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Metabolomic profiling of Akebia species: Comparative analysis of bioactive compounds in the pulp of *A. trifoliata*, *A. trifoliata* ssp. australis, and *A. quinata*

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ABSTRACT

Akebia species including, *A. trifoliata* (AKT), *A. trifoliata* ssp. australis (AKA), and *A. quinata* (AKQ) are popular for their sweet, aromatic fruits and pharmacological applications. Despite their commercial and medicinal importance, the metabolomic profiles of Akebia pulp remain largely unexplored. This study employs UPLC-MS/MS and GC–MS techniques to comprehensively analyze the chemical composition of the pulp from these three species. Among the 1429 metabolites detected and putatively identified, terpenoids, amino acids, flavonoids, and phenolics were predominant. AKT shows the richest bioactive compound accumulation, including significant levels of L-Isoleucyl-L-Aspartate, 6-C-Methylquercetin-3-O-rutinoside, and Madasiatic acid, suggesting its high nutritional and medicinal value. AKA has the lowest metabolite accumulation, while AKQ displays an intermediate profile with notable antioxidants like Quercetin-3-O-xyloside. These findings provide a foundation for optimizing the cultivation, harvesting, and utilization of Akebia fruits and underscore their potential for developing functional foods and nutraceuticals.

1. Introduction

Akebia, a genus within the Lardizabalaceae family, stands out for its distinctive fruiting attributes, which significantly contribute to both ecological variety and agricultural interests. Species like *A. trifoliata*, *A. trifoliata* ssp. *australis*, and *A. quinata* are cultivated not only for their pleasing, sweet, and fragrant fruits but also for their notable medicinal properties (Jiang et al., 2012; L. Liu & Qian, 2002; Nazir et al., 2024), long recognized in traditional Chinese medicine (Huang et al., 2022; Li et al., 2021; X. Liu et al., 2023; Maciąg et al., 2021). These fruits are prized for their complex metabolic profiles, including a variety of bioactive compounds like flavonoids, polyphenols, and vitamins, which are advantageous for health, offering anti-inflammatory, antioxidant, and anticancer benefits (Zou et al., 2023). Recent studies have highlighted *Akebia*'s potential as a promising new fruit crop (Zou et al., 2023; Zou, Yao, Zhong, Zhao, & Huang, 2018), with attributes such as high unsaturated fatty acids in seeds up to 39 %, useful in producing edible oils, and pectin in fruit peels for commercial food applications. Despite their increasing popularity and commercial potential, there is a scarcity of comprehensive studies on their chemical composition and comparative metabolomic analyses, which are essential for maximizing their agricultural and therapeutic capacities.

The pulp of the *Akebia* fruit is highly valued for its flavor and health benefits (Jiang et al., 2012). However, the chemical profiles of the pulp across different species have not been extensively studied. Previous studies have primarily focused on isolated bioactive compounds, such as flavonoids, saponins, and alkaloids, but a comprehensive metabolomic approach to examine the full range of metabolites in these fruits has been lacking (Li et al., 2021). Metabolomics, utilizing advanced techniques like mass spectrometry and nuclear magnetic resonance (NMR), provides an effective tool to analyze the entire spectrum of metabolites in plant tissues, offering a more holistic view of their biochemical composition (Deborde et al., 2017; Eisenreich & Bacher, 2007; Emwas et al., 2019).

Moreover, recent genomic studies have enhanced our understanding of the molecular basis of *Akebia* species' traits. The chromosome-level genome of *A. trifoliata* has been sequenced, revealing valuable insights into the genetic makeup of this species and potentially guiding breeding efforts for improving fruit quality and medicinal properties (Zhong et al., 2022). Such genomic advancements pave the way for more targeted

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metabolomic analyses, enabling a deeper understanding of how genetic variation influences the chemical profiles of *Akebia* fruit pulp.

Metabolomics, the study of small molecules involved in cellular processes, offers a powerful approach to investigate the full spectrum of metabolites present in biological samples (Dona et al., 2016). By employing techniques like mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Dias et al., 2016; Takis, Ghini, Tenori, Turano, & Luchinat, 2019), it is possible to gain a detailed and comprehensive understanding of the chemical composition of fruit pulp, which could significantly inform agricultural practices, enhance product development, and aid in the identification of bioactive compounds for potential therapeutic applications. Despite this, no study has yet explored the comparative metabolomic profile of *Akebia* pulp from these three important species.

This research aims to fill this gap by conducting a thorough metabolomic analysis of the pulp from *A. trifoliata* (AKT), *A. trifoliata* ssp. australis (AKA), and *A. quinata* (AKQ) by utilizing advance chromatographic and mass spectrometric techniques. By identifying and comparing the metabolite compositions across these species, we aim to highlight their chemical diversity and provide a deeper understanding of the factors influencing their commercial and medicinal value. Such insights will contribute to optimizing the cultivation, harvesting, and utilization of *Akebia* fruits, paving the way for future studies on their nutritional and pharmacological potentials.

2. Materials and methods

2.1. Sample collection

Pulp samples from three species of *Akebia*—*A. trifoliata* (AKT), *A. trifoliata* ssp. *australis* (AKA), and *A. quinata* (AKQ)—were collected at Lushan botanical Garden, Nanchang, China, grown under similar environmental conditions. The plants were established concurrently in 2022 and grown under identical field conditions, allowing for open pollination. Fruits were harvested in September 2023 at a consistent ripening stage. This stage was defined physiologically as the initial appearance of natural longitudinal cracking along the ventral suture, which typically occurs upon ripening in this genus (S.-Y. Zou, C. Feng, P.-X. Gao, T.-J. Li, T.-J. Jia, & H. Huang, 2023). For each species, nine representative fruits exhibiting this specific ripeness criterion were collected. The pulp tissue was carefully excised from these fruits. To prepare biological replicates, pulp material from three individual fruits was pooled. This resulted in a total of three independent biological replicates for each of the three species ($n = 3$ per group) used for subsequent metabolomic analysis. Fresh samples were immediately flash-frozen in liquid nitrogen and stored at −80 °C until further analysis.

Frozen pulp samples were ground to a fine powder using a grinder (MM 400, Retsch) operated at 30 Hz for 30 s, with the grinding jar and steel beads pre-cooled in liquid nitrogen. Precisely 50 mg of the resulting powder was weighed using an electronic balance (MS105DM). To each weighed sample, 600 µL of pre-cooled 70 % methanol:water (*v*/v) solution, containing internal standard extract, was added. The mixture was vortexed vigorously for 30 s, repeated 6 times in total, and then allowed to stand at −20 °C for 30 min.

Following incubation, the samples were centrifuged at 12,000 rpm for 3 min. The resulting supernatant was carefully aspirated and filtered through a 0.22 µm microporous membrane filter into an injection vial. The filtered extracts were then stored appropriately (e.g., at −80 °C or prepared for immediate analysis) prior to UPLC-MS/MS analysis.

2.2. Metabolite profiling

The comprehensive metabolite profiling using UPLC-MS/MS and GC–MS platforms was carried out as a service by MetWare Biotechnology Co., Ltd. (Wuhan, China). Procedural blanks were included during sample preparation, and Quality Control (QC) samples (prepared by pooling aliquots of all extracts) were injected periodically (e.g., every 10 samples) throughout the analytical runs to monitor instrument stability, assess data quality, and check for contamination.

