first proved in the paper. This study provided a scientific basis for establishing a novel commodity specification and grade of AR for clinical rational drug use.

[KEY WORDS] Astragali Radix; growth rings; chemical analysis; anti-heart failure

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were mainly included the Hunyuan and Shanxi provinces of China. In these areas, the growing areas are mainly located on slopes with barren and porous soil, abundant sunshine during summer, arid (rainless) climate, large temperature differences between day and night, and cold winters. WAR grows for more than six years under such conditions. By contrast, TAR are cultivated on selected managed land and are harvested two years later. WAR suffers from drought, cold, nutrient deficiency, and other environmental stresses, whereas TAR is less affected by environmental stressors.

In the 1980s, the longer growth period of WAR caused the situation of demand exceeding supply. With the appearance of TAR with the short period (2-3 years), market demand was alleviated but the WAR resources were declining seriously [1]. As the qualified standard of RA, the Chinese Pharmacopoeia (2015 edition) specifies that the contents of astragaloside IV and isoflavone glucoside should not be less than 0.04% and 0.02% respectively. However, previous studies [4] showed that as the increases of the content of Astragaloside IV, diameter and commercial grades decreased. In the same study, it found that the content of flavonoids had no correlation with different grades. Tu [5] found that the content of calycosin glucoside of two/three levels of AR was not lower than the first levels. However, the international market does not accept TAR, and some veteran TCM physicians still use WAR. These conflicts show that the current quality evaluation method cannot be used for accurate assessment of the quality of AR. Therefore, a more rational evaluation method is urgently needed for the development of genuine medicinal materials.

The commodity specification and grade of traditional Chinese medicine is an important reference index to evaluate the better quality of herb. Commodity AR is traded only by grade, with diameter as the main index. A large diameter indicates high grade and price [6]. The commodity specification and grades of the medicine plants’ roots should reflect not only the quality but also the growth years. Long life expectancy leads to considerable accumulation of secondary metabolites and high efficacy [7]. Taking ginseng as an example, the longer the growing year, the better the quality is. The growth of ginseng can be determined by the number of rhizome [8], which does not exist in 3- and 4-year-old ginseng while exists in the 5- and 6-year-old samples. However, the appearance of AR cannot directly reflect the growth years due to the fact that removing the reed head and tail of AR and cutting the lateral roots mixed into the main root. Consequently, different parts of AR with different growth years may mix together by diameter in the processing for commodity (Fig. 1). Therefore, accurate judgement of the growth years of AR is of great significance for establishing reasonable and scientific commercial grades of AR.

As "Herb-chronology" has received more and more attention, Dietz [9-11] found that most of them existed growth ring structure representing growth years in the roots of 35 dicotyledonous herbs. Peng [12] thought that the growth ring of AR can be used as the basis for the identification of growth years. The present work not only investigated differences of growth ring between different years AR and the upper and lower of the same root based on observation of the growth ring of AR by paraffin sections coupled with bare-hand sections, but also summarized the relativity between growth ring, diameter and growth years to establish an identification method of growth years of AR based on the number of growth ring. Additionally, a segmentation method was established according to the feature that the number of growth ring of AR root was gradually decreased from top to bottom. Then, the corresponding relation was analyzed between two kinds of AR and 12 kinds of indicators by HPLC-UV-ELSD to compare the accumulation trends of flavonoids and saponins between WAR (4-year-old) and TAR based on the segmentation method. In additional, our group found that AR has obvious anti-heart failure effect in the early stage [13], so the anti-heart failure effects of WAR (4-year-old) and TAR were compared with rat models of heart failure induced by doxorubicin in order to build a Potential quality evaluation approach for the absolute growth years’ wild and transplanted Astragal Radix based on anti-heart failure efficacy.

Overall, this study will not only provided a scientific basis for establishing a novel commodity specification and grade of AR for clinical rational drug use, but also points out the direction for protecting and developing the resources of AR.

Materials and methods

**Materials and Chemicals**

WAR was obtained from Hunyuan, Shanxi, and TAR was collected from Longxi, Gansu, both of which were identified as the roots of Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao by Professor Xue-Mei Qin. The voucher specimens were deposited in the herbarium of the Modern Research Center for Traditional Chinese Medicine of Shanxi University.

