

RESEARCH ARTICLE

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Metabolomic traits of *Amomum villosum* Lour. fruits from different areas in Asia

Zhihang Duan^{1†}, Changlian Gan^{1†}, Jianjun Yue¹, Pengfei Shen¹, Dongxiu Ning², Yegao Chen^{3*} and Xiaohua Li^{1*} 

Abstract

Amomum villosum Lour. is an essential plant for both consumption and healing purposes. Among all the regions in China where *A. villosum* is grown, Yangchun City is widely regarded as the most ideal habitat for this plant. However, there were rare studies that explored the differential metabolites between Yangchun and other regions of *A. villosum* fruits. The current study analyzed the metabolic profiles of *A. villosum* fruit samples cultivated in four representative producing areas. As a result, 260 differentially accumulated metabolites were identified in the GDYC-F versus LW-F. A total of 224 differentially accumulated metabolites were identified in the GDYC-F versus MD-F. A total of 285 differentially accumulated metabolites were identified in the YNML-F versus LW-F. A total of 262 differentially accumulated metabolites were identified in the YNML-F versus MD-F. Moreover, we identified the biomarkers to distinguish samples from different regions, including 9,12,13-TriHOME, 2-Ketobutyric acid, alpha-linolenic acid, nobiletin, sodium deoxycholate, gluconolactone and adipic acid, mifepristone, Qing Hau Sau and p-Coumaroylquinic acid, coumarin, ciliatine, methyl benzoate, 1,3-Benzenediol, (2'E,4'Z,8E)-Colneleic acid and beta-D-Fructose 6-phosphate, phenylethylamine, 4-Nitrophenol and geranyl acetate. KEGG enrichment analysis revealed that 'Linoleic acid metabolism' and 'Phenylpropanoid biosynthesis' were significant metabolic pathways among the different comparisons. The research uncovered metabolic variances in *A. villosum* fruits between authentic (Yangchun) and non-authentic regions across various nations, providing a scientific foundation for evaluating habitat appropriateness and introducing superior *A. villosum*. Subsequent research ought to confirm this study's findings by employing more sampling sites and gathering *A. villosum* fruits across successive years.

Keywords *Amomum villosum* Lour. metabonomics, Geo-authentic area, LC–MS

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Introduction

Amomum villosum Lour., also known as ‘Sha Ren,’ is a perennial herb belonging to the Zingiberaceae family in traditional Chinese medicine (Wei et al. 2024). The name of this plant was further checked in world flora online (www.worldfloraonline.org), accessed on August 08, 2023. Among the four most renowned traditional Chinese medicines (TCMs), it is widely recognized in Chinese folklore along with *Areca catechu* L., *Morinda officinalis* How and *Alpinia oxyphylla* Miq (Liang et al. 2017). The desiccated and fully grown fruits of *A. villosum* have been documented for their medicinal properties. Traditionally, because of its flavor, it has been incorporated into tea, wine and herbal drinks, which are utilized for addressing gastrointestinal ailments like inflammation, ulcers and fortifying the intestinal mucosal barrier (Suo et al. 2018; Yue et al. 2021). *A. villosum* can be found primarily in South and East Asia, encompassing regions such as southern China, Laos, Vietnam, Myanmar, Cambodia and Thailand. Provinces like Guangdong, Guangxi, Fujian and Yunnan in China are where it is grown (Cai et al. 2021). Yangchun was recognized as the original habitat of *A. villosum* among various regions in China, known for producing authentic and top-quality *A. villosum* as documented in the 1822 book Guangdong Tong Zhi (Li et al. 2024). However, there is relatively little research on the differences in the population of *A. villosum* from different regions. Various regions produce *A. villosum* of varying quality, resulting in a diverse range of *A. villosum* varieties with differing qualities available in the market (Liyun et al. 2012). The purpose of this study was to quantitatively evaluate and compare the fruits of domestic and overseas *A. villosum*.

Recent pharmacological research has shown that the primary constituents of *A. villosum* consist of essential oil, flavonoids, organic acids and polysaccharides. Following identification, the primary bioactive volatile oils found in the fruit of *A. villosum* are bornyl

acetate, borneol and camphor (Wei et al. 2024; Xue et al. 2015). In the Chinese Pharmacopoeia, *A. villosum* fruit is required to meet the quality standard of bornyl acetate (Commission 2020). Using only one chemical component as a quality control measure for traditional Chinese medicine is insufficient, as medicinal plants contain a diverse range of active ingredients and complex chemical compounds (Xiao et al. 2022). Plant metabolomics offers a thorough examination of chemical constituents, aligning with the intricate nature of medicinal substances and aiding in the authentication and assessment of their quality (Xiao et al. 2022). This study aims to identify the metabolites of *A. villosum* and investigate the variations in metabolites across various production regions, as the genuineness and excellence of *A. villosum* are heavily reliant on its metabolic constituents. LC–MS metabolomics analysis offers excellent sensitivity and specificity, allowing for the simultaneous detection of numerous endogenous compounds (Dettmer et al. 2007; Raftery 2014). It has been widely used in plant metabolite research (Ni et al. 2017), biomarker screening (Lee et al. 2019), medicinal material quality evaluation (Yoshitomi et al. 2020) and other fields (Table 1).

Various geographical elements such as climate, precipitation and soil quality can influence the creation and storage of plant metabolites, ultimately impacting the medicinal and biological properties of plants used for medicine (Karimi et al. 2020). To date, no studies have reported the differential metabolites in domestic and overseas *A. villosum* fruits. Comprehending the varied metabolite compositions of *A. villosum* fruits is crucial for evaluating their habitat appropriateness, introduction and growth of superior *A. villosum*. In this study, *A. villosum* fruits were gathered from four regions (Guangdong–Yangchun, Yunnan–Mengla, Laos and Myanmar) and along with relevant environmental data in the research. Metabolic clustering analysis was conducted using LC–MS/MS and multivariate analysis techniques to differentiate fruit samples from various

Table 1 Climatic and geographical information of sampling points of *A. villosum*

No	Location	Province	Code	Latitude (N)	Longitude (E)	Annual precipitation (mm)	Climate
1	Yangchun city	Guangdong	GDYC-F	21°50′36″–22°41′01″	111°16′12″–112°09′22″	2380	Subtropical monsoon climate zone
2	Mengla city	Yunnan	YNML-F	21°32′–21°52′	101°6′–101°29′	1616	Subtropical monsoon climate zone
3	Phongsali province	Laos	LW-F	21°41′	102°07′	2000	Tropical monsoon climate
4	Mongla	Myanmar	MD-F	21°38′–21°51′	99°57′–100°18′	1341	Tropical and subtropical southwest monsoon climate