2.2.1. UPLC-MS/MS analysis

Sample extracts were analyzed using a UPLC-ESI-MS/MS system comprising an ExionLC™ AD UPLC (SCIEX, Framingham, MA, USA) coupled to an Applied Biosystems 6500 Q TRAP MS/MS (SCIEX). Separation was performed on an Agilent SB-C18 column (1.8 µm, 2.1 mm * 100 mm). The mobile phase consisted of solvent A (pure water with 0.1 % formic acid) and solvent B (acetonitrile with 0.1 % formic acid). The gradient elution program was as follows: 5 % B maintained for 0–9 min with a linear increase to 95 % B, 95 % B held for 1 min (9–10 min), returned to 5 % B within 1.1 min (10–11.1 min), and equilibrated at 5 % B for 2.9 min (11.1–14 min). The flow rate was 0.35 mL/min, the column oven temperature was maintained at 40 °C, and the injection volume was 2 µL.

The ESI source was operated in both positive and negative ion modes. Key parameters were: source temperature 500 °C, ion spray voltage (IS) +5500 V (positive) / -4500 V (negative), ion source gas I (GSI) 50 psi, gas II (GSII) 60 psi, and curtain gas (CUR) 25 psi. Collision-activated dissociation (CAD) gas was set to medium. Metabolite quantification was performed in Multiple Reaction Monitoring (MRM) mode using optimized declustering potential (DP) and collision energy (CE) for each precursor-product ion transition. The system monitored specific MRM transitions for metabolites based on the MetWare database (MWDB), which contains retention times and MS/MS spectra (secondary spectra) for numerous compounds, including common adducts (e.g., [M + H]+, [M + Na]+, [M + NH4]+, [M-H]-). Operating in MRM mode enhances precision, reproducibility, and sensitivity by selectively monitoring specific ion transitions.

2.2.2. GC–MS analysis

Samples (500 mg powder in 20 mL headspace vial with saturated NaCl) were incubated at 60 °C for 5 min with continuous agitation (CTC Analytics Agitator). Volatiles were extracted using a 120 µm DVB/CWR/PDMS fiber (Agilent Technologies, Santa Clara, CA, USA) exposed to the headspace for 15 min at 60 °C, performed using a CTC Analytics SPME autosampler. GC–MS was conducted on an Agilent Model 8890 GC coupled to an Agilent 7000D mass spectrometer.

The extracted volatiles were desorbed from the fiber in the GC injection port (250 °C) for 5 min in splitless mode. Separation was achieved on a DB-5MS capillary column (30 m × 0.25 mm × 0.25 µm, 5 % phenyl-polymethylsiloxane; Agilent J&W Scientific). Helium was used as the carrier gas at a constant linear velocity of 1.2 mL/min. The oven temperature program was: initial 40 °C held for 3.5 min, ramped at 10 °C/min to 100 °C, then at 7 °C/min to 180 °C, then at 25 °C/min to 280 °C, and held at 280 °C for 5 min.

Electron ionization (EI) was performed at 70 eV. Mass spectra were recorded across a specified *m/z* range (e.g., 50–500 amu). The ion source, quadrupole mass detector, and transfer line temperatures were maintained at 230 °C, 150 °C, and 280 °C, respectively. Selected Ion Monitoring (SIM) mode was utilized for enhanced sensitivity and selectivity in the identification and quantification of target analytes where applicable.

2.3. Data processing and analysis

Raw UPLC-MS/MS data files were processed using SCIEX Analyst software (v1.6.3). MRM peak areas were extracted and integrated using MultiQuant software (v3.0.3, SCIEX). Raw GC–MS data were processed using Agilent MassHunter Workstation software (v10.0). Peak detection, integration, and spectral deconvolution (where necessary) were performed. The areas of the peaks were normalized against internal standard (3-Hexanone-2,2,4,4-d4), and metabolite identification was based on the comparison of retention times and mass spectra with known

libraries. Multivariate analysis was performed using R software (v.4.1.2, www.r-project.org) to visualize clustering and differential accumulation patterns. Principal Component Analysis (PCA) was performed using the prcomp function from the R base stats package after Unit Variance scaling. Pearson correlation coefficients (PCC) were calculated between samples using the "cor" function in R to assess reproducibility within groups and distinctness between groups. Correlation heatmaps were generated using the ComplexHeatmap R package (v2.9.4).

2.4. Metabolite identification and classification

Metabolite identification and annotation were performed using stringent criteria for both UPLC-MS/MS and GC–MS data, primarily leveraging the proprietary MetWare database (MWDB, MetWare Biotechnology Co., Ltd.) alongside publicly available databases and spectral libraries.

2.4.1. UPLC-MS/MS metabolite identification

Non-volatile metabolites detected via UPLC-MS/MS were putatively identified (corresponding primarily to Metabolomics Standards Initiative [MSI] Level 2 or 3) by matching their characteristics against the MWDB database. The criteria included:

Precursor Ion Mass: Matching the accurate *m/z* of the precursor ion within a specified tolerance (e.g., $\leq \pm 10$ ppm).

Retention Time (RT): Matching the chromatographic retention time within a narrow window (e.g., $\pm$ 0.1 min) compared to the database entry under identical analytical conditions.

MS/MS Fragmentation Pattern: Comparing the acquired tandem mass spectra (MS/MS or secondary spectra) with the reference spectra in the MWDB, requiring a high similarity score (e.g., $\geq$ 0.7). Adduct information (e.g., [M + H]+, [M-H]-, [M + Na]+) was also considered.

Multiple Reaction Monitoring (MRM) mode was employed for the quantification of selected metabolites. Peak areas were integrated from the resulting Extracted Ion Chromatograms (XICs Fig. S1), which were generated based on precursor-product ion transitions defined using the MetWare database (MWDB) and potentially confirmed with standards (corresponding to MSI Level 1 confidence for confirmed analytes).

2.4.2. GC–MS metabolite identification

Volatile metabolites detected via GC–MS were putatively identified (MSI Level 2/3) based on:

Mass Spectrum Matching: Comparing the acquired Electron Ionization (EI) mass spectrum against reference spectra in standard public libraries such as the National Institute of Standards and Technology (NIST) database and potentially the Fiehn RI library or other relevant mass spectral libraries. A high spectral similarity score (e.g., $>$ 700 out of 1000) was typically required.

Retention Index (RI): Comparing the experimental Kovats retention index (RI), calculated relative to a homologous series of n-alkanes, with RI values reported in the referenced libraries. A match within a defined tolerance window ($\pm$ 20 RI units) was considered acceptable.

2.5. Differential accumulation of metabolites

Differentially accumulated metabolites (DAMs) between the *Akebia* species (AKT, AKA, and AKQ) were identified through pairwise comparisons using Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), a supervised multivariate statistical method. This analysis was performed using the R package 'MetaboAnalystR' (v1.0.1). Metabolites were considered significantly differentially accumulated if they met two criteria derived from the OPLS-DA model: a Variable Importance in Projection (VIP) score greater than 1, and an absolute Log_2 Fold Change (|Log2FC|) of 1.0 or greater, ensuring a substantial difference in abundance. Differentially accumulated metabolites were then mapped to metabolic pathways using KEGG pathway enrichment analysis, providing insight into the biological processes most impacted by these metabolic changes. The Differential Abundance Score (DA Score) was calculated for each pathway, reflecting the overall upregulation or downregulation of metabolites within that pathway. Pathway analysis further helped identify key metabolic networks that were distinctly regulated in each species, highlighting the unique metabolic profiles of AKT, AKA, and AKQ. Data quality was controlled using QC samples to ensure that only reliable metabolites were included in the analysis.