Phloroglucinol was purchased from Dezhou Runxin Experimental Instrument Co., Ltd. (Dezhou, China). FAA fixative (70% ethanol : methanol : acetic acid = 90 : 5 : 5). Doxorubicin (DOX) was purchased from Shanxi Pude Pharmaceutical Co., Ltd. (Shanxi, China). Digoxintablet was purchased from Xinyi Pharmaceutical Co., Ltd. (Shanghai, China). Calycosin-7-O-β-D-glucoside, Ononin, (6aR,11aR)-9,10- Dimethoxypterocarp, 8,2’-Dihydroxy-3,4-Dimethoxy isoflavan-7-O-β-D-glucopyranoside, Calycosin, Formononetin, 4-Dimethoxyisoflavan-7-O-β-D-glucopyranoside, (6aR, 11aR)-9,10-Dimethoxypterocarp, 8,2’-Dihydroxy-3,4-Dimethoxyisoflavan, Astragaloside IV, Astragaloside III, Astragaloside II and Astragaloside I were obtained from Shanghai...
After AR was infiltrated with water, the root was cut into 2-3 mm sections. Concentrated hydrochloric acid phloroglucinol was used to dye the sections of AR and observe the number of growth ring of the sections under a stereomicroscope. The materials were cut into 20 μm sections after fixation, dehydrated, and embedded using paraffin. Safranine and Fast Green were used to dye the sections of AR and observe the ring structure with the stereomicroscope.

**HPLC-UV-ELSD analysis**

Samples of Calycosin-7-O-β-D-glucoside, Ononin, (6αR,11αR)-9,10-Dimethoxypterocarpan-3-O-β-D-glucopyranoside, 8,2′-Dihydroxy-3,4-Dimethoxyisoflavan-7-O-β-D-glucopyranoside, Calycosin, Formononetin, 3-hydroxy-9,10-Dimethoxypter can, 8,2′-Dihydroxy-3,4-Dimethoxyisoflavan, Astragaloside IV, III, II and I were precision weighed. The method validation was include precision, stability, repeatability, recovery and linear regression, and the result was shown in Supplementary Table S1.

**Extract preparation**

Dried WAR and TAR samples were soaked for 4 h and extracted 3 times with 10 times the amount of water under backflow for each 1.5 h. The combined extract was concentrated under reduced pressure to 1 g/mL for a further animal experiment.

**Animals and treatment**

A total 40 male Sprague–Dawley rats (weighing 200 ± 20g) were provided from Beijing Vital River Laboratories Co., Ltd. (SCCXK (Jing) 2017-0012, Beijing, China). The environment was maintained at 25 ± 1 °C with a relative humidity of 50%-60% and a 12 h: 12 h light-dark cycle. All rats had free access to diet and water. Rates were allowed to adapt to the new environment for 7 days. The experiment was approved in accordance with the National Guidelines for Experimental Animal Welfare (MOST, China, 2006) at the Center for Animal Experiments, which has full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International.

Rats were randomly divided into five groups (n = 8): control group (NS), model group (MS), positive control group (DG), transplanted Astragali Radix group (TAR), and wild Astragali Radix group (WAR). The model group and treatment groups were intraperitoneally injected with DOX, and the dosage regimens were 1, 3 days, 1 mg/kg; 5, 7 days, 2 mg/kg; 9, 11 days, 3 mg/kg for DOX. For the control group, rats received equal volume of physiological saline. Rats in the treatment groups were administered AR (7.5 g/kg body weight). The positive control group was orally gavaged with digoxin (0.03 mg/kg), whereas the control and model groups were only administered the same amount of water every day. This procedure lasted for 11 days.

**Determination of Echocardiography**

48 hours after the DOX administration, all rats were checked to Echocardiography after anesthesia with chloral hydrate (4%, 0.8 ml/100 g). IVSs (inter-ventricular septum thickness of systolic), IVSd (inter-ventricular septum thick-
ness of diastolic), LVIDs (left ventricular systolic diameter), LVIDd (left ventricular diastolic diameter), LVPWs (left ventricular systolic wall thickness), and LVPWd (left ventricular diastolic wall thickness) were measured. The EF(ejection fraction) and FS (fractional shortening) were determined by functional calculation software.

