

## QUANTITATIVE METABOLOMICS-DRIVEN ELUCIDATION OF FLAVONOID DIVERSITY AND NOVEL QUALITY ASSESSMENT STRATEGY IN *Artemisia argyi* Levl. ET VAN GERMPLASMS

Lanjie Xu<sup>1,2</sup>  <https://orcid.org/0000-0001-8355-9640>

Sufang An<sup>1,2</sup>  <https://orcid.org/0009-0003-1599-1759>

Yongliang Yu<sup>1,2</sup>  <https://orcid.org/0009-0006-2922-2813>

Wei Dong<sup>1,2</sup>  <https://orcid.org/0009-0001-4162-0316>

Huizhen Liang<sup>1,2</sup>  <https://orcid.org/0009-0008-4146-9303>

Qing Yang<sup>1,2</sup>  <https://orcid.org/0009-0008-1068-7332>

Xiaohui Wu<sup>1,2</sup>  <https://orcid.org/0009-0009-0554-9493>

<sup>1</sup> Institute of Chinese Herbal Medicines, Henan Academy of Agricultural Sciences, Zhengzhou 450002, China

<sup>2</sup> Provincial Key Laboratory of Conservation and Utilization of Traditional Chinese Medicine Resources in Henan, Zhengzhou 450002, China

✉ yyl790721@126.com

### ABSTRACT

This study employed quantitative metabolomics to conduct a comprehensive and systematic analysis of the diversity and accumulation patterns of flavonoid compounds in the leaves of six different genotypes of *Artemisia argyi* Levl. et Van (*A. argyi*) germplasms. The aim was to establish a metabolite-marker-based quality evaluation system and provide theoretical underpinnings for germplasm conservation and targeted development. Flavonoids were quantitatively analyzed using ultra-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS). A combination of principal component analysis (PCA) and hierarchical cluster analysis (HCA) of heatmaps was applied to reveal the disparities in metabolic profiles among different germplasms. Orthogonal partial least squares discriminant analysis (OPLS-DA) was utilized to identify differential metabolites, followed by Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to explore the underpinnings of metabolic pathways. The findings demonstrated that a total of 76 flavonoids belonging to 11 categories were identified. Flavones (24 compounds) and flavonols (20 compounds) were the predominant classes, accounting for 57.9% of the total. Aa36 and Aa60 all displayed the highest diversity with 64 compounds. The total flavonoid content ranged from 8.70 to 14.01 µg/g, and Aa41 had the highest content. Seven flavonoids of jaceosidin, eriodictyol, eupatorin, hispidulin, chrysosplenetin, scutellarin, quercimeritrin consistently ranked among the top 10 components in six germplasms, thereby constituting the common pharmacodynamic foundation. PCA and HCA classified six germplasms into two metabolic types. Group I, composed of Aa9, Aa13, Aa36, Aa38, and Aa41, was abundant in methoxylated flavonoids. Group II, only Aa60, had a distinctive profile dominated by scutellarin, which accounted for 52.4% of the total content (34.7 µg/g). The differential metabolites were significantly enriched in the secondary metabolite biosynthesis pathway (ko01110), flavonoid biosynthesis pathway (ko00941), and flavone/flavonol biosynthesis pathway (ko00944), which uncovered the regulatory mechanisms. The seven identified core flavonoids can function as stable metabolic markers for the quality assessment of germplasms. Meanwhile, the scutellarin dominant profile of Aa60 offers a distinct resource orientation for the development of *A. argyi* cultivars with cardio-cerebrovascular protective functions.

**Keywords:** *Artemisia argyi* Levl. et Van, flavonoid diversity, differential metabolites, quality assessment

## INTRODUCTION

*Artemisia argyi* Levl. et Van (*A. argyi*), a perennial herbaceous plant within the Asteraceae family, has a medicinal application history extending over 3,000 years. The dried leaves of *A. argyi* are rich in flavonoids, amino acids and volatile oils, and possess antioxidant, anti-cancer and antibacterial activities [Moacă et al. 2019, Wei et al. 2024, Orege et al. 2023, Al-Hajj et al. 2025, Bsharat et al. 2025a, 2025b]. Modern pharmacological studies suggest that the medicinal value of *A. argyi* is closely correlated with its rich flavonoid content.

Flavonoids, including quercetin, rutin, kaempferitrin and apigenin have been isolated and identified from *A. argyi* [Wang et al. 2024]. These flavonoids exhibit multiple biological activities, such as antioxidant, anti-inflammatory, antibacterial and antitumor effects, through the regulation of signaling pathways like NF- $\kappa$ B and MAPK [Xiang et al. 2018, Lee et al. 2018, Zhang et al. 2023, Wang et al. 2024]. For instance, quercetin significantly inhibits the lipopolysaccharide-induced secretion of inflammatory factors TNF- $\alpha$  and IL-6 in RAW264.7 macrophages, with a half maximal inhibitory concentration (IC<sub>50</sub>) of 12.3  $\mu$ M [Liu et al. 2022]. Meanwhile, rutin enhances antioxidant capacity by activating the Nrf2/ARE pathway, and its 2,2-diphenyl-1-picrylhydrazyl radical scavenging efficiency (half-maximal effective concentration, EC<sub>50</sub> = 0.18 mg/mL) is significantly superior to that of vitamin C [Li et al. 2024]. Flavonoids are considered the core pharmacodynamic substances of *A. argyi*, and their content and composition directly determine the quality grade of *A. argyi* medicinal materials.

With the further implementation of the “Healthy China” strategy, the *A. argyi* industry has witnessed explosive growth. Statistical data indicate that the market scale of *A. argyi* products in China reached 18.2 billion yuan in 2023, with an annual demand exceeding 500,000 tons [Yu et al. 2023]. Therefore, conducting a systematic analysis of the flavonoid composition in *A. argyi* and comprehensively exploring the differential accumulation patterns of natural products among different provenances hold significant importance for the quality assessment, conservation of genetic resources, efficient development and utilization, and breeding of high quality varieties of *A. argyi* resources. In light of this, the present study selected six representative *A. argyi* germplasms from different genotypes. Ultra performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) technology was employed to conduct untargeted metabolomics analysis and systematically establish a quantitative fingerprint of flavonoid components in its leaves. In combination with OPLS-DA analysis and cluster analysis, characteristic differential metabolites capable of distinguishing different germplasms were screened. Through KEGG pathway enrichment analysis, the synthetic biological basis of its metabolic differences was disclosed.