3. Results

3.1. Overview of metabolic profiling

The metabolic profiling of three *Akebia* species—AKT, AKA, and AKQ—reveals significant differences in their metabolite compositions (Table S1). Class distribution of the 1429 compounds (1302 identified through UPLC-MS/MS and 126 through GC/MS) was identified showed that terpenoids (19.79 %) were the most abundant class, followed by amino acids and derivatives (8.39 %), flavonoids (8.16 %), and lipids (8.25 %). Other prominent classes included alkaloids (7.44 %), phenolic acids (7.21 %), and nucleotides and derivatives (3.38 %) (Fig. 1A). Principal Component Analysis (PCA) was performed to assess the overall variation in the metabolic profiles of the three species. The PCA plot (Fig. 1B and Fig. S2) clearly demonstrated that the metabolic profiles of the species were distinct, with samples from each species clustering separately along the first two principal components (PC1: 39.98 %, PC2: 38.04 %). This suggests that each species exhibits a unique metabolic signature, although variability was observed within each species. Furthermore, a correlation plot (Fig. 1C) was generated to examine the relationships between metabolites within the samples. Strong positive correlations were observed among metabolites within each species, with correlation coefficients approaching 1. This indicates that each species follows a consistent metabolic pattern, while the inter-species correlations were lower, further supporting the distinct metabolic profiles of the three *Akebia* species.

3.2. Differential accumulation of metabolites

The analysis of differentially accumulated metabolites (DAMs) across AKA, AKT, and AKQ revealed significant metabolic differences among the species (Figs. 2 and 3). In the AKA vs. AKT comparison, a total of 1012 DAMs were identified, with 597 downregulated and 415 upregulated in AKA compared to AKT (Fig. 2A). Similarly, in the AKQ vs. AKA comparison, 976 DAMs were detected, of which 377 were downregulated and 599 were upregulated in AKQ relative to AKA. In the AKQ vs. AKT comparison, 960 DAMs were identified, with an almost equal distribution of 481 downregulated and 479 upregulated metabolites in AKQ compared to AKT. These results highlight the significant biochemical diversity among the three *Akebia* species and reflect differences in their metabolic pathways and ecological adaptations.

In the AKA vs. AKT comparison (Fig. 3A and S3), the metabolites with the highest fold changes included Asperosaponin VI and Hederagenin-3-O-glucosyl(1–2)glucosyl(1–4)arabinoside, which showed strong downregulation, with log_2FC exceeding −9. Conversely, metabolites such as Luteolin 7-O-(6″-malonylglucoside) and Plantaginin were among the most upregulated, with log_2FC exceeding 7. Additional notable metabolites included various kaempferol derivatives and saponins, which contributed significantly to the metabolic differences between AKA and AKT. Moreover, the KEGG enrichment analysis associated with the DAMs suggested significant enrichment of flavonoid biosynthesis, nucleotide metabolism, starch and sucrose metabolism, phenylpropanoid biosynthesis, biosynthesis of nucleotide sugars, anthocyanin biosynthesis, and arginine biosynthesis pathways including others (Fig. 4A).

In the AKQ vs. AKA comparison (Fig. 3B and S4), Hederagenin-3-O-Beta-D-Glucopyranoside was the most upregulated metabolite, with a log_2FC of 10.4, followed by Dopamine with a log2 fold change of 8.91.

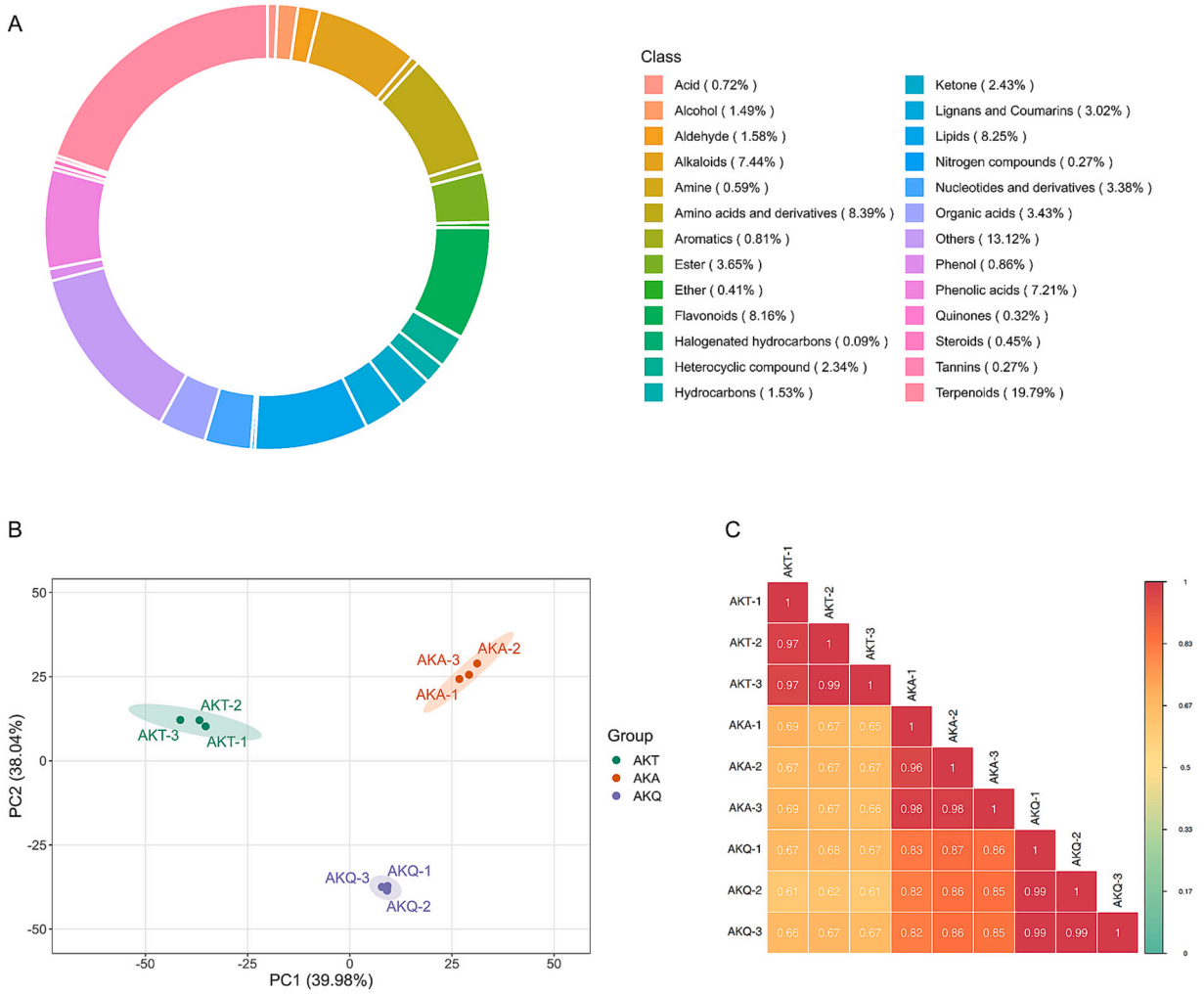

Fig. 1. Metabolic Profiling of *Akebia* Species. A) Class distribution of identified metabolites in the pulp of *A. trifoliata* (AKT), *A. trifoliata* ssp. australis (AKA), and *A. quinata* (AKQ). The most abundant classes include terpenoids (19.79 %), amino acids and derivatives (8.39 %), flavonoids (8.16 %), and lipids (8.25 %), among others. **B)** Principal Component Analysis (PCA) of the metabolic profiles of the three *Akebia* species. The first two principal components (PC1: 39.98 %, PC2: 38.04 %) clearly separate the species, indicating distinct metabolic profiles for each species. **C)** Correlation plot showing the relationships between metabolites within the samples. Strong positive correlations are observed within each species, suggesting consistent metabolic patterns within species, while inter-species correlations are lower, supporting the uniqueness of each species' metabolic profile.