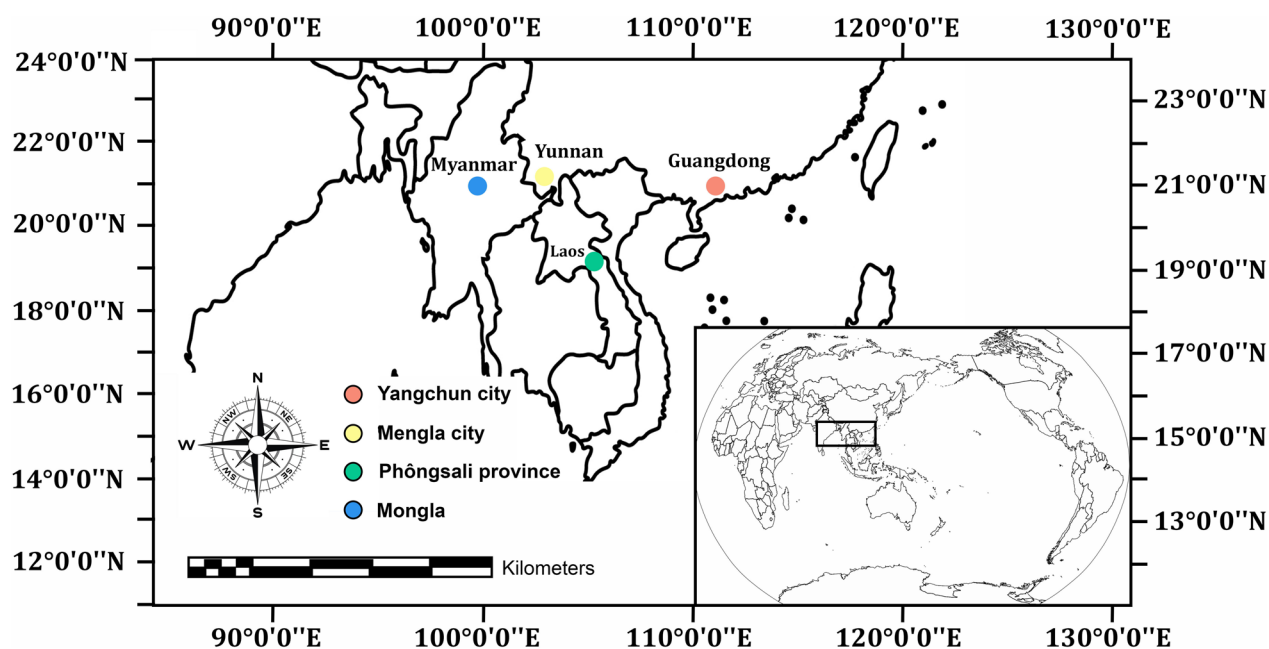


Fig. 1 Four study regions of *A. villosum* in world. The locations of samples are illustrated on the map using longitudes and latitudes

regions and pinpoint unique metabolites specific to each region. Principal component analysis (PCA) was used to observe the differences in unique metabolites specific to each region. Finally, we have successfully pinpointed a group of biomarkers that can differentiate samples based on their geographical origins, including 2-Ketobutyric acid, alpha-linolenic acid, nobiletin, sodium deoxycholate, gluconolactone and adipic acid. This study aimed to reveal the metabolic differences among *A. villosum* fruits from the geo-authentic (Yangchun) and non-authentic producing areas in different countries that provides a guide for the application of *A. villosum* plants in the food and pharmaceutical industries, which also presents an effective method for identifying disruptions in the natural habitat of *A. villosum*, and lays a scientific foundation for evaluating the appropriateness of the habitat and the introduction of superior *A. villosum* (Fig. 1).

Results

Widely targeted metabolome analyses between GDYC-F versus LW-F

Metabolic profiles of *A. villosum* fruit from four distinct areas were obtained through LC-MS/MS analysis. Fold change (FC) ≥ 2 , VIP > 1 and fold change (FC) $\leq 1/2$, VIP > 1 were the criteria for the upregulated and downregulated metabolites, respectively. To compare the different metabolites of fruits between Guangdong-Yangchun and Laos, the heatmap analysis was carried out for GDYC-F versus LW-F (Fig. 2A), some metabolites of

GDYC-F were upregulated, but downregulated of LW-F, suggesting that *A. villosum* fruits from different regions might have different metabolites. In total, 260 differential metabolites were identified in the GDYC-F versus LW-F. Among them, the content of 121 metabolites in Yangchun was significantly higher than that in Laos. Likewise, 139 metabolites in Yangchun were significantly decreased than in Laos (Fig. 2B). The top 20 differentially abundant metabolites in GDYC-F versus LW-F are listed in Table 2. To compare the metabolites among different regions of *A. villosum*, principal component analysis (PCA) analysis was utilized on the 260 metabolites. Analysis has demonstrated a distinct separation of metabolites from the two regions in the score plots, with PC1 plotted against PC2. PC1 and PC2 represented 47% and 14.6% of the total variation, respectively (Fig. 2C). In Fig. 2D, KEGG enrichment analysis revealed that 'Linoleic acid metabolism' and 'Phenylpropanoid biosynthesis' were significant metabolic pathways. *A. villosum* in the Yangchun area exhibited notably elevated levels of 9,12,13-Tri-HOME, 2-Ketobutyric acid, alpha-linolenic acid, nobiletin, sodium deoxycholate, gluconolactone and adipic acid content than those in the Laos region (Fig. 3).

Widely targeted metabolome analyses between GDYC-F versus MD-F

Despite sharing a similar latitude, Myanmar and Guangdong exhibit significant differences in longitude, resulting in distinct region-specific metabolites found in *A. villosum* fruits between the two locations. The heatmap