Biochemistry assays and examination of histopathology

After Echocardiography, rats were sacrificed under 4% chloral hydrate (0.8 ml/100 g). Blood were collected and centrifuged at 4000 rpm for 15 min at 4.0 °C. The resultant serum were obtained and stored at −80 °C. The heart tissues were quickly removed, and one part of myocardial tissues was cut for the Histopathological examination.

The serum levels of creatine kinase (CK) and lactate dehydrogenase (LDH) were determined with an automatic biochemical analysis tester. The serum level of brain natriuretic peptide (BNP) was determined with the corresponding enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions.

Formalin-fixed heart tissues were embedded in paraffin wax and cut transversely into sections of 4-5 μm. These sections were then stained with routine hematoylin-cosin staining (H&E). Images were obtained and studied under light microscopy (Olympus BX53, Tokyo, Japan).

Preparation of sample and analysis of \(^1\text{H}\)-NMR spectroscopy

200 mg heart tissue were weight into 10mL centrifuge tube and mixed with 900 μL methanol-water solution, homogenization. The samples were centrifuged at 13000 rpm for 20 min at 4 °C, and the supernatants were collected and concentrated to dry. Then samples were mixed with PBS (D_2O, 0.1 mol/L, K_2HPO_4/NaH_2PO_4, PH 7.4) and centrifuged for 15 min at 13000 rpm, 4 °C. 600 μL of the supernatants were transferred into 5 mm NMR tubes for \(^1\text{H}\)-NMR analysis.

\(^1\text{H}\)-NMR spectra were recorded at 298 K on a Bruker 600-MHz AVANCE III NMR spectrometer (Bruker BioSpin, Bremen, Germany) equipped with a Bruker 5 mm PABBO probe operated at 600.13 MHz \(^1\text{H}\) frequency. One-dimensional spectra were recorded using noesygpprd sequence. Each \(^1\text{H}\)-NMR spectra of serum consisted of 64 scans requiring a 2.654 s acquisition time with the following parameters: spectral width of 12 345.7 Hz, spectral size of 65536 points, and a relaxation delay (RD) of 1.0 s.

Statistical analysis

The \(^1\text{H}\)-NMR spectra of heart samples were manually phased and baseline corrected using MestReNova software (version 8.0.1, Mestrelab Research, Santiago de Compostella, Spain). The peaks of heart tissue spectra were referenced internally at δ 0.00 ppm. The spectral regions of δ 0.5–9.0 were integrated into equal width of 0.01 ppm. The regions contained residual water signals δ 4.68–5.06 were excluded to eliminate the effect of imperfect water saturation. The remaining spectra were normalized to the total sum of integral area prior to subsequent data analysis. The normalized integral values were then pare to centered and subjected to multivariate pattern recognition analysis using the SIMCA-P 13.0 software (Umetrics, Umeå, Sweden). Orthogonal partial least squares discriminate analysis (OPLS-DA) was utilized to reveal the differences between two groups. Potential biomarkers were extracted from S-plots based on their contribution to the variation.

All values were presented as mean ± SD. The results were compared by t-test using SPSS 21.0 software, and \(P < 0.05\) were considered significant.

Results and discussion

Establishment of a rapid identification approach of the growth years for WAR

As shown in Fig.2A, the cross-sectional structure of WAR conformed to the secondary structure of dicotyledonous roots and consisted of peritoneum, phloem, vascular cambium, secondary xylem, and primary xylem. Cambium, which was easily broken by external forces, was the dividing line between xylem and phloem and the beginning of the growth ring. Secondary xylem, due to the seasonal changes, had grown the big catheter group, small catheter group, and structure of wood fibers, which were regularly arranged to form growing rings.

The bare-handed section results showed that the number of growth ring, except that in the hollow part, was consistent with the actual growth period of AR and the paraffin-cut section dose, which indicated that the use of bare-handed section could accurately determine the growth years of WAR. This research inspired us to develop a simple and rapid approach of identification the years of AR in the market for selection of high quality commodity. The method avoids many of the problem that the experiment process was complicated (fixation, washing and dehydration, transparency, wax dipping, embedding, slicing and patching, dewaxing, dyeing, dehydration, transparency, sealing), time-consuming and costly in the paraffin-cut section.