Markedly distinct from conventional methodologies in experimental design, research framing and analytical rigor, this study advances beyond the technical constraints of traditional flavonoid profiling strategies. Whereas prior approaches have typically depended on spectrophotometry to measure total flavonoid content or high-performance liquid chromatography to quantify a restricted set of known analytes—techniques hampered by limited detection throughput and narrow metabolite coverage—the present work overcomes these drawbacks to achieve a holistic characterization of flavonoid metabolic diversity [Kabr e et al. 2023, Gong et al. 2019]. For the first time, this study integrates UPLC-ESI-MS/MS with an untargeted metabolomics strategy, enabling high-throughput and systematic analysis of both known and unknown flavonoids in *A. argyi* and breaking through the technical limitations of conventional targeted analysis. In terms of research perspective, previous studies have mostly focused on the effects of exogenous factors such as producing area and harvesting period on chemical components [Gong et al. 2019, Nie et al. 2019, Zhang et al. 2023], while insufficient attention has been paid to the genetic differences among germplasms. Taking six *A. argyi* germplasms with clear genetic backgrounds and distinct geographical origins as research objects, this study explores the natural variation patterns of flavonoid accumulation from the perspective of genetic essence, providing metabolome-based chemical classification criteria for variety identification and breeding. In terms of analytical depth, previous studies have been largely confined to descriptive comparisons or quantification of individual components, lacking a holistic interpretation of metabolic differences [Jiang et al. 2009, Dong et al. 2016, Gong et al. 2019]. By employing OPLS-DA analysis and hierarchical clustering analysis, this study screens out differential metabolic markers with discriminative significance. Combined with KEGG pathway enrichment analysis, it systematically reveals the underlying biosynthetic and regulatory networks. This approach elevates the evaluation of *A. argyi* germplasms from superficial chemical description to a mechanistically linked, index-based discrimination level, providing a quantifiable and interpretable new strategy for breeding high-value varieties and standardizing quality.

## MATERIAL AND METHODS

**Plant materials.** The experiment was carried out on April 20, 2025. Six *A. argyi* germplasms (codes: Aa09, Aa13, Aa36, Aa38, Aa41, Aa60) were cultivated in the modern agricultural research and development base of Henan Province. On May 20, 2025, the upper middle leaves of robust and disease-free plants were harvested, with three biological replicates for each germplasm (a total of 18 samples). Subsequent to sampling, these leaves were rapidly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent utilization. The leaf morphology of each material is presented in Figure 1, and the germplasm origins are Bainiu Town, Dengzhou City, Henan Province (Aa09); Tongbai County, Nanyang City, Henan Province (Aa13); Potou Town, Jiyuan City, Henan Province (Aa36); Caohe Town, Qichun County, Hubei Province (Aa38); Guanyao Town, Qichun County, Hubei Province (Aa41); Tangyin County, Anyang City, Henan Province (Aa60).

**Main instruments.** Liquid chromatography tandem mass spectrometry (LC-MS/MS) (model: QTRAP 6500+, SCIEX); high speed refrigerated centrifuge (model: 5424R, Eppendorf); precision electronic balance (model: AS60/220.R2, RADWAG); ball mill (model: MM400, Retsch); multitube vortex oscillator (model: MIX-200, Shanghai Jingxin); ultrasonic cleaner (model: KQ5200E, Kunshan Shumei).

**Main reagents.** Methanol (Chromatographic grade, Merck); acetonitrile (Chromatographic grade, Shanghai Xingke); formic acid (Chromatographic grade, Sigma-Aldrich); flavonoid standard substances (Purity > 98%, Med Chem Express); 70% methanol (Containing internal standard working solution with an internal standard concentration of 4000 nmol/L).

**Extraction of flavonoid compounds.** Leaves ( $1 \times 2$  cm) samples preserved at  $-80^{\circ}\text{C}$  was retrieved and transferred to a ball mill pre-cooled with liquid nitrogen (30 Hz). Subsequently, the samples were comprehensively ground for 1.5 minutes until a powdered form was achieved. Subsequently, 20.0 mg of the powder was accurately weighed into an EP tube, and 10  $\mu\text{L}$  of 4000 nmol/L internal standard mixed working solution and 500  $\mu\text{L}$  of 70% methanol aqueous solution were added. Ultrasonic extraction was carried out for 30 min under ice-bath conditions. The extract was then centrifuged at  $4^{\circ}\text{C}$  and 12,000 r/min for 5 min. The supernatant was aspirated, filtered through a 0.22  $\mu\text{m}$  microfiltration membrane, and transferred to a sample vial for subsequent LC-MS/MS analysis.

**Chromatographic conditions.** Column waters ACQUITY UPLC HSS T3 C18 column (1.8  $\mu\text{m}$ ,  $100 \times 2.1$  mm); column temperature of  $40^{\circ}\text{C}$ ; mobile phase-phase A is a 0.05% formic acid aqueous solution, and phase B is an acetonitrile solution containing 0.05% formic acid; flow rate of 0.4 mL/min; injection volume of 4  $\mu\text{L}$ . Gradient elution program: at 0 min (90%A:10%B), at 1.0 min (80%A:20%B), at 9.0 min (30%A:70%B), at 12.5 min (5%A:95%B), at 13.5 min (5%A:95%B), at 13.6 min (90%A:10%B), at 15.0 min (90%A:10%). Mass spectrometry conditions were as

**Figure 1.** Leaf morphology of 6 *Artemisia argyi* germplasm



follows: the ion source was electrospray ionization, with an ion source temperature of 550 °C. In the positive ion mode, the ion spray voltage was 5500 V, while in the negative ion mode, it was 4500 V. The curtain gas pressure was set at 35 psi, the nebulizer gas pressure at 55 psi, and the auxiliary gas pressure at 55 psi. The scanning mode employed was multiple reaction monitoring (MRM). The declustering voltage (DP) and collision energy (CE) corresponding to specific analytes were determined in accordance with the optimized parameters for each compound.