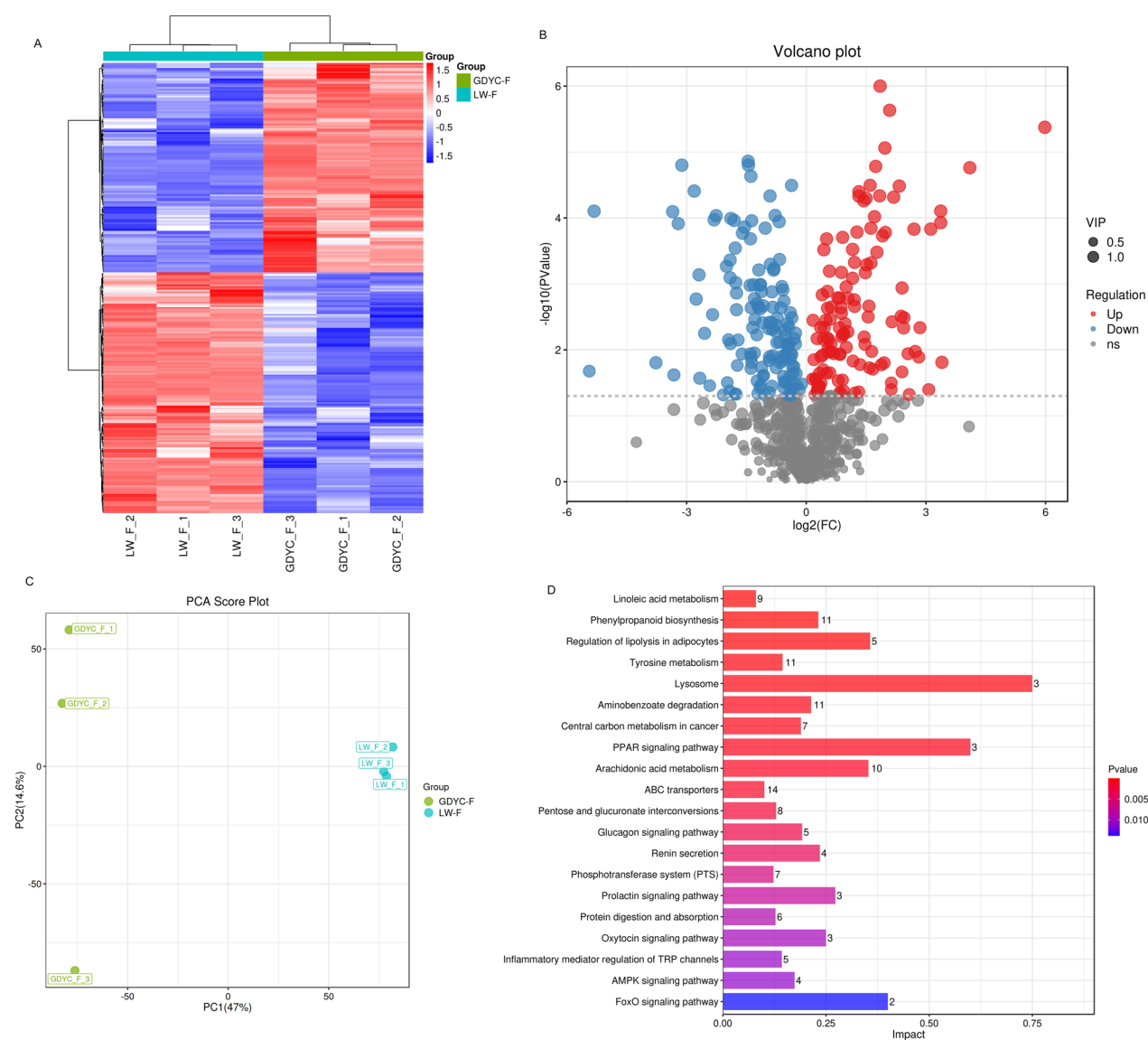


Fig. 2 Integrative metabolomic phenotypes showing cultivation-region specificity in GDYC-F versus LW-F. **A** The heatmap of differentially abundant metabolites in GDYC-F versus LW-F. **B** Metabolite profiles are visualized using volcano plots. Red dots represent differential metabolites whose relative contents were significantly higher in Yangchun than Laos region. Blue dots represent differential metabolites with relative contents that were significantly lower in Yangchun than Laos region. **C** PCA analysis of all metabolites identified in GDYC-F versus LW-F. **D** Results of KEGG pathways analysis (top 20 terms)

analysis was carried out for GDYC-F versus MD-F (Fig. 4A); some metabolites of GDYC-F were upregulated, but downregulated of MD-F, suggesting that *A. villosum* fruits from different regions might have different metabolites. A total of 224 differential metabolites were identified in the GDYC-F versus MD-F. Among them, 128 metabolites were upregulated and 96 were downregulated (Fig. 4B). The top 20 differentially abundant metabolites in GDYC-F versus MD-F are listed in Table 2. Additionally, research has demonstrated that

metabolites from distinct areas were distinctly segregated in the score graphs, with PC1 plotted against PC2. PC1 and PC2 represented 44.5% and 17.1% of the total variation, respectively (Fig. 4C). In Fig. 4D, KEGG enrichment analysis revealed that ‘Tyrosine metabolism,’ ‘Arachidonic acid metabolism’ and ‘Linoleic acid metabolism’ were significant metabolic pathways. In particular, *A. villosum* in the Yangchun region had significantly higher 9,12,13-TriHOME, SR95531, Mifepristone, Qing Hau Sau and p-Coumaroylquinic acid content than those in the

Table 2 Identification of top 20 differentially abundant metabolites in the four comparisons

Group	Compounds	p-value	VIP	Log ₂ FC	Regulation
GDYC-F versus LW-F	Acetylphosphate	1.00E-06	1.457814223	1.85	Up
	Terephthalate	2.33E-06	1.432929712	2.09	Up
	9,12,13-TriHOME	4.21E-06	1.456488048	5.98	Up
	4-Methylamino-4-de(dimethylamino)anhydrotetracycline	8.68E-06	1.455483028	1.97	Up
	Thymine	1.38E-05	1.454459141	-1.46	Down
	N-Ethylmaleimide	1.58E-05	1.454150527	-1.45	Down
	15-Deoxy-d-12,14-PGJ2	1.58E-05	1.454385947	-3.12	Down
	Phthalic acid	1.65E-05	1.429777331	1.74	Up
	2-Ketobutyric acid	1.73E-05	1.429776996	4.1	Up
	Kojibiose	2.33E-05	1.453113764	-1.39	Down
	1,3-Benzenediol	3.20E-05	1.428172598	1.6	Up
	(-)-Epigallocatechin	3.21E-05	1.428287784	-0.37	Down
	Thiamine	3.28E-05	1.452115608	2.33	Up
	Retinoyl b-glucuronide	3.9E-05	1.451347089	-2.81	Down
	Procyanidin B2	4.00E-05	1.451524721	1.32	Up
	(6Z)-Octadecenoic acid	4.61E-05	1.426537271	1.84	Up
	Myristoleic acid	4.64E-05	1.426917482	-0.91	Down
	Oxoglutaric acid	4.66E-05	1.450939422	1.32	Up
	2-Furoate	4.84E-05	1.426737242	2.19	Up
	4-Nitrophenol	5.03E-05	1.426255581	1.5	Up
GDYC-F versus MD-F	Coumarin	2.32E-06	1.498539381	-1.67	Down
	9,12,13-TriHOME	5.13E-06	1.497917567	5.88	Up
	SR95531	7.82E-06	1.497062959	2.1	Up
	Mifepristone	1.78E-05	1.495380721	3.69	Up
	Qing Hau Sau	1.79E-05	1.495258278	2.76	Up
	p-Coumaroylquinic acid	3.15E-05	1.408329615	1.55	Up
	15-Deoxy-d-12,14-PGJ2	4.68E-05	1.492241762	-2.14	Down
	Methoxamine	5.11E-05	1.491698504	1.02	Up
	Adenine	5.74E-05	1.405388106	0.95	Up
	Bufotalin	6.14E-05	1.491114874	-4.74	Down
	Dehydroepiandrosterone sulfate	7.20E-05	1.490178006	-2.73	Down
	1-O-Vanilloyl-beta-D-glucose	7.36E-05	1.405248445	-3.33	Down
	Acetylphosphate	1.13E-04	1.487381967	1.2	Up
	Cerulenin	1.21E-04	1.487392674	1.12	Up
	EPA (d5)	1.22E-04	1.487316274	1.35	Up
	Nobiletin	1.38E-04	1.486226323	4.12	Up
	(6Z)-Octadecenoic acid	1.40E-04	1.400326825	1.38	Up
	Diosmetin	1.67E-04	1.484898727	1.69	Up
	Isocorypalmine	1.78E-04	1.484553771	-4.26	Down
	Azelaic acid	1.96E-04	1.398362262	-1.27	Down