The research indicated that the diameter of annual WAR root was less than 0.5 cm. Only the primary xylem structure existed in the central region, the vessel arrangement was irregular, and the growth ring was not formed (Fig. 2B). The 2-year-old AR presented two clear growth rings (Fig. 2C). Three growth rings existed in the 3-year-old AR (Fig. 2D), and the number of growth ring increased consequently from 4-year-old to 6-year-old AR (Fig. 2E, F, and G).

The results showed that the numbers of growth ring on the same WAR were inconsistent, and the number of growth rings gradually decreased from the upper to lower. For the 6-year-old WAR, for example, the numbers of growth ring, except in the hollow part, was consistent with the actual growth period of AR (Fig. 3A). The number of growth rings presented a difference of one year, when the diameter differed by approximately 0.3 cm (Fig. 3B). The difference was two years when the diameter differed by approximately 0.6 cm (Fig. 3C). All the rest might be deduced by analogy that as the cross-sectional diameter decreased, the number of growth rings decreased (Fig. 3D, E, and F). The numbers of growth
ring on the same WAR xylem gradually decreased from the upper to lower, which indicated that the growth rings of xylem can be used as a basis to determine the actual growth years of WAR.

Analysis of 12 chemical components in different absolute growth years WAR

Flavonoids and saponins are the main index chemical components of AR, and HPLC-UV-ELSD spectra can simultaneously detect their components, which is the effective means of quality control and evaluation of AR. The HPLC-UV-ELSD spectra of the standard and samples are shown in Fig. 4, in which the flavonoid spectra were obtained by a UV detector (Fig. 4A, 4C), and saponins were displayed by an evaporating photodetector (Fig. 4B, 4D).

The content of 8 flavonoids was added to obtain the content of total flavonoids, and the contents of the four saponins were added to obtain the total saponin content. The content of total flavonoids and total saponins in Radix Astragali, the content of total flavonoids in Astragali Radix grown for 4-years was obviously higher than that of other growth years and the content of total flavonoids fluctuated with different years (Fig. 5A). The contents of total saponin reached its peak in three years of growth and contents of AR decreased slowly with the growth years excepted 3-years AR (Fig. 5B).

Analysis of 12 chemical components in WAR and TAR

In Fig. 6, the quality results of WAR and TAR indicated that the contents of Calycosin-7-O-β-D-glucoside, Ononin, (6αR,11αR)-3-Hydroxy-9,10-Dimethoxypterо carpan, 8,2′-Dihydroxy-3,4-Dimethoxyisoflavan, Astragaloside I and III in WAR was significantly higher than those in TAR. The content of astragaloside IV of TAR was higher than that of WAR, but no significant difference was observed. The content of total flavonoids in WAR was significantly higher than that in TAR, but no significant difference was found in total saponins (Fig. 5C).

Differences of the 12 chemical components between the WAR and TAR were compared through multivariate statistic-

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**Fig. 2** Structure anatomy and growth rings of different years of WAR root. (A) Structure anatomy of WAR root (P-periderm; Ph-phloem; Vc-vascular cambium; Xy-secondary xylem; Px-primary xylem); (B) Growth ring of annual WAR; (C) Growth ring of 2-years WAR; (D) Growth ring of 3-years WAR; (E) Growth ring of 4-years WAR; (F) Growth ring of 5-years WAR; (G) Growth ring of 6-years WAR.
al analysis. The PCA score plot showed that there was a significant separation between the two type of growth patterns of AR (Fig. 5D). The PLS-DA model was validated using the response of the permutation test through 200 permutations, and $R^2$ and $Q^2$ values were lower than the original ones, which informed the validity of the established discriminant model (Fig. 5E). Therefore, the chemical components difference did exist between the WAR and TAR groups.

**Effects of WAR and TAR on rats body weight**

Efficacy is the gold standard of Chinese medicines quality evaluation [16]. Intraperitoneal injection of DOX is a frequently established heart failure model method, which presents simple operation and low mortality [17]. This method was used in this study to evaluate the efficacy of WAR and TAR. During the experiment, the weight of rats in the control group continued to increase (Fig. 7). The weight of rats in the model control group first increased and then decreased until reaching its maximum on the 9th day. Significant differences were observed between the control and model groups from the 9th day ($P < 0.05$). The drug treatment group exerted a significant inhibitory effect on DOX-induced weight loss in rats, and the body weight of the WAR rats was signi-

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**Fig. 3** Growth ring numbers of different diameters from 6 year old WAR root.