On the other hand, metabolites such as Astralanosaponin B and Norarjunolic acid showed strong downregulation, with log_2FC of −8.7 and − 8.59, respectively. Other key metabolites like Asiatic acid, Madasiatic acid, and various saponins also showed substantial differential accumulation, further emphasizing the distinct metabolic profiles of AKQ and AKA. Moreover, the KEGG enrichment analysis associated with the DAMs suggested significant enrichment of ABC transporters, anthocyanin biosynthesis, tryptophan metabolism, zeatin biosynthesis, purine metabolism, lysine biosynthesis, and nicotinate and nicotinamide metabolism pathways among others (Fig. 4B).

In the AKQ vs. AKT comparison (Fig. 3C and S5), the most upregulated metabolites included Met-Thr-His and 1-Methylnicotinamide, with log_2FC of 10.0 and 9.92, respectively. Other upregulated metabolites included N-Methylnicotinate and Quercetin-3-O-xyloside (Reynoutrin), with log2 fold changes above 7. Conversely, metabolites like 1-O-p-Coumaroylquinic acid and Coumaroyl Quinic Acid exhibited strong downregulation, with log_2FC of −8.18. Additional significant metabolites included flavonoids, phenolic acids, and saponins, which played a key role in differentiating the metabolic profiles of AKQ and AKT. Moreover, the KEGG enrichment analysis associated with the DAMs suggested significant enrichment of flavone and flavonol biosynthesis, arginine biosynthesis, sulfur metabolism, porphyrin metabolism, purine, and pyrimidine metabolism pathways among others (Fig. 4C).

The analysis of differentially accumulated metabolites (DAMs) revealed a total of 309 common DAMs shared across the three pairwise comparisons: AKA vs. AKT, AKQ vs. AKA, and AKQ vs. AKT. These common DAMs, visualized in the Venn diagram (Fig. 2B), represent a core set of metabolites consistently differentially accumulated among the three *Akebia* species, reflecting key biochemical similarities and differences.

The chemical distribution of these 307 common DAMs is shown in the circular chart (Fig. 2C). Among the identified categories, Terpenoids were the most abundant, accounting for 31.4 % of the total DAMs, followed by Flavonoids (11.7 %) and Phenolic acids (10.0 %). Other notable categories included Amino acids and derivatives (7.4 %), Alkaloids (7.4 %), and Lipids (5.8 %), with minor contributions from Lignans and Coumarins (3.2 %), Nucleotides and derivatives (2.9 %), and Organic acids (2.6 %). These findings highlight the chemical diversity of the common DAMs, emphasizing their potential biological importance and the complexity of metabolic differences among AKA, AKT, and AKQ.

To further understand the differential regulation of overlapped DAMs, we categorized the DAMs according to classes and characterized their accumulation pattern as below

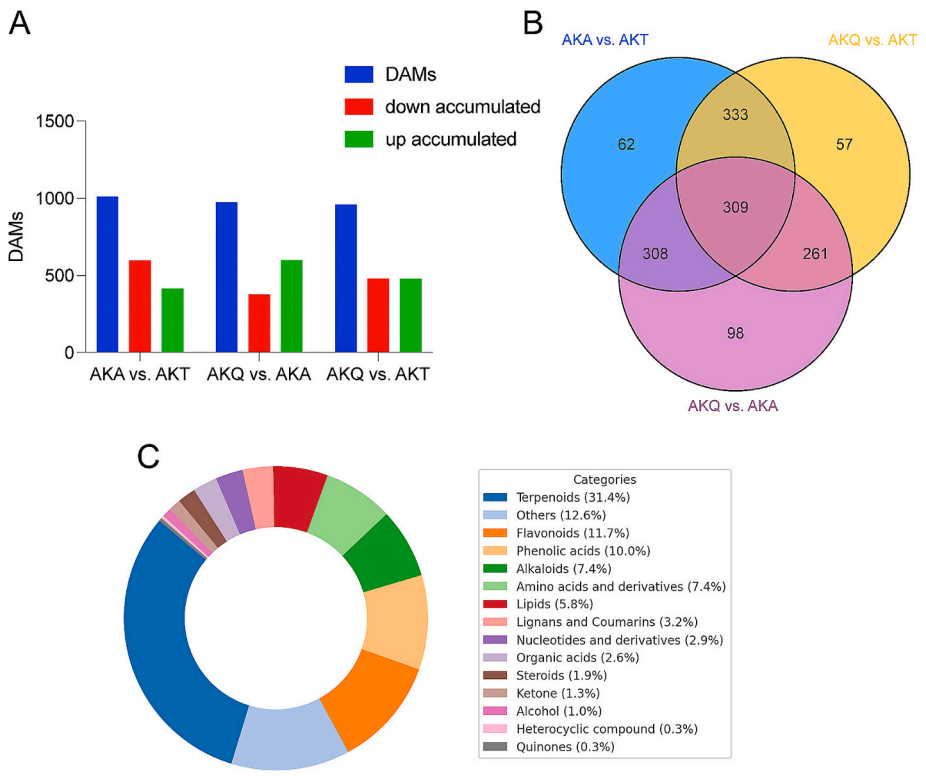

Fig. 2. Differentially Accumulated Metabolites (DAMs) across AKA, AKT, and AKQ Comparisons. A) Bar plot showing the number of DAMs identified in each pairwise comparison: AKA vs. AKT, AKQ vs. AKA, and AKQ vs. AKT. Blue bars represent the total number of DAMs, red bars indicate downregulated metabolites, and green bars represent upregulated metabolites in the respective comparisons. **B)** Venn diagram illustrating the overlap of DAMs among the three pairwise comparisons. Shared and unique metabolites are displayed for AKA vs. AKT (blue), AKQ vs. AKA (purple), and AKQ vs. AKT (yellow). **C)** Circular chart depicting the percentage distribution of common DAMs by their chemical categories, with Terpenoids (31.4 %) being the most abundant, followed by Flavonoids (11.7 %), Phenolic acids (10.0 %), and other classes such as Amino acids, Lipids, and Alkaloids. The chart highlights the diverse chemical nature of the DAMs across the comparisons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Species-specific terpenoid metabolism in Akebia

The differential accumulation of terpenoid metabolites across the three species (represented by AKT, AKA, and AKQ) reveals significant species-specific metabolic strategies (Fig. 5). The terpenoids identified include Monoterpenoids, Sesquiterpenoids, Diterpenoids, Triterpenes, and Triterpene Saponins. Each of these classes shows distinct accumulation patterns, which highlight the unique metabolic adaptations of each species to its ecological niche.

In the Monoterpenoid category, Russujaponol E and 13-Nor-desacylcynaropicrin accumulate significantly more in AKA than in AKT and AKQ, indicating a specialized metabolic pathway in AKA that favors these compounds. Conversely, Blumenol B Glucoside shows higher accumulation in AKT, suggesting that AKT might be more adept at synthesizing or storing this metabolite. Interestingly, 13-Nor-desacylcynaropicrin also accumulates more in AKQ, pointing to a unique metabolic profile in AKQ that could be influenced by environmental or ecological factors.

In the Sesquiterpenoid category, metabolites such as 3-Oxooleana-11,13(18)-dien-28-oic acid and Rubuminatus A accumulate predominantly in AKA, suggesting that this species may have evolved to favor these compounds in response to specific environmental pressures. On the other hand, 3-Hydroxylup-20(29)-en-28-al (Betulinaldehyde) is more abundant in AKQ, indicating that AKQ may rely on this metabolite for defense or other physiological functions. Blumenol B Glucoside also shows higher levels in AKA, suggesting its potential role in the physiology of AKA.