Table 2 (continued)

Group	Compounds	p-value	VIP	Log ₂ FC	Regulation
YNML-F versus LW-F	4-Hydroxyphenylethanol	4.39E-08	1.42612582	0.49	Up
	Ciliatine	2.95E-07	1.433924085	− 1.67	Down
	Methyl benzoate	1.04E-06	1.433506718	− 0.19	Down
	1,3-Benzenediol	1.43E-06	1.425165841	− 1.79	Down
	(2'E,4'Z,8E)-Colneleic acid	2.62E-06	1.432820592	− 1.86	Down
	beta-D-Fructose 6-phosphate	3.00E-06	1.424360736	− 1.12	Down
	10-Deoxymethynolide	3.03E-06	1.432770798	2.69	Up
	2-trans,6-trans-Farnesal	3.63E-06	1.432391485	− 1.99	Down
	2-Aminobenzoic acid	3.79E-06	1.432475332	− 1.04	Down
	Hispidulin	3.93E-06	1.424269102	− 2.94	Down
	N-Acetyl-L-phenylalanine	4.30E-06	1.424067281	0.76	Up
	L-Homoserine	5.73E-06	1.423664446	− 1.5	Down
	Terephthalate	6.85E-06	1.423164857	1.15	Up
	Thymine	7.06E-06	1.431377083	− 1.3	Down
	Corticosterone	7.2E-06	1.431716998	− 0.91	Down
	Glycitein	9.09E-06	1.422858487	− 1.62	Down
	myo-Inositol	1.0E-05	1.422925172	− 1.2	Down
	D-Xylose	1.22E-05	1.422579334	− 1.46	Down
	4-Nitrophenol	1.39E-05	1.422142006	− 2.06	Down
	Phenylethylamine	1.54E-05	1.422095316	− 1.93	Down
YNML-F versus MD-F	(S)-Norlaudanine	4.368E-06	1.456689601	3.81	Up
	1,3-Benzenediol	5.72319E-06	1.402131725	− 2.72	Down
	Syringic acid	6.37108E-06	1.400667395	2.54	Up
	Phenylethylamine	1.16162E-05	1.399838336	− 2.16	Down
	4-Nitrophenol	2.64039E-05	1.399523036	− 3.06	Down
	Geranyl acetate	2.71976E-05	1.452589481	− 1.67	Down
	Glycitein	2.85506E-05	1.396586543	− 2	Down
	(2'E,4'Z,8E)-Colneleic acid	3.59851E-05	1.451268288	− 1.05	Down
	5-Hydroxyconiferyl alcohol	3.93229E-05	1.451062798	− 0.32	Down
	Guanosine	4.85969E-05	1.396454377	3.16	Up
	Barbituric acid	5.6295E-05	1.450388546	− 1.76	Down
	1-Arachidonoylglycerol	6.00444E-05	1.449994263	2.45	Up
	L-Homoserine	6.62397E-05	1.396373109	− 1.77	Down
	3-Dehydroshikimate	6.79548E-05	1.448765419	− 0.91	Down
	4-Hydroxy-3-methoxy-benzaldehyde	7.11288E-05	1.395392928	− 1.58	Down
	Menthyl pyrrolidone carboxylate	7.70895E-05	1.448290794	2.35	Up
	Levopimarinal	8.01856E-05	1.448831766	− 2.03	Down
	Falcarindiol	8.10348E-05	1.448817995	1.54	Up
	trans-Ferulic acid	9.45568E-05	1.394226924	− 2.4	Down
	Ribonic acid	0.000106384	1.447282978	− 1.06	Down