- **A**
- **B**
- **C**
- **D**
- **E**
- **F**
significantly regulated compared with that of the model group on the 13th day ($P < 0.05$).

**Results of the organ indexes of rats**

In this study, we determined the levels of the body weight, heart weight, and heart index of all rats. As shown in Table 2, the body weight of the model group decreased significantly compared with that of the control group, and the ratio of heart/body weight increased significantly. The WAR and DG groups exhibited significantly decreased levels of heart/body weight ratio relative to those in the MS group ($P < 0.05$). Thus, WAR enhanced tolerance to doxorubicin (DOX)-induced heart failure.

**Results of Echocardiographic examination**

The heart is the power pump of the entire body. Heart failure often leads to pathophysiological and morphological changes, which lead to the abnormality of the function and structure of the heart. Echocardiography, directly reflecting the ejection capacity, contractile and diastolic capacity of cardiac function, has been indispensably used in the diagnosis of heart failure. EF and FS can reflect the extent of heart damage. The left ventricular Echocardiography M-type curve and two-dimensional spectra of each group of rats are shown in Fig. 8A. In Table 3, compared with the control group, the levels of FS, EF, LVPWs, LVPWd, IVSs, and IVSd were decreased in the model group, whereas the levels of LVIDd and LVIDs were increased. The results indicated that the heart failure model was successfully replicated. Unlike the model group, the WAR and TAR groups exerted sig-
significant effects on FS, EF, LVIDs, IVSs, and LVPWs. Compared with the TAR group, the effect of WAR group on FS, EF, and LVIDs was significant ($P < 0.05$). This result showed that DOX caused cardiac structure and dysfunction, and the WAR group showed a better protective effect than that of the TAR group.

Results of histopathological examination

H&E staining was applied to investigate the effects of AR on heart tissue histological changes in the heart-failure rats (Fig. 8B). The results indicated that the morphology of the myocardial cells was normal, the myocardial fibers were pink, and the cytoplasm was clear in the control group. In the model group, we observed the typical pathological characteristic that the myocardial fibers was swollen and broken, the outline was not clear, some myocardial cells sarcoplasm were condensed, and karyopyknosis and mesenchymal blood existed. The myocardial lesions in each treatment group were sig-

Table 2  Body weight, heart weight, and heart index of rats. (mean ± SD, n=8)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Heart weight (g)</th>
<th>Heart/weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>281 ± 9.85</td>
<td>361.4 ± 31.24</td>
<td>1.18 ± 0.11</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>MS</td>
<td>281 ± 8.90</td>
<td>278.0 ± 31.56</td>
<td>1.31 ± 0.44</td>
<td>0.47 ± 0.12 ***</td>
</tr>
<tr>
<td>DG</td>
<td>281 ± 9.83</td>
<td>299.8 ± 29.85</td>
<td>1.02 ± 0.18</td>
<td>0.34 ± 0.04 *</td>
</tr>
<tr>
<td>WAR</td>
<td>281 ± 8.75</td>
<td>291.5 ± 21.83</td>
<td>1.02 ± 0.11</td>
<td>0.35 ± 0.03 *</td>
</tr>
<tr>
<td>TAR</td>
<td>281 ± 8.83</td>
<td>288.2 ± 19.19</td>
<td>1.12 ± 0.22</td>
<td>0.39 ± 0.13</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001 compared with control group; *p < 0.05, **p < 0.01, ***p < 0.001 compared with model group
Table 3  Effects of AR treatment on Echocardiography parameters. (mean ± SD, n=8)