**Data processing.** The raw mass spectrometry data were gathered and processed by means of the analyst 1.6.3 software. Qualitative analysis was carried out based on the MWDB (Metware database) of Wuhan Metware Biotechnology Co., Ltd. Quantitative analysis employed the MRM mode, and standard curves were plotted by analyzing the MRM chromatographic peak intensities of standard products at each concentration gradient. The peak areas of each chromatographic peak in the samples were inserted into the corresponding standard curves to compute their actual contents (unit: µg/g).

**Principal component analysis (PCA).** This method was employed to assess the overall disparities and within group variations of flavonoids among distinct germplasm samples. OPLS-DA analysis was utilized to screen differential metabolites between pairwise groups of different germplasms. The screening criteria were as follows: the variable importance projection value > exceeded 1.0, and the fold change (FC) was either greater than or equal to 2.0 or less than or equal to 0.5. Heat map and hierarchical clustering analysis (HCA): based on the euclidean distance of flavonoid contents and ward's clustering strategy, this approach was used to visualize the similarity and differential accumulation patterns among germplasms. KEGG enrichment analysis: the KEGG database was applied to conduct metabolic pathway enrichment analysis on significantly differential metabolites, with a significant enrichment threshold of  $p < 0.05$ . Bar charts were generated using Excel 2007.

## RESULTS

**Overall composition of flavonoid compounds in *Artemisia argyi*.** A total of 76 flavonoid compounds, categorized into 11 groups, were identified from the leaves of six *A. argyi* germplasms through UPLC-MS/MS (Figure 2). An examination of the compound category distribution indicated that flavones (24 compounds) and flavonols (20 compounds) were the most prevalent groups, accounting for 31.58% and 26.32% of the total identified compounds respectively, and jointly constituted the main framework of flavonoid metabolism. The sub-prevalent groups encompassed dihydroflavones (8 compounds, 10.53%), isoflavones and chalcones (6 compounds each, 7.89%), and dihydroflavonols (4 compounds, 5.26%). Flavanols, xanthonones, flavonoid C-glycosides, and other categories displayed lower abundances, with 1–2 compounds detected in each category (accounting for ≤ 2.63%).

**Flavonoid compound types of different *Artemisia argyi* germplasms.** As presented in Table 1, eleven categories of flavonoid compounds were detected in the leaves of all six *A. argyi* germplasms, yet notable disparities were observed in the composition of each category. Regarding the total number of compounds, the leaves of Aa36 and Aa60 exhibited the highest number of flavonoid categories, both reaching 64. The Aa13 samples contained 63 types of flavonoids, while the leaves of Aa13, Aa41 and Aa38 harbored 62 flavonoid compounds.

An analysis of the distribution characteristics of key categories indicated that flavones and flavonols were the predominant groups among all six germplasms. Specifically, the quantities of flavones detected in Aa36, Aa9, Aa13, Aa41, Aa38 and Aa60 were 20, 21, 22, 22, 21 and 21 respectively, accounting for 31.25%, 33.87%, 34.92%, 35.48%, 33.87% and 32.81% of their total identified compounds. The quantities of flavonols detected in Aa36, Aa9, Aa13, Aa41, Aa38 and Aa60 were 17, 17, 18, 16, 15, 2 and 18 respectively, accounting for 26.56%, 27.42%, 28.57%, 25.81%, 25.81%, 24.19% and 28.31% of their total identified compounds. Dihydroflavones (7–8 species), chalcones (4–5 species) and dihydroflavonols (3–4 species) were sub-predominant groups, accounting for 4.76–12.90%. Flavonoid C-glycosides, xanthonones, etc., were rare groups, with only 1–2 species detected (accounting for ≤ 3.23%).

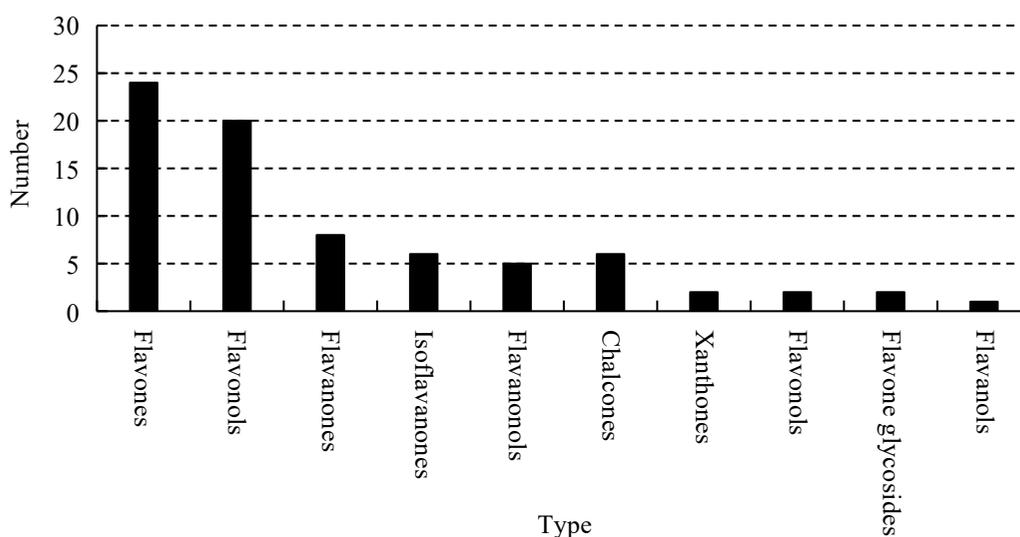
The specific category differences among the six germplasms were as follows: flavonol glycosides were not detected in sample Aa13, whereas one species was detected in the other five germplasms. Regarding chalcones, five species were detected in each of Aa36, Aa38 and Aa41, while four species were detected in each of Aa9, Aa13 and Aa60. For dihydroflavones, eight species were detected in each of Aa9, Aa36 and Aa38, and seven species were detected in each of Aa13, Aa41 and Aa60. In the case of dihydroflavone glycosides, only one species was detected in germplasm Aa36. Concerning flavones, the largest number of detected species (22) was observed in Aa13 and Aa41, followed by Aa9, Aa38 and Aa60 (21), and the fewest (20) was in Aa36. With respect to flavonols, the most de-

tected species (18) were found in Aa13 and Aa60, followed by Aa9 (17) and fewer (16 or 15) were in Aa41, Aa36 and Aa38. Other flavonoid compounds also demonstrated certain variations among the germplasm (Table 1). These differences suggest that *A. argyi* germplasm with distinct genotypes display specific synthesis and accumulation patterns of flavonoid compounds.