The Diterpenoid category, while less prominent in the dataset, follows similar trends to the other terpenoid classes, with species-specific accumulation patterns suggesting different biosynthetic routes. These compounds likely serve specific ecological or physiological roles within each species, but further study is needed to fully elucidate their functions.

In the Triterpene category, compounds such as Asiatic acid and Madasiatic acid show significantly higher accumulation in AKT, highlighting AKT's central role in synthesizing these compounds. These triterpenes are often involved in stress responses and defense mechanisms, suggesting that AKT may be better suited to dealing with abiotic stress. In contrast, metabolites like Norarjunolic acid and Cordianol B accumulate more in AKA, emphasizing the unique metabolic adaptations of AKA. Rubuminatus A, another triterpene, is more abundant in AKQ, further suggesting that each species has evolved a distinct set of metabolites to address its ecological challenges.

The Triterpene Saponins category shows even more pronounced differences, with higher accumulation in AKA, especially for compounds such as Methyl jasmonate and Oleanolic acid saponins. These metabolites are likely involved in the defense mechanisms of AKA, especially given their known roles in herbivore deterrence and pathogen resistance. However, AKQ also accumulates significant levels of triterpene saponins, which further supports the idea that this species employs these compounds for similar defensive purposes.

Overall, the analysis underscores the remarkable species-specific variation in terpenoid metabolism. Each species—AKT, AKA, and AKQ—accumulates a distinct set of terpenoids, reflecting their unique ecological niches and adaptive strategies. These differences are likely driven by a combination of genetic factors and environmental influences, which shape the metabolic pathways each species employs. The findings highlight the ecological significance of these metabolites in defense, stress response, and other physiological processes. Further studies on the functional roles of these terpenoids will provide deeper

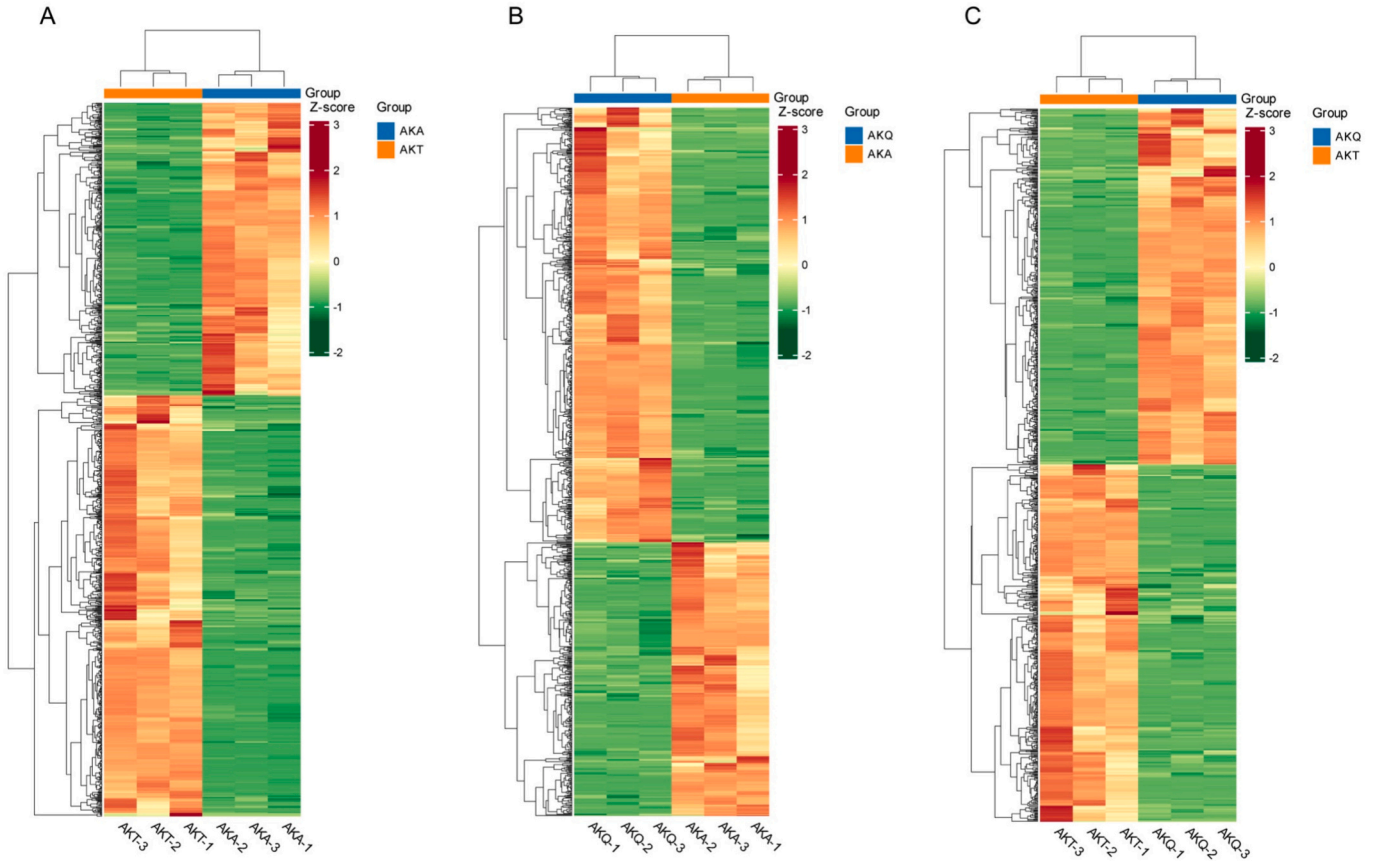

Fig. 3. Differential accumulation of metabolites in *Akebia* species. A) AKA vs. AKT B) AKQ vs. AKA C) AKQ vs. AKT. Color intensity reflects standardized metabolite abundance (Z-scores), with warmer colors indicating higher accumulation and cooler colors indicating lower accumulation. Each row represents a metabolite, and each column corresponds to a biological replicate within the compared aging groups.

insights into how each species uses these compounds to thrive in its respective environment.

3.4. Comparative evaluation of key compounds in Akebia species

The differential accumulation of alkaloids across the three species—AKT, AKA, and AKQ—reveals distinct patterns of upregulation and downregulation in metabolite levels (Fig. 6A). The AKT species generally shows higher accumulation of alkaloids, particularly 1-Methylnicotinamide, N-Methylnicotinate, and Hexanoate, which are significantly more abundant in AKT compared to the other species, indicating that AKT has a higher capacity for producing or retaining these metabolites. In contrast, the AKA species tends to show lower accumulation of these same alkaloids, particularly 1-Methylnicotinamide and N-Methylnicotinate, suggesting a reduced metabolic activity for these compounds. However, some metabolites like Cis-Zeatin riboside and Carboxylic acid accumulate more in AKA compared to AKQ. The AKQ exhibits a mixed pattern, with compounds such as Cis-Zeatin riboside and Hexanoate showing higher levels in AKQ compared to AKT and AKA, while other metabolites like 1-Methylnicotinamide and N-Methylnicotinate are downregulated in AKQ. Overall, AKT is characterized by the highest accumulation of most alkaloids, suggesting it is the most metabolically active, while AKA shows generally lower levels, and AKQ displays a more varied accumulation profile with certain metabolites being more abundant.