<table>
<thead>
<tr>
<th>parameter</th>
<th>NS</th>
<th>MS</th>
<th>DG</th>
<th>WAR</th>
<th>TAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>0.54 ± 0.06</td>
<td>0.33 ± 0.04***</td>
<td>0.44 ± 0.07***</td>
<td>0.48 ± 0.07***</td>
<td>0.43 ± 0.07***</td>
</tr>
<tr>
<td>EF</td>
<td>0.88 ± 0.04</td>
<td>0.68 ± 0.05***</td>
<td>0.80 ± 0.07***</td>
<td>0.84 ± 0.06***</td>
<td>0.79 ± 0.08***</td>
</tr>
<tr>
<td>LVPWs</td>
<td>3.18 ± 0.32</td>
<td>2.26 ± 0.19***</td>
<td>2.73 ± 0.26***</td>
<td>2.93 ± 0.24***</td>
<td>2.82 ± 0.42***</td>
</tr>
<tr>
<td>LVIDd</td>
<td>2.14 ± 0.41</td>
<td>1.49 ± 0.19***</td>
<td>1.88 ± 0.35</td>
<td>1.84 ± 0.36</td>
<td>1.85 ± 0.31</td>
</tr>
<tr>
<td>LVIDs</td>
<td>5.94 ± 0.52</td>
<td>6.53 ± 0.36***</td>
<td>6.06 ± 0.36</td>
<td>6.12 ± 0.57</td>
<td>6.31 ± 0.46</td>
</tr>
<tr>
<td>IVSs</td>
<td>3.30 ± 0.35</td>
<td>4.30 ± 0.33***</td>
<td>3.40 ± 0.41***</td>
<td>3.06 ± 0.62***</td>
<td>3.46 ± 0.65**</td>
</tr>
<tr>
<td>IVSd</td>
<td>2.09 ± 0.43</td>
<td>2.12 ± 0.33***</td>
<td>2.50 ± 0.29</td>
<td>2.84 ± 0.29***</td>
<td>2.68 ± 0.29***</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001 compared with control group; *p < 0.05, **p < 0.01, ***p < 0.001 compared with model group

Table 4  Effects of AR on serum biochemical parameters in rats. (mean ± SD, n=8)

<table>
<thead>
<tr>
<th>parameter</th>
<th>NS</th>
<th>MS</th>
<th>DG</th>
<th>WAR</th>
<th>TAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>2642.1 ± 746.9</td>
<td>4506.6 ± 1211.1**</td>
<td>2429.6 ± 614.3***</td>
<td>2123.4 ± 574.7***</td>
<td>3879.9 ± 820.1*</td>
</tr>
<tr>
<td>LDH</td>
<td>487.9 ± 114.1</td>
<td>959.2 ± 193.7***</td>
<td>503.6 ± 53.1***</td>
<td>593.1 ± 125.6**</td>
<td>652.1 ± 148.2*</td>
</tr>
<tr>
<td>BNP</td>
<td>87.99 ± 12.74</td>
<td>106.51 ± 22.56**</td>
<td>91.01 ± 13.87**</td>
<td>98.17 ± 12.18</td>
<td>102.15 ± 12.45</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001 compared with control group; *p < 0.05, **p < 0.01, ***p < 0.001 compared with model group

The model group (P < 0.05).

Analysis of 1H-NMR spectroscopy of heart samples

Representative 1H-NMR spectra of the heart from the control group. 29 metabolites were assigned according to public accessible metabolomic databases, such as HMDB (http://www.hmdb.ca/) and BMRB (http://www.bmrb.wisc.edu/). (Fig. S1, Table S2)

Multivariate statistical analysis

Further, in order to find the main endogenous metabolites that caused the difference between the control group and the model group, a multivariate statistical analysis method was used for analysis. PLS-DA model (Fig. 9A) was firstly established to inspect the separation of two groups. The result showed that rats in the model group have completely separated from the control group. Validity of the model (Fig. 9B) was evaluated in the response of permutation test with 200...
permutations. The model $Q^2$, $R^2$ value obtained from the permutation model was lower than the original points, which was deemed to be of great predictive ability and reliability.

In this work, two-component OPLS-DA models (Fig. 9C) were constructed to reveal the differential metabolites between the model group and the control group. Clear separation was observed in the score plot between the control group and the model group, indicating that there were significant differences between the heart metabolite profiles of two groups. Potential biomarkers were extracted from S-plot (Fig. 9D) based on their contributions to the differences. S-plot is a tool used to visualize the covariance and correlation between the metabolites and the modeled class. Those variables far from the origin contributed to the clustering significantly. The further the variable deviated from the origin, the higher the value of VIP. As a result, variables with VIP > 1 were obtained from the S-plot. Based on the VIP > 1 and $P < 0.05$, 13 metabolites were identified as the potential biomarkers contributed to the clustering.