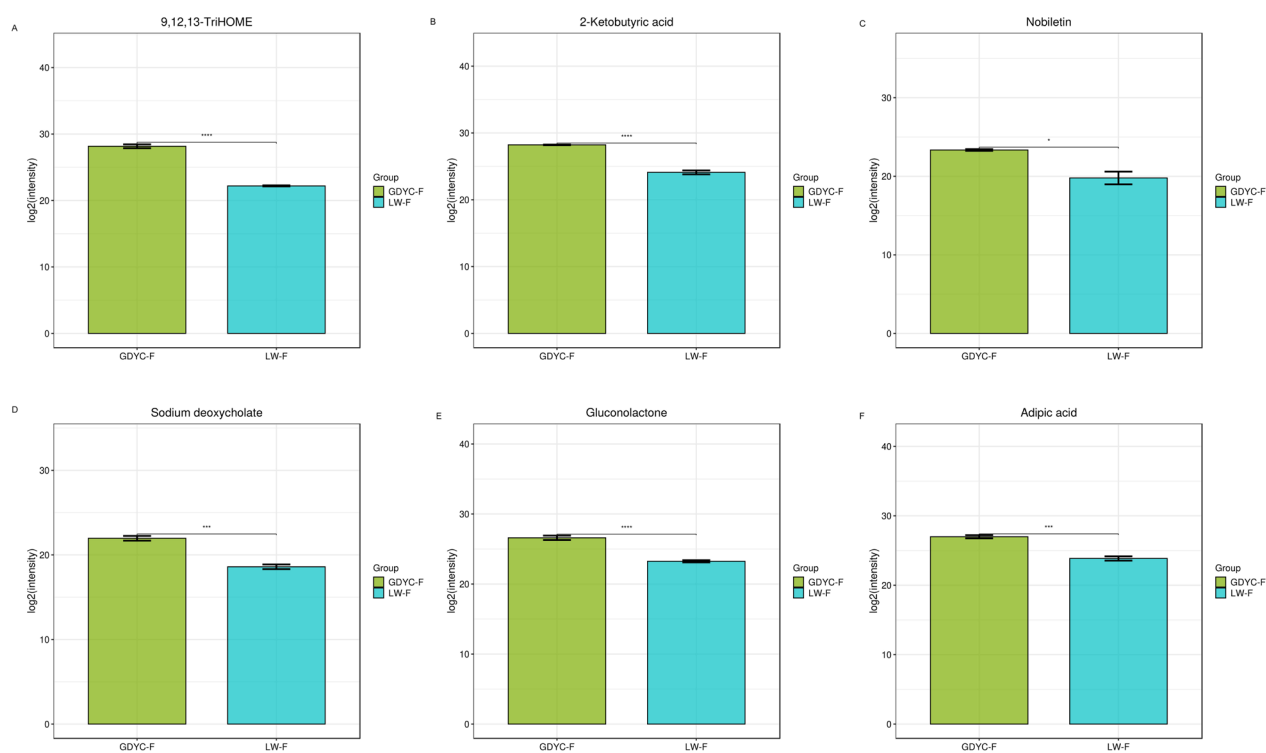


Fig. 3 Identified a biomarker cluster to distinguish samples from Guangdong-Yangchun and Laos region. **A** Coumarin, **B** 9,12,13-TriHOME, **C** nobiletin, **D** sodium deoxycholate, **E** gluconolactone, **F** adipic acid

Myanmar region. However, the content of Coumarin in the Myanmar region was significantly increased than in Yangchun region (Fig. 5).

Widely targeted metabolome analyses between YNML-F versus LW-F

To compare the different metabolites of fruits between Yunnan-Mengla and Laos, the heatmap analysis was carried out for YNML-F versus LW-F (Fig. 6A). A total of 285 differential metabolites were identified in the YNML-F versus LW-F. Among them, 114 metabolites were upregulated and 171 were downregulated (Fig. 6B). To compare the metabolites among different regions of *A. villosum*, principal component analysis (PCA) analysis was utilized on the 285 metabolites. The top 20 differentially abundant metabolites in YNML-F versus LW-F are listed in Table 2. PC1 and PC2 represented 48.6% and 15.2% of the total variation, respectively (Fig. 6C). In Fig. 6D, KEGG enrichment analysis revealed that 'Aminobenzoate degradation', 'Phenylpropanoid biosynthesis', 'Arachidonic acid metabolism' and 'Tyrosine metabolism' were significant metabolic pathways. Furthermore, the contents of ciliatine, methyl benzoate, 1,3-Benzenediol, (2'E,4'Z,8E)-Colneleic acid and beta-D-Fructose 6-phosphate were significantly increased in Laos region than in Yunnan-Mengla region. However, the content of

4-Hydroxyphenylethanol in the Yunnan-Mengla region was significantly increased than in Laos region (Fig. 7).

Widely targeted metabolome analyses between YNML-F versus MD-F

To compare the different metabolites of fruits between Yunnan-Mengla region and Myanmar region, the heatmap analysis was carried out for YNML-F versus MD-F (Fig. 8A), some metabolites of YNML-F were upregulated, but downregulated of MD-F, suggesting that *A. villosum* fruits from different regions might have different metabolites. A total of 262 differential metabolites were identified in the YNML-F versus MD-F. Among them, 114 metabolites were upregulated and 148 were downregulated (Fig. 8B). The top 20 differentially abundant metabolites in YNML-F versus MD-F are listed in Table 2. To compare the metabolites among different regions of *A. villosum*, principal component analysis (PCA) analysis was utilized on the 224 metabolites. PC1 and PC2 represented 47.1% and 16.6% of the total variation, respectively (Fig. 8C). In Fig. 8D, KEGG enrichment analysis revealed that 'Phenylpropanoid biosynthesis', 'Aminobenzoate degradation', 'Linoleic acid metabolism' and 'Tyrosine metabolism' were significant metabolic pathways. In particular, *A. villosum* in the Myanmar region had significantly higher 1,3-Benzenediol, phenylethylamine, 4-Nitrophenol and