**Table 1.** Categories and quantities of flavonoid compounds in different *Artemisia argyi* germplasm

Type	Aa36	Aa9	Aa13	Aa41	Aa38	Aa60
Flavonol glycoside	1	1	0	1	1	1
Chalcone	5	4	4	5	5	4
Flavanon	8	8	7	7	8	7
Dihydroflavonol	4	4	3	3	4	4
Flavanon glycoside	1	0	0	0	0	0
Flavone	20	21	22	22	21	21
Flavonol	17	17	18	16	15	18
C-Glycosyl flavone	1	2	2	1	2	2
Flavanols	1	1	1	1	1	1
Xanthone	2	1	1	1	1	1
Isoflavone	4	3	5	5	4	5
Total	64	62	63	62	62	64

**Figure 2.** Categories of flavonoid compounds in *Artemisia argyi* germplasm



**Flavonoid compound contents in *Artemisia argyi*.** The mean content of flavonoid compounds in different *A. argyi* germplasm spanned from 8.70 to 14.01  $\mu\text{g/g}$ , specifically presenting the following order: Aa41 (14.01  $\mu\text{g/g}$ ) > Aa13 (12.80  $\mu\text{g/g}$ ) > Aa38 (10.25  $\mu\text{g/g}$ ) > Aa60 (9.84  $\mu\text{g/g}$ ) > Aa36 (9.23  $\mu\text{g/g}$ ) > Aa9 (8.70  $\mu\text{g/g}$ ). The average flavonoid content in Aa41 was notably higher than that in other germplasm ( $p < 0.05$ ), whereas there was no significant disparity between Aa60 and Aa36 (Figure 3).

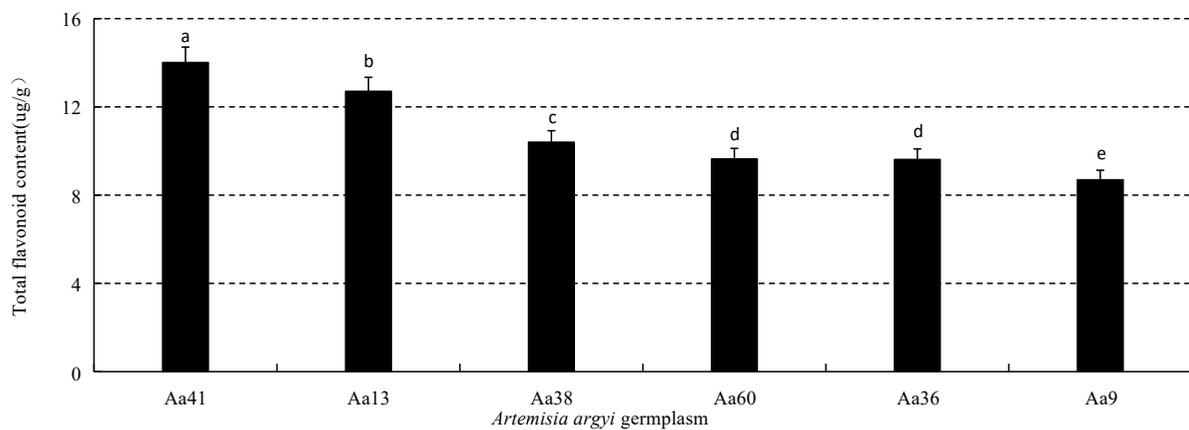
Analysis of the top ten flavonoid compounds in each sample indicated that both the contents and specific compositions of the top ten compounds differed among germplasm (Figure 4). Seven compounds consistently emerged in the top ten of all six germplasm: jaceosidin, eriodictyol, eupatorin, hispidulin, chrysopterin, scutellarin, and quercimeritrin, which reflects the relative conservatism of the dominant flavonoid components in *A. argyi*.

Certain flavonoids were only present within the top 10 rankings of specific germplasms. Specifically, luteolin was not among the top 10 in Aa60, being substituted by kaempferol 3-neohesperidoside. Cynaroside failed to rank among the top 10 in Aa41 and Aa9, replaced by hyperoside and diosmin respectively. Rutin did not make it into the top 10 in Aa36 and Aa38.