The peak areas of 13 metabolites were statistically analyzed. The results are shown in Table 5. Compared with the control group, the content of methionine, glutamine, creatine, taurine and alanine increased in the model group. The content of proline, isoleucine, glutamic acid, succinic acid, aspartic acid, phosphatidylcholine, glycerol and lactic acid decreased. Compared with the model group, the WAR group and the TAR group could call back them.

The rate of change of the different metabolites were calculated in WAR and TAR groups compared with the model group and expressed as heat maps (Fig. 9E). The results showed that metabolites such as proline, creatine, methionine and phosphatidylcholine had a higher change ratio in WAR group. The rate of change was calculated according to the following formula (1):

$$ C_R = \frac{(C_1 - C_0)}{C_0} $$

$C_R$ stands for the Rate of Change. $C_1$ stands for the peak area of treat group. $C_0$ stands for the peak area of model group.

**Conclusion**

WAR, which is direct seeding and in which the root has...
approximately 1 m length, grows for at least 6 years, which results in difficulty of dredging and resource shortage. Regarding the development of the 1980s transplanted methods (seedling transplanting, the main roots lying on the ditch, continue to grow down), their low-cost growth characteristics are attributed to the rapid expansion of the production of AR to ease the market demand and impact the wild AR resources [32]. The planting patterns of TAR are cultivated with sources – 11 –

Table 5  Potential biomarkers identified in the rat heart sample. (mean ± SD, n = 8)

<table>
<thead>
<tr>
<th>No.</th>
<th>Metabolites</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>1</td>
<td>Proline</td>
<td>0.108 ± 0.019</td>
</tr>
<tr>
<td>2</td>
<td>Isoleucine</td>
<td>0.085 ± 0.014</td>
</tr>
<tr>
<td>3</td>
<td>Glutamate</td>
<td>0.207 ± 0.032</td>
</tr>
<tr>
<td>4</td>
<td>Methionine</td>
<td>0.609 ± 0.065</td>
</tr>
<tr>
<td>5</td>
<td>Glutamine</td>
<td>0.916 ± 0.101</td>
</tr>
<tr>
<td>6</td>
<td>succinate</td>
<td>0.072 ± 0.007</td>
</tr>
<tr>
<td>7</td>
<td>Aspartic acid</td>
<td>0.104 ± 0.020</td>
</tr>
<tr>
<td>8</td>
<td>Creatine</td>
<td>4.882 ± 0.642</td>
</tr>
<tr>
<td>9</td>
<td>Phosphatidylcholine</td>
<td>0.553 ± 0.108</td>
</tr>
<tr>
<td>10</td>
<td>Taurine</td>
<td>0.610 ± 0.206</td>
</tr>
<tr>
<td>11</td>
<td>glycine</td>
<td>0.284 ± 0.047</td>
</tr>
<tr>
<td>12</td>
<td>Alanine</td>
<td>1.038 ± 0.067</td>
</tr>
<tr>
<td>13</td>
<td>Lactic acid</td>
<td>0.143 ± 0.018</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001 compared with control group; *p < 0.05, **p < 0.01, ***p < 0.001 compared with model group

In conclusion, quality stability and uniformity are the keys and foundations to ensure the quality, the safe and effective clinical use of traditional Chinese medicine [28]. The chemical composition of Chinese herb medicine is the material basis for exerting efficacy and is susceptible to various factors, such as origin, producer, environment, growth period, and planting methods. The study on the composition and efficacy of medicinal plants based on absolute growth years is the hot spot in the quality evaluation of traditional Chinese medicine. The present study obtained absolute 4-year-old WAR segmentation based on the number of growth rings and combined it with the chemical analysis and anti-heart failure effects to compare the difference between WAR and TAR. This study provided a positive and practical significance for further study on the medicinal characteristics and clinical application of AR and its “quality evaluation through morphological” connotation. At the same time, this study provided a scientific basis for establishing the specification grade based on the absolute growth years and clinical rational drug use of AR.

Conflict of interest

We confirm that this manuscript has not been published by another journal. All authors have approved the manuscript and agree with its submission to your journal. The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