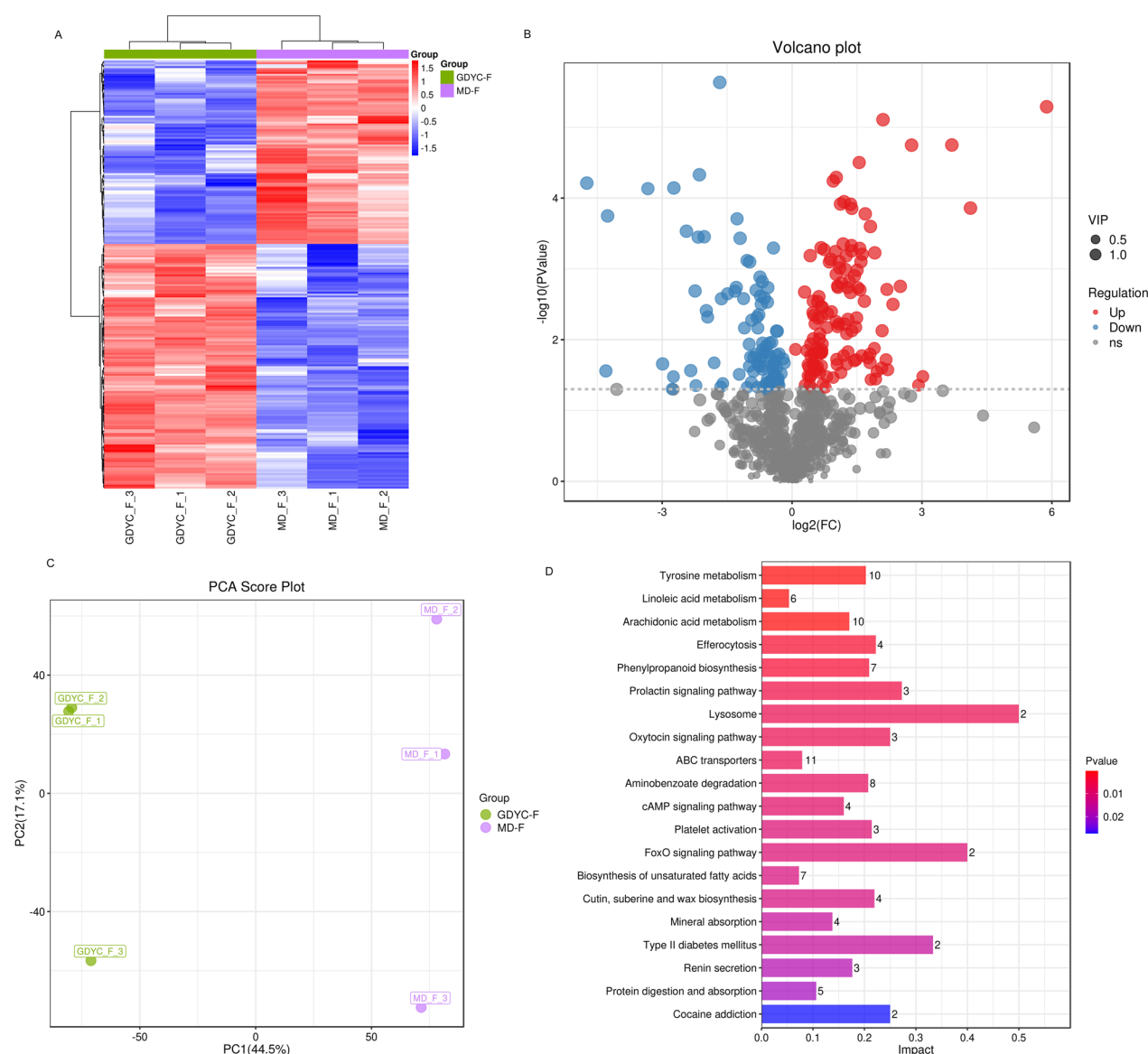


Fig. 4 Integrative metabolomic phenotypes showing cultivation-region specificity in GDYC-F versus MD-F. **A** The heatmap of differentially abundant metabolites in GDYC-F versus MD-F. **B** Metabolite profiles are visualized using volcano plots. Red dots represent differential metabolites whose relative contents were significantly higher in Yangchun than Myanmar region. Blue dots represent differential metabolites with relative contents that were significantly lower in Yangchun than Myanmar region. **C** PCA analysis of all metabolites identified in GDYC-F versus MD-F. **D** Results of KEGG pathways analysis (top 20 terms)

geranyl acetate content than those in the Yunnan-Mengla region. However, the content of (S)-Norlaudanine and syringic acid in the Yunnan-Mengla region was significantly increased than in Myanmar region (Fig. 9).

Discussion

A. villosum is essential medicine for dissipating dampness and invigorating the spleen in traditional Chinese medicine. It is also a kind of medicinal material with the same origin as medicine and food (Wei et al. 2024; Karunarathne et al. 2014). It can be used as condiment,

healthcare products, tonic diet, etc., so its annual consumption is huge (Sone et al. 2021). The fruits of *A. villosum* are the main clinical medicinal parts of *A. villosum*. Dai medical used rhizome of *A. villosum* for palpitations, irregular heartbeat, overall discomfort, weakness and loss of appetite (Cui et al. 2019). Within China’s various cultivation zones, Yangchun, situated in Guangdong Province’s southwest, is recognized as the authentic habitat for *A. villosum*, noted for its authentic and superior quality production. Nonetheless, limited research has delved into the variances in *A. villosum* populations

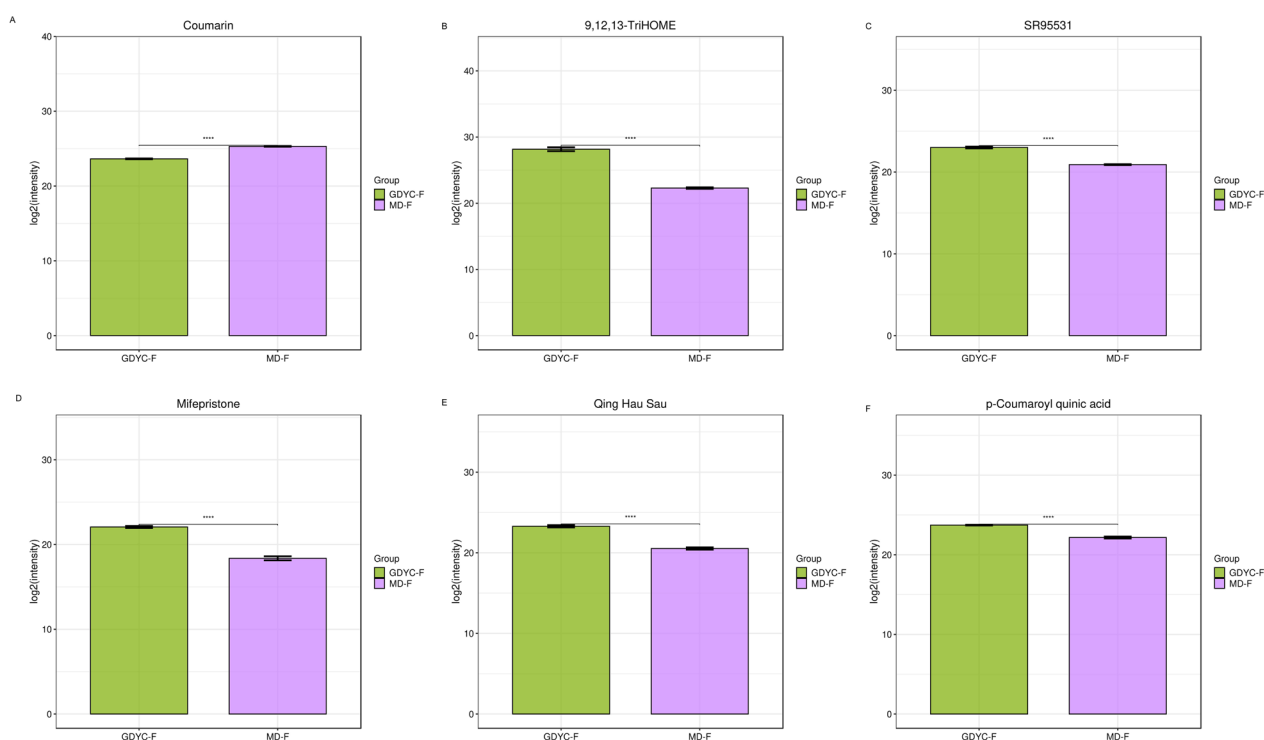


Fig. 5 Identified a biomarker cluster to distinguish samples from Guangdong-Yangchun and Myanmar region. **A** 9,12,13-TriHOME, **B** 2-Ketobutyric acid, **C** SR95531, **D** Mifepristone, **E** Qing Hau Sau, **F** p-Coumaroylquinic acid