The comparative analysis of phenolic compound accumulation reveals distinct patterns of upregulation and downregulation (Fig. 6B). AKT consistently shows higher accumulation of phenolic compounds compared to both AKA and AKQ, indicating a higher metabolic activity or capacity for these compounds. For example, compounds like carboxylic acid and 1-Caffeoyl-beta-D-glucose are significantly more abundant in AKT, reflecting its stronger metabolic synthesis of phenolics. In contrast, AKA shows lower accumulation levels for most phenolic compounds, such as Calceolarioside A and Desrhamnosylacteoside, indicating reduced metabolic activity. AKQ, on the other hand, exhibits an intermediate accumulation pattern, with some compounds like Caffeoylquinic acid being more abundant than in AKA but still lower than in AKT. Other compounds, such as Desrhamnosylacteoside, are upregulated in AKQ compared to AKA but downregulated relative to AKT. Overall, AKT demonstrates the highest accumulation of phenolic compounds, AKA the lowest, and AKQ shows a more balanced but generally intermediate profile of phenolic accumulation, highlighting the distinct metabolic regulation across the species.

The analysis of flavonoid accumulation reveals distinct pasterns of upregulation and downregulation (Fig. 6C). AKT consistently shows higher accumulation of flavonoids compared to both AKA and AKQ, indicating that AKT has a greater capacity for producing or retaining these compounds. Key flavonoids such as Kaempferol, Quercetin, and Luteolin are significantly more accumulated in AKT than in the other species. In contrast, AKA shows lower accumulation of flavonoids relative to both AKT and AKQ, suggesting reduced metabolic activity for these compounds in AKA. Flavonoids like Myricetin and Apigenin show particularly lower levels in AKA. AKQ exhibits an intermediate accumulation profile, with higher flavonoid levels than AKA but still lower than AKT, with Rutin and Chrysoeriol being among the more accumulated compounds in AKQ compared to AKA. Overall, AKT is the most metabolically active in terms of flavonoid accumulation, while AKA has the lowest levels across the board, and AKQ shows moderate accumulation, falling between the other two species. These patterns highlight the differential regulation of flavonoid metabolism among the species.

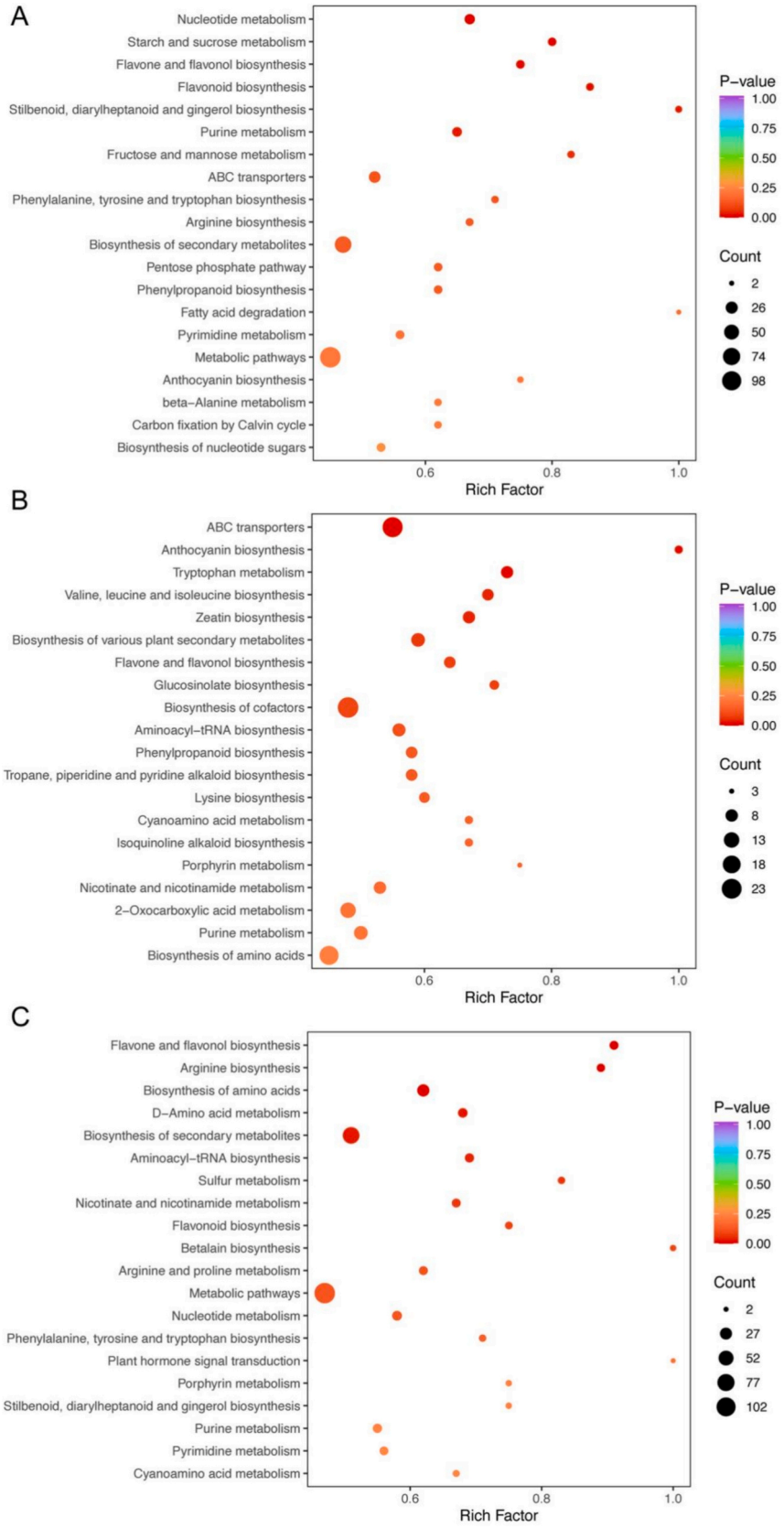

Fig. 4. KEGG enrichment analysis of DAMs in three pairwise comparisons A) AKA vs. AKT, B) AKQ vs. AKA, and C) AKQ vs. AKT. The x-axis ('Rich Factor') indicates the ratio of the number of differentially accumulated metabolites mapped to a specific pathway to the total number of metabolites that map to that pathway. The y-axis lists the metabolic pathways involved. The color gradient from green to red represents the significance levels of the pathways (*p*-values), with red indicating higher significance. The size of each dot corresponds to the number of DAMs involved in each pathway. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