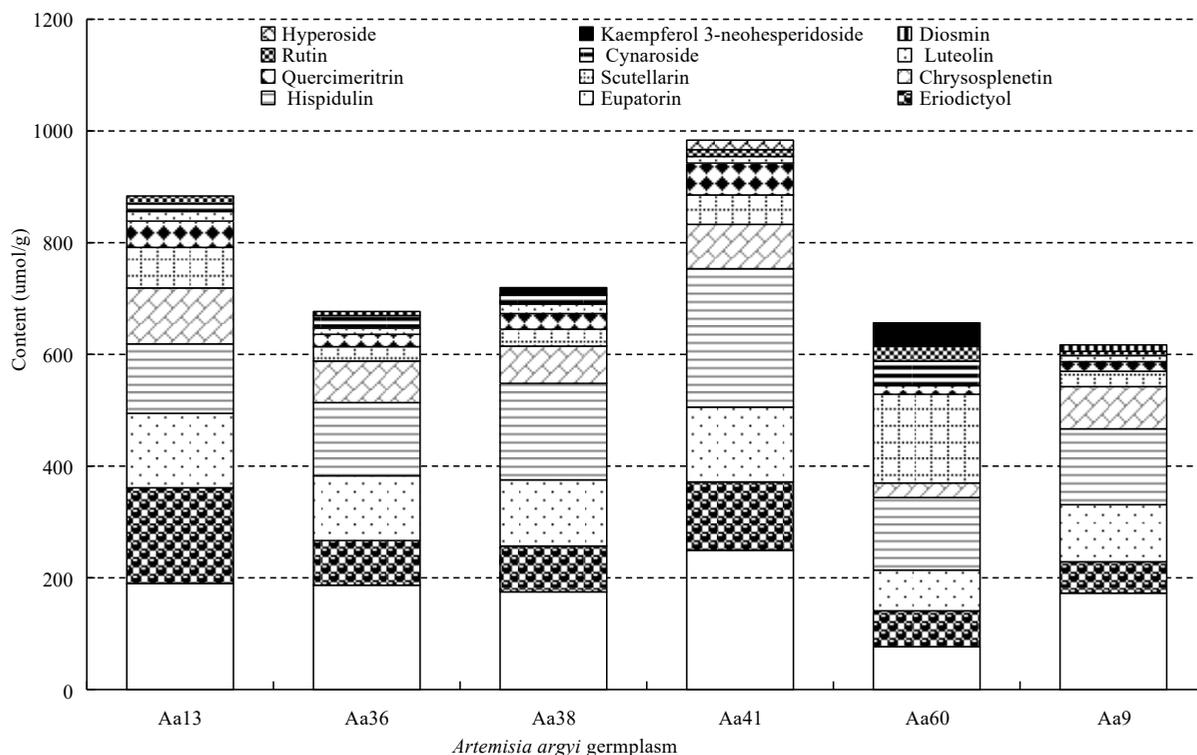
**PCA analysis.** PCA demonstrated that the flavonoid metabolic profiles of the six *A. argyi* germplasms were genotype-dependent (Figure 5). The cumulative contribution rate of the first principal component (PC1) and the second principal component (PC2) attained 56.88%, where PC1 accounted for 36.52% and PC2 for 20.36%.

Distinct separation trends were discerned among diverse germplasm groups. Along the PC1 of Aa9, Aa36 and Aa38 group was characterized by an enrichment of methoxylated flavonoids, including jaceosidin, hispidulin and eupatilin in the upper – right quadrant; the Aa13 and Aa41 group was predominantly composed of jaceosidin,

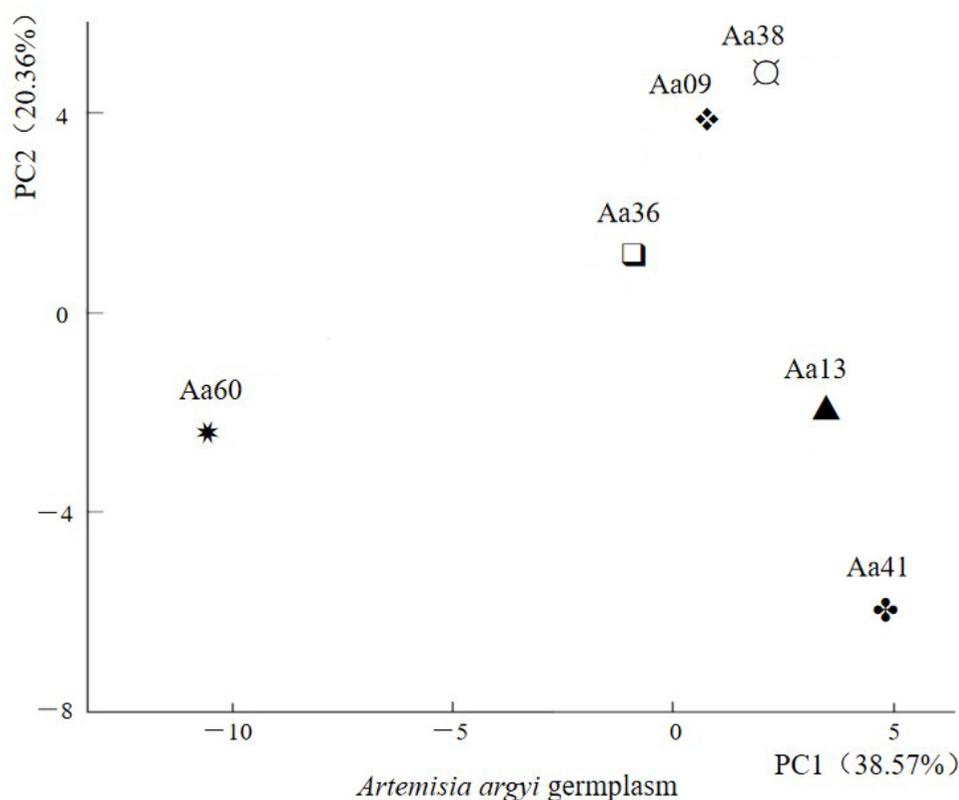
**Figure 3.** The content differences of total flavonoids in different *Artemisia argyi* germplasm (different *A. argyi* germplasm indicate significant differences in treatments ( $p < 0.05$ ))



**Figure 4.** The tip 10 types and their contents of flavonoid compounds in leaves of *Artemisia argyi*



**Figure 5.** PCA analysis of flavonoid accumulation patterns in leaves of different *Artemisia argyi* germplasm



eriodictyol and eupatilin in the lower-right quadrant. Along the PC2 of Aa60 was separately distributed on the far-left side, which exhibited a marked distinction from the other five germplasm, primarily driven by scutellarin with a loading value of 0.92. The scutellarin content in Aa60 reached 158.73  $\mu\text{g/g}$ , accounting for 52.4% of the total flavonoids, which was 2.19–6.17 times higher than that in other germplasm, thereby contributing to its unique metabolic profile.