across various production zones. The current research aimed to explore variations in the metabolites of *A. villosum* fruits across various production areas (Yangchun County, Mengla County, Phongsali province and Mongla). To begin our research, we obtained the metabolic profiles of *A. villosum* fruit samples grown in four key production areas using LC-MS/MS. In addition, we investigated potential biomarker compounds that could differentiate samples from various regions and discovered metabolites specific to each production location. As a result, 260 differential metabolites were identified in the GDYC-F versus LW-F. Among them, the content of 121 metabolites in Yangchun was significantly higher than that in Laos. Likewise, 139 metabolites in Yangchun were significantly decreased than in Laos. A total of 224 differential metabolites were identified in the GDYC-F versus MD-F. Among them, 128 metabolites were upregulated and 96 were downregulated. A total of 285 differential metabolites were identified in the YNML-F versus LW-F. Among them, 114 metabolites were upregulated and 171 were downregulated. A total of 262 differential metabolites were identified in the YNML-F versus MD-F. Among them, 114 metabolites were upregulated and 148 were downregulated.

This study's findings enhance our overall comprehension of the genuine geographical origins of *A. villosum*.

Metabolic characteristics of *A. villosum* fruits grown in four distinct areas were examined to distinguish their origins of cultivation. In addition, we have identified a group of biomarkers that can be used to differentiate samples from various regions, including 9,12,13-TriHOME, 2-Ketobutyric acid, alpha-linolenic acid, nobiletin, sodium deoxycholate, gluconolactone and adipic acid, mifepristone, Qing Hau Sau and p-Coumaroylquinic acid, coumarin, ciliatine, methyl benzoate, 1,3-Benzenediol, (2'E,4'Z,8E)-Colneleic acid and beta-D-Fructose 6-phosphate, phenylethylamine, 4-Nitrophenol and geranyl acetate. Some of these compounds are linked to biological activity and therapeutic benefits. For instance, the antifungal mechanism of 2-ketobutyric acid extracted from tea flowers was further investigated by RNA-seq (Chen et al. 2023).

Guangdong-Yangchun Region: The prominent metabolites of *A. villosum* fruits from Yangchun City were mainly related to anti-inflammatory effects, such as the notably elevated levels of 2-Ketobutyric acid, alpha-linolenic acid, nobiletin, sodium deoxycholate, gluconolactone and adipic acid. This aligns with the therapeutic properties of *A. villosum* and could indicate the geographical authenticity of Yangchun City in cultivating authentic and premium *A. villosum*. In addition, according to reports, the quality of *A. villosum* is related to precipitation (Lu et al. 2017). Alpha-linolenic acid may decrease the risk

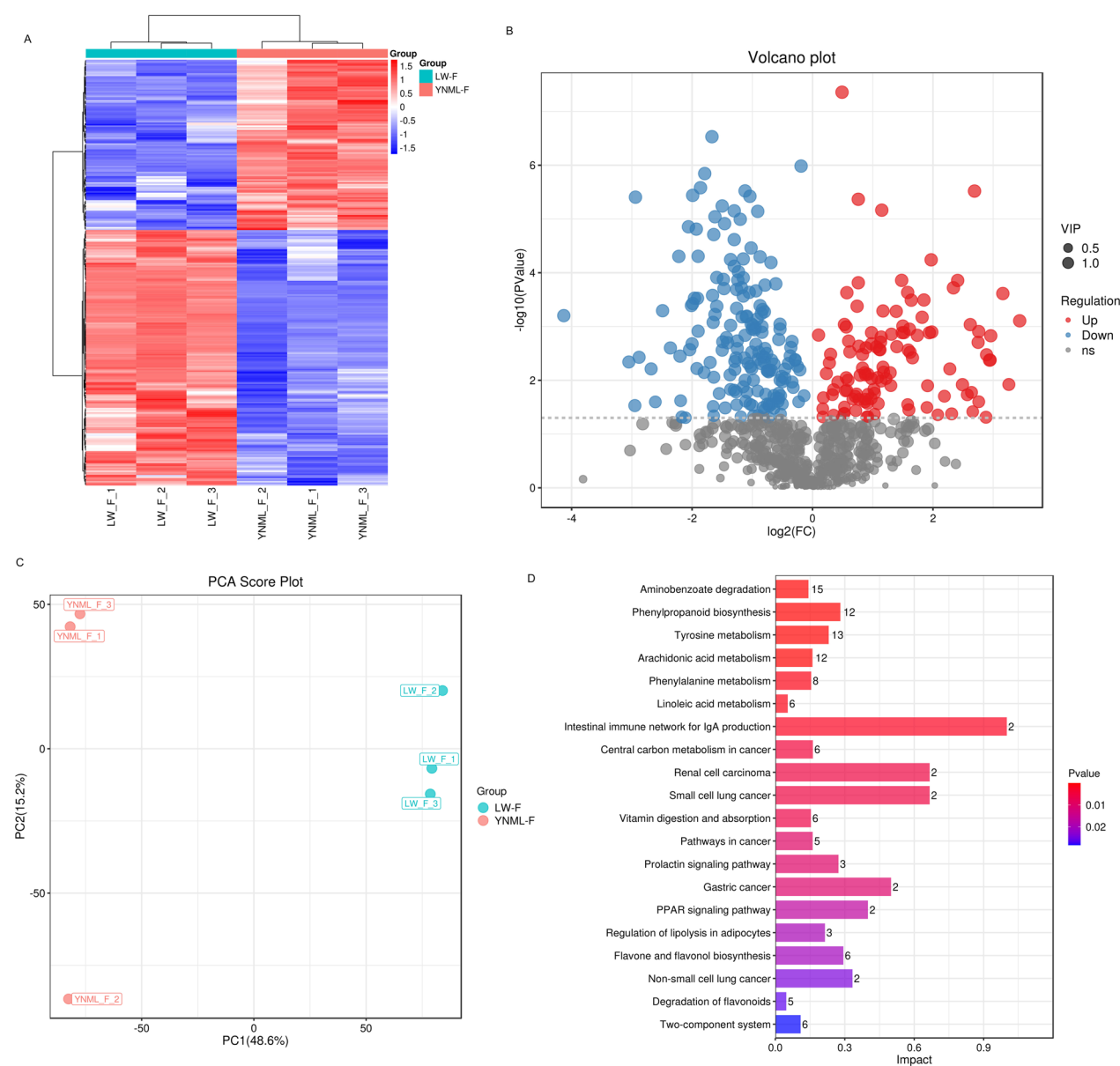


Fig. 6 Integrative metabolomic phenotypes showing cultivation-region specificity in YNML-F versus LW-F. **A** The heatmap of differentially abundant metabolites in YNML-F versus LW-F. **B** Metabolite profiles are visualized using volcano plots. Red dots represent differential metabolites whose relative contents were significantly higher in Mengla than Laos region. Blue dots represent differential metabolites with relative contents that were significantly lower in Mengla than Laos region. **C** PCA analysis of all metabolites identified in YNML-F versus LW-F. **D** Results of KEGG pathways analysis (top 20 terms)