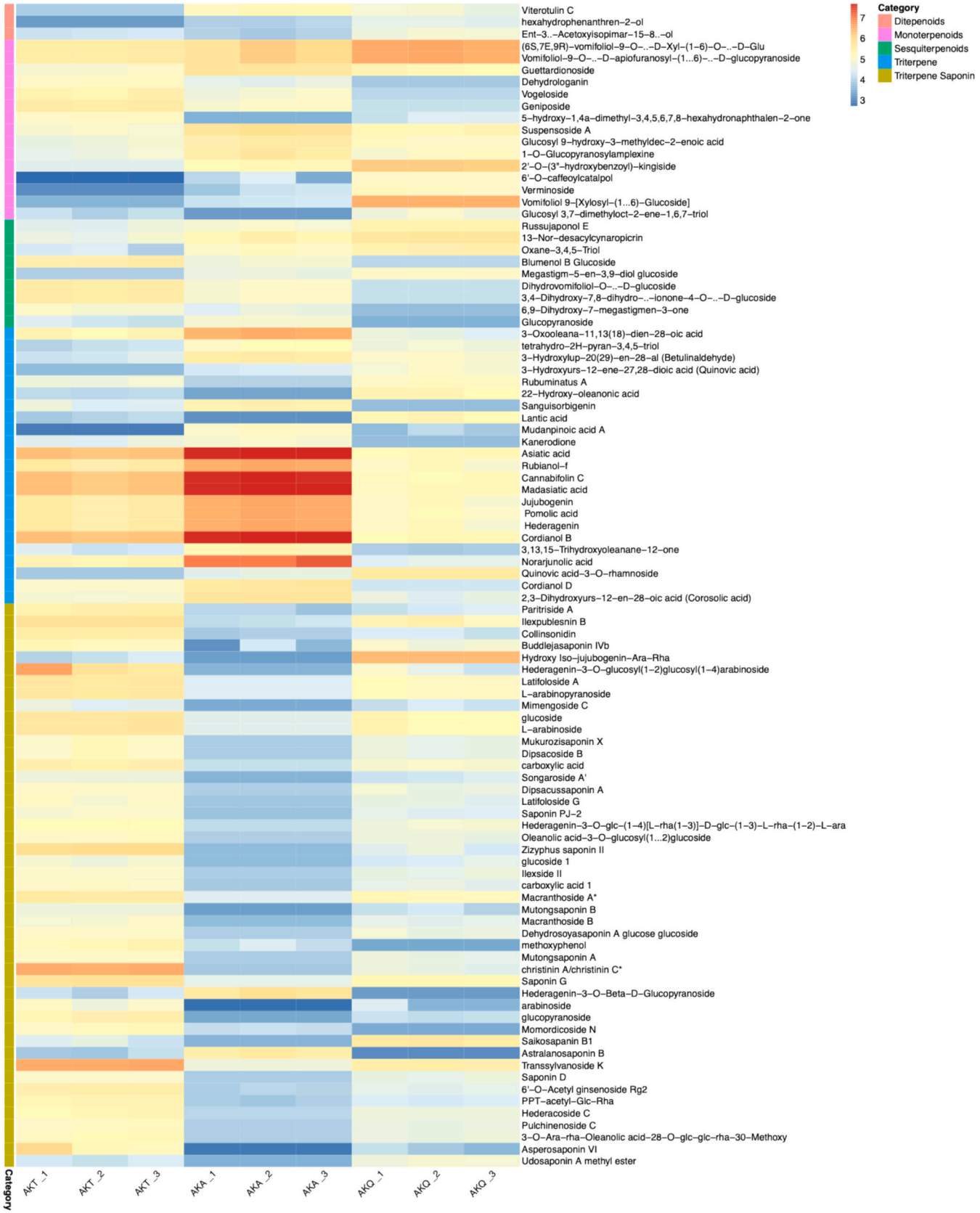

Fig. 5. Differential accumulation of terpenoids. Heatmap color intensity represents accumulation levels: warmer color (red) indicates higher expression, and cooler color (blue) represent lower expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

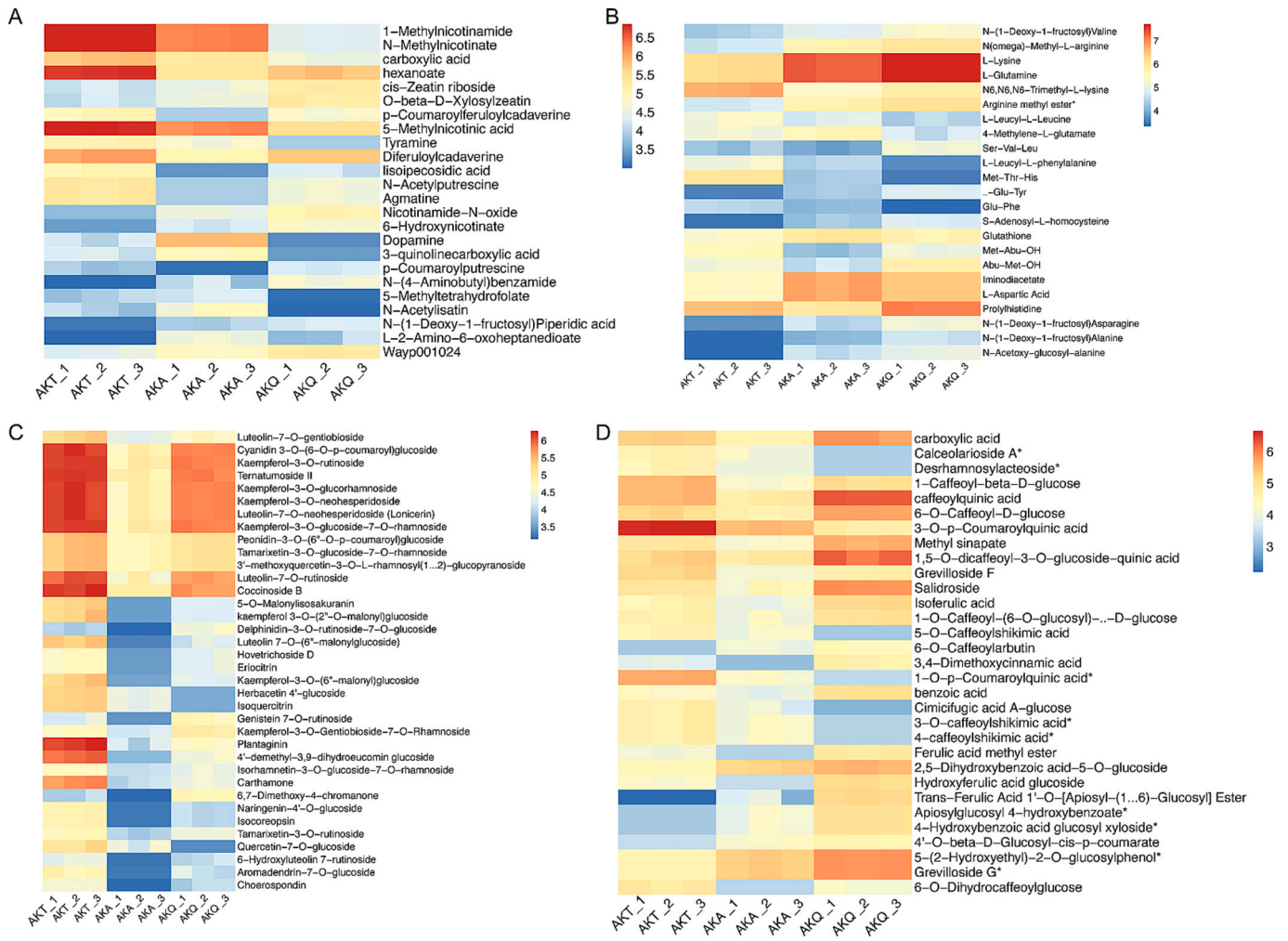

Fig. 6. Differential accumulation of A) alkaloids, B) flavonoids, C) phenolics, and D) amino acids. Heatmap color intensity represents accumulation levels: warmer color (red) indicates higher expression, and cooler color (blue) represent lower expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The comparative analysis of amino acids and derivatives accumulation reveals distinct patterns of upregulation and downregulation (Fig. 6D). AKT shows downregulated accumulation of most amino acids and derivatives compared to both AKA and AKQ, with compounds such as N-(1-Deoxy-1-fructosyl)Valine, N(omega)-Methyl-L-arginine, and L-Lysine accumulating at significantly lower levels. AKA also exhibits downregulated accumulation for most compounds when compared to AKT and AKQ, with L-Glutamine and N6,N6,N6-Trimethyl-L-lysine showing particularly low levels. In contrast, AKQ displays a more intermediate pattern, with certain compounds like N6,N6,N6-Trimethyl-L-lysine being more abundant than in AKA, but still lower than in AKT. Overall, AKT tends to accumulate fewer amino acids and derivatives than AKQ, and AKA consistently shows the lowest levels across most compounds, indicating reduced metabolic activity for these compounds.