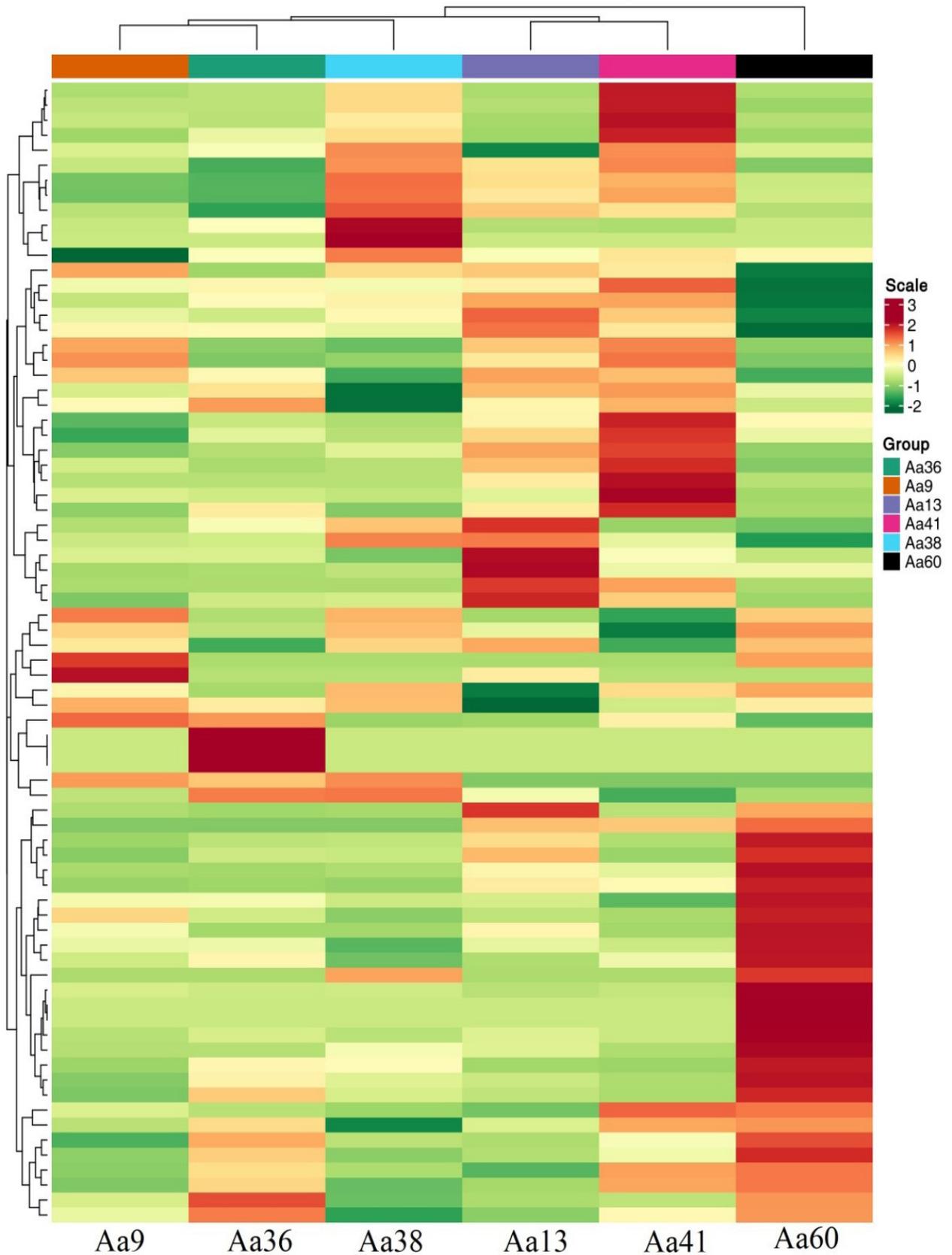
**Cluster analysis.** Cluster analysis based on the contents of 76 flavonoid compounds, indicated that the six *A. argyi* germplasm were classified into two major distinct groups (Figure 6). Main group I encompassed five germplasm of Aa9, Aa13, Aa36, Aa38 and Aa41, which could be further partitioned into two subgroups. Subgroup 1 consisted of Aa9 and Aa36 originated from Henan, sharing 9 characteristic flavonoids, including methoxylated derivatives such as jaceosidin and eupatilin (average content  $>7.77 \mu\text{g/g}$ ). Subgroup 2 included Aa13 (from Henan), Aa38 (from Hubei) and Aa41 (from Hubei), characterized by the co-accumulation of eriodictyol and acacetin (correlation coefficient  $r = 0.53$ ). Despite the influences of regional environments, metabolic convergence was propelled by the conservatism of key flavonoid synthase genes.

Main group II contained only germplasm Aa60, with its core distinguishing attribute being that scutellarin alone accounted for over 52% of the total flavonoids (8.3-fold higher than group I).