of stroke and coronary heart disease by preventing the development of atherosclerosis and enhancing cognitive function in the brain. Alpha-Linoleic acid exhibits positive therapeutic benefits for both obesity and non-alcoholic fatty liver disease (Rajna et al. 2018). Studies have shown that supplementing mice with Alpha-Linoleic acid can greatly decrease their obesity rate, and human trials have demonstrated that individuals who were given linolenic acid experienced a reduction in body fat and weight. Alpha-linolenic acid has a good therapeutic effect

on tumors (Blondeau 2016; Naghshi et al. 2021). In our results, *A. villosum* in the Yangchun region had significantly higher alpha-linolenic acid content than those in the Laos region. Our results also indicated that ‘Linoleic acid metabolism pathway’ was significantly enriched in GDYC-F versus LW-F, GDYC-F versus MD-F and YNML-F versus MD-F comparisons. *A. villosum* in the Yangchun area exhibited notably elevated levels of alpha-linolenic acid compared to specimens in the Laos region in our findings. In most reported polymethoxyflavones

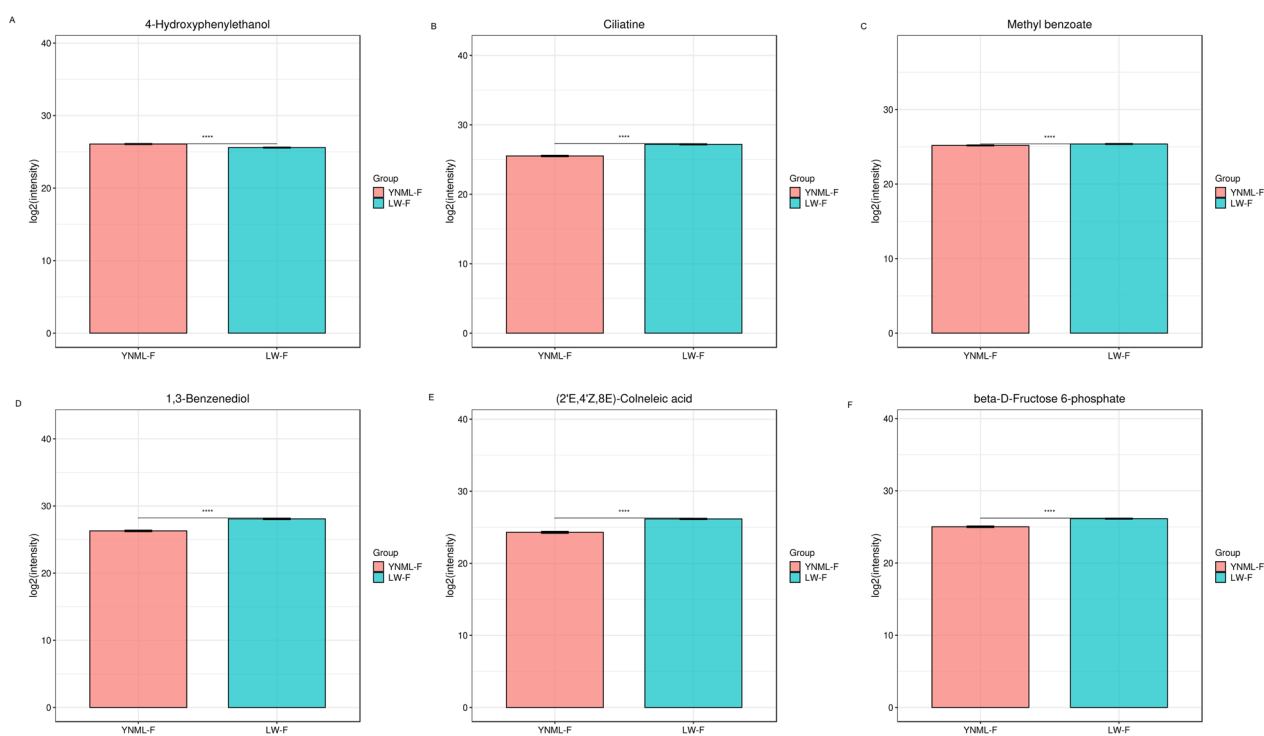


Fig. 7 Identified a biomarker cluster to distinguish samples from Yunnan-Mengla and Laos region. **A** 4-Hydroxyphenylethanol, **B** ciliatine, **C** methyl benzoate, **D** 1,3-Benzenediol, **E** (2'E,4'Z,8E)-Colneleic acid, **F** beta-D-Fructose 6-phosphate

(PMFs) containing citrus peel extracts (CPEs), nobiletin is widely present and abundant in content (Li et al. 2006). With recent identification and availability of sources, metabolites of nobiletin have also been found to have many important biological properties, such as anti-inflammatory, anti-tumor and neurotrophic activity, and typically have stronger pharmacological activity than their parent compound, nobiletin (Li et al. 2007). *A. villosum* in the Yangchun area exhibited notably elevated levels of nobiletin compared to specimens in the Laos area in our findings. A previous investigation revealed that the latitude had the strongest association with *A. villosum* fruit metabolites among environmental factors (Li et al. 2024). In addition, there was a strong correlation between latitude and the proportions of metabolites specific to different regions in the GDYC-F versus LW-F and YNML-F versus LW-F groups. Previous research has also shown changes in metabolite levels along latitudinal gradients (Demasi et al. 2018; Lee et al. 2021). Different climatic environments and soil conditions lead to significant differences in the quality of *A. villosum*, thus forming the concept of authentic medicinal materials with strong regional characteristics and outstanding therapeutic effects in clinical practice (Li et al. 2020). Yangchun City has the highest precipitation among other production areas, potentially contributing to its high concentration of anti-inflammatory metabolites (high-quality). It is