3.5. Comparative metabolic profiling of Akebia species highlights the superior value of A. trifoliata pulp

Based on the metabolite profiling data, AKT pulp appears to be the most nutritionally valuable among the three *Akebia* species. It shows the highest accumulation of bioactive compounds, particularly amino acids, flavonoids, and terpenoids, which are crucial for health benefits. For instance, amino acids like L-Isoleucyl-L-Aspartate contribute to protein synthesis, while flavonoids such as Luteolin and 6-C-Methylquercetin-3-O-rutinoside enhance antioxidant activity, offering potential anti-inflammatory and cellular protection properties. Additionally, AKT contains important terpenoids, such as Russujaponol E, which are linked to antimicrobial and anti-cancer effects. In contrast, AKA exhibits lower accumulation of key metabolites, particularly amino acids and derivatives, suggesting a more limited nutritional value. AKQ presents an intermediate nutritional profile, with a moderate presence of antioxidants like Quercetin and Rutin, which contribute to its antioxidant and cardiovascular benefits, but its overall nutritional content is lower than that of AKT. Thus, AKT pulp stands out as the most nutritionally rich, followed by AKQ, while AKA appears to have the lowest nutritional value among the species.

4. Discussion

This study presents a comprehensive metabolomic comparison of the pulp from three species of *Akebia*: *A. trifoliata* (AKT), *A. trifoliata* ssp. australis (AKA), and *A. quinata* (AKQ). By employing advanced UPLC-MS/MS and GC–MS techniques, we identified 1429 metabolites and uncovered distinct species-specific metabolic signatures. The findings contribute significantly to the understanding of *Akebia* metabolomics, building upon and addressing gaps in previous research.

Previous studies on *Akebia* have primarily focused on isolated bioactive compounds such as flavonoids, saponins, and alkaloids, which are known for their anti-inflammatory, antioxidant, and anticancer properties (Huang et al., 2022; X. Liu et al., 2023). However, these

studies lacked a comprehensive metabolomic approach to characterize the full spectrum of metabolites in *Akebia* fruits. For instance, Li et al. (2019) identified individual flavonoids and alkaloids in *A. trifoliata* but did not explore their broader metabolomic context or interspecies differences (Gong et al., 2019; Li et al., 2021). Similarly, Gong et al. (2019) highlighted the pharmacological potential of saponins in *Akebia* but limited their analysis to specific compounds, omitting other metabolite classes such as terpenoids and phenolics (Gong et al., 2019).

This study provides the first comprehensive metabolomic comparison of the pulp from three *Akebia* species, highlighting their distinct chemical profiles and potential applications. AKT demonstrated the most nutritionally and pharmacologically rich profile, with high levels of terpenoids (e.g., Russujaponol E, known for diverse biological activities (Tholl, 2015; Zwenger & Basu, 2008)), flavonoids (e.g., Luteolin 7-O-(6″-malonylglucoside), contributing to antioxidant capacity (Panche, Diwan, & Chandra, 2016; Qian et al., 2023)), and amino acids (e.g., L-Isoleucyl-L-Aspartate, essential building blocks (Daniel, 2006; Hou & Wu, 2018)). These are critical for health benefits such as antioxidant activity, protein synthesis, and anti-inflammatory effects (Daniel, 2006; Qian et al., 2023; Sun et al., 2023). The significant enrichment of flavonoid biosynthesis and terpenoid pathways in AKT emphasizes its potential for therapeutic applications and superior nutritional value.

In contrast, AKA exhibited lower accumulation of bioactive compounds, suggesting limited nutritional and medicinal utility, corroborating findings from earlier studies that suggested a limited pharmacological potential for this subspecies (Jiang et al., 2012). Meanwhile, AKQ displayed an intermediate metabolic profile, with moderate levels of antioxidants and terpenoids, aligns with prior research indicating its moderate antioxidant and medicinal properties (Maciąg et al., 2021). These findings suggest that genetic and environmental factors shape the metabolomic diversity among these species, reflecting their ecological adaptations.

Monoterpenoids such as Russujaponol E and 13-Nor-desacylcynaropicrin, which accumulate more in AKA, are known for their antimicrobial and anti-inflammatory properties (Balkrishna, Sharma, Srivastava, Chaudhary, & Arya, 2024). Systemic inflammation is a key factor in the development of atherosclerosis (Libby, 2021), and compounds that reduce inflammation may help maintain vascular integrity. Additionally, their antioxidant properties can mitigate oxidative stress, which is strongly linked to endothelial dysfunction and cardiovascular disease progression (Münzel, Daiber, Ullrich, & Mülsch, 2005). Blumenols, known to be involved in plant signaling pathways, also exhibit antioxidant activity in human systems, which may protect cardiovascular tissues from oxidative damage and improve overall vascular health (Han & Bakovic, 2015; Mindt, Wang, Schäfer, Halitschke, & Baldwin, 2019). The significant presence of triterpenes like Asiatic acid and Madasiatic acid in AKT reflects its therapeutic potential (Nagoor Meeran et al., 2018; Witkowska, Paczkowska-Walendowska, Garbiec, & Cielecka-Piontek, 2024). Asiatic acid, in particular, is recognized for its ability to reduce oxidative damage and inflammation, two major contributors to cardiovascular disease. It has also been reported to enhance collagen synthesis and support wound healing, which could contribute to vascular repair and maintenance (Nagoor Meeran et al., 2018). These properties suggest that the consumption of AKT fruit pulp could have protective effects on cardiovascular tissues, aiding in the prevention of heart-related conditions. Similarly, the higher levels of Rubuminatus A in AKQ might contribute to its defensive capabilities against herbivory or fungal infections, suggesting a functional specialization (Moses, Papadopoulou, & Osbourn, 2014; War et al., 2012). The pronounced accumulation of triterpene saponins, particularly Methyl jasmonate and Oleanolic acid saponins, in AKA underscores their importance in modulating cholesterol metabolism and reducing lipid accumulation in the arteries (Ayeleso, Matumba, & Mukwevho, 2017; Lin, Wen, & Sun, 2016). Saponins are known for their ability to lower cholesterol levels by inhibiting its absorption in the gastrointestinal tract (Francis, Kerem, Makkar, & Becker, 2002). Moreover, these compounds have potent anti-inflammatory and antioxidant properties that can protect against vascular damage caused by free radicals and inflammation (Farooq et al., 2016; Gunjegaonkar Shivshankar et al., 2022; Reyes-Díaz et al., 2016).

The identification of species-specific metabolites, such as Russujaponol E in AKT and Norarjunolic acid in AKA, underscores the unique metabolic strategies of each species. These compounds not only serve as markers for distinguishing the species but also hold potential for targeted pharmacological investigations.

The findings have significant implications for the cultivation and utilization of *Akebia* fruits. By leveraging the genomic and metabolomic insights, breeders can enhance the nutritional and medicinal properties of these species. Additionally, the study offers valuable guidance for developing functional foods and nutraceuticals, particularly from AKT.

The distinct metabolic profiles of each *Akebia* species bring unique advantages for various applications, whether in agriculture, nutrition, or medicine. This study's findings highlight the importance of recognizing and utilizing these differences to harness the full potential of each species. By understanding the specific contexts in which these plants offer benefits, we can better inform cultivation practices and medicinal uses.

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CRediT authorship contribution statement

Mian Faisal Nazir: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Tianjiao Jia:** Visualization, Methodology, Investigation. **Jie Xu:** Software, Methodology, Investigation. **Longyu Dai:** Visualization, Validation, Methodology, Investigation. **Yafang Zhao:** Validation, Resources, Methodology, Data curation. **Shuaiyu Zou:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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