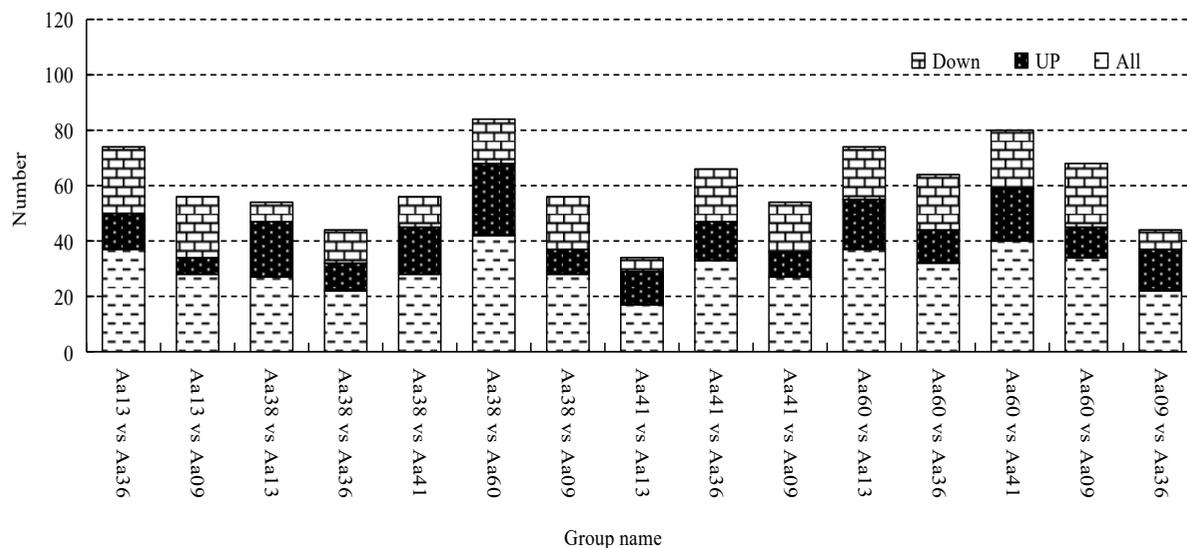
**Screening of differentially accumulated flavonoid metabolites in *Artemisia argyi* germplasm.** Differentially accumulated metabolites (DAMs) were screened among paired germplasm through an OPLS-DA model ( $\text{VIP} > 1$  &  $|\log_2\text{FC}| \geq 1$ ). Noticeable disparities were observed in the quantity of DAMs across diverse germplasm comparisons. The Aa38 vs Aa60 group exhibited the highest number of DAMs (42), succeeded by the Aa60 vs Aa41 group (40) and the Aa13 vs Aa36 group (37). In each comparison group, the numbers of up-regulated and down-regulated metabolites were approximately balanced (e.g., Aa38 vs Aa60: 26/26, Aa60 vs Aa41: 21/19, Aa13 vs Aa36: 24/13); see Figure 7.

Twenty DAMs exhibiting the most substantial fold-change disparities between groups were further compared. Key DAMs encompassed apigenin-7-O-glucuronide, apigenin-7-glucoside, hispidulin glycoside, apigenin dimethyl ether, astilbin, sakuranetin, isorhamnetin-3-O-glucoside, etc., demonstrating highly significant accumulation differences within specific groups:

**Figure 6.** Heat map cluster analysis of different *Artemisia argyi* germplasm based on the content of flavonoids



**Figure 7.** Distribution pattern of flavonoids among *Artemisia argyi* germplasm



Aa13 vs Aa36: in Aa36 leaves, only apigenin dimethyl ether and naringenin chalcone were up-regulated, whereas the others were down-regulated.

Aa13 vs Aa9: in Aa9 leaves, only epimedin B and (-)-catechin gallate were down-regulated, while the others were up-regulated. Among them, apigenin-7-glucoside and apigenin-7-O-glucuronide exhibited the most pronounced up-regulation.

Aa13 vs Aa36: in Aa36 leaves, astilbin, sakuranetin, hispidulin glycoside, isorhamnetin-3-O-glucoside and catechin gallate were down-regulated, and the others were up-regulated. Apigenin-7-O-glucuronide showed the highest degree of up-regulation.

Aa38 vs Aa9: in Aa9 leaves, catechin gallate, linarin and apigenin dimethyl ether were down-regulated, and the others were up-regulated. Hispidulin glycoside presented the most significant up-regulation.

Aa38 vs Aa13: in Aa13 leaves, only hispidulin glycoside was up-regulated, and the others were down-regulated. Apigenin dimethyl ether and apigenin-7-O-glucuronide had the most notable down-regulation.

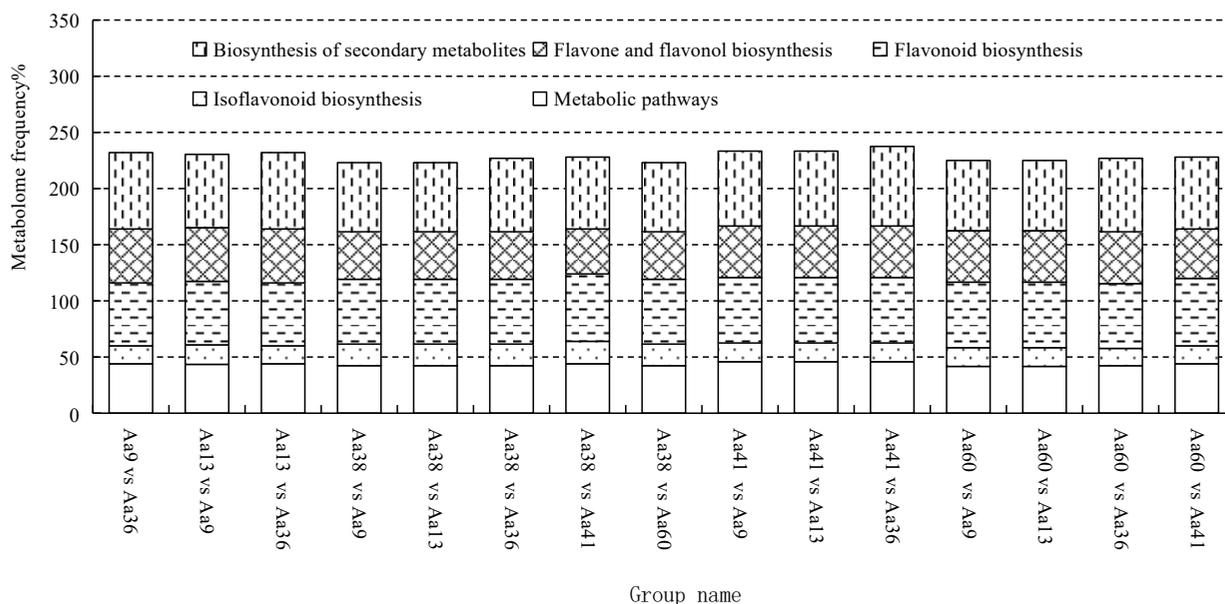
**KEGG enrichment analysis of flavonoid differential metabolites in *Artemisia argyi* germplasm.** KEGG metabolic pathway enrichment analysis was conducted on all the previously screened significant DAMs1 (Figure 8). The findings indicated that the differential metabolites were significantly enriched ( $p < 0.05$ ) in five core metabolic pathways. The enrichment intensity, ranked in descending order of the rich factor, was as follows: secondary metabolite biosynthesis (ko01110, Rich Factor (RF) = 0.38,  $p = 4.2 \times 10^{-5}$ ), flavonoid biosynthesis (ko00941, RF = 0.32,  $p = 8.7 \times 10^{-4}$ ), flavone and flavonol biosynthesis (ko00944, RF = 0.28,  $p = 0.006$ ), phenylpropanoid biosynthesis (ko00940, RF = 0.25,  $p = 0.013$ ), and isoflavonoid biosynthesis (ko00943, RF = 0.21,  $p = 0.039$ ).

## DISCUSSION

Flavonoids, as one of the principal active constituents of *A. argyi*, make substantial contributions to its traditional therapeutic effects, such as warming the meridians to arrest bleeding and dispelling cold to alleviate pain [Chen et al. 2017, Zhang et al. 2023, Yang et al. 2024]. In this study, the diversity of flavonoids in six *A. argyi* germplasms was comprehensively analyzed via quantitative metabolomics. A total of 11 categories of flavonoids, encompassing the major branches of the flavonoid metabolic pathway, were identified, including flavones, flavonols, dihydroflavones, isoflavones and chalcones. Specifically, 24 flavones and 20 flavonols were detected, which is consistent with the previous research findings that “flavones and flavonols are the most predominant classes of flavonoid compounds in plants” [Shen et al. 2022].

Further analysis revealed seven core flavonoids that dominated the content in all six germplasm: eupatilin, eriodictyol, eupalin, hispidulin, chrysosplenetin, scutellarin and quercetin-7-O- $\beta$ -D-glucoside. These core components exhibit clear biological activities: quercetin derivatives show significant anti-inflammatory effects [Li et al. 2016];

**Figure 8.** Results of KEGG enrichment analysis for differentially accumulated flavonoids



hispidulin has attracted attention for its neuroprotective [Wang et al. 2015, An et al. 2018], scutellarin is widely studied for its cardiovascular and neuroprotective effects [Chen et al. 2020]; eupatilin is renowned for its potent anti-inflammatory, gastric mucosa-protective and potential antitumor activities [Park et al. 2018, Hong et al. 2023]. Thus, these seven compounds likely constitute the key material basis of *A. argyi* efficacy, and their high abundance and stability may serve as core markers for quality evaluation of *A. argyi*. Notably, scutellarin had the highest content in germplasm Aa60, suggesting its potential for developing cardiovascular protective varieties [Chen et al. 2020].

Notable disparities in the quantities of specific flavonoid categories and sub-classes were discerned among distinct genotypes. For instance, dihydroflavone glycosides were solely detected in Aa36, and flavonoid C-glycosides were concentrated in Aa60, which suggests subtle genetic differentiation within the species. These disparities are directly associated with the regulation of key enzyme gene expression, and Aa36 might possess a dominant allelic variation of this gene. The number of flavonols also differed among three germplasm species (18 in Aa41 compared to 21 in Aa60), indicating that the regulation of flavonoid biosynthesis pathways is genotype-dependent, mirroring the genetic diversity of *A. argyi* germplasm [Zhou et al. 2022].

Although germplasm Aa60 (originating from Tangyin, Henan) did not exhibit the highest average flavonoid content, PCA and HCA revealed its distinctive flavonoid accumulation profile, which was clearly distinguishable from those of the other five germplasms. This distinctiveness may be attributed to its unique genetic background or adaptive disparities to the micro ecological environment of its provenance.

According to relevant research, the disparities in flavonoids predominantly stem from the biosynthetic pathway of secondary metabolites, particularly the flavonoid and its downstream flavone/flavonol branch pathways. There exist allelic variations and spatio-temporal expression differences in the key synthase genes (such as the chalcone synthase gene, chalcone isomerase gene, flavone synthase gene, flavanone 3-hydroxylase gene and flavonol synthase gene) or modifier enzyme genes (such as glycosyltransferase genes) involved in flavonoid synthesis [Tohge et al. 2013, Doyle et al. 2024a, 2024b].

While this study systematically revealed the accumulation patterns and potential biosynthetic pathway differences of flavonoids among *A. argyi* germplasm resources through UPLC-ESI-MS/MS untargeted metabolomics, two main limitations remain. First, the identification of metabolites is highly dependent on the coverage of existing mass spectrometry databases, and the structural confirmation of rare or novel flavonoids lacking reference standards still requires orthogonal techniques such as nuclear magnetic resonance. Second, although the study uncovered germplasm-specific metabolic phenotypic differences, it has not yet established a direct causal link at the molecular level between genetic variations in key enzyme genes and flavonoid biosynthesis phenotypes using techniques such as transcriptomics, genome-wide association analysis, or gene editing. As genomic resources

for *Artemisia* species continue to expand [Wang et al. 2023], future research could integrate multi-omics and functional genomics approaches (e.g., GWAS, CRISPR) to precisely identify the genetic loci regulating the synthesis of key flavonoids [Doyle et al. 2024a, 2024b, Chen et al. 2014], thereby advancing the in-depth elucidation of *A. argyi* quality formation mechanisms and promoting the practice of molecular design breeding.

## CONCLUSIONS

Based on quantitative metabolomics analysis, this study systematically revealed the accumulation patterns and regulatory mechanisms of flavonoids in leaves of six *A. argyi* germplasm: 11 categories and 76 species of flavonoids were identified, and seven core flavonoid clusters stably and highly expressed in all germplasm were defined for the first time (eupatilin, eriodictyol, eupalin, hispidulin, chryso-splenetin, scutellarin, quercetin-7-O- $\beta$ -D-glucoside). This cluster covers key active components (anti-inflammatory, antioxidant, neuroprotective) of *A. argyi* efficacy in warming meridians and dispelling cold, and can serve as universal metabolic markers for cross-germplasm quality evaluation. Additionally, Aa60 exhibited a unique scutellarin-dominant metabolic profile, providing characteristic resources for developing neuroprotective *A. argyi* varieties.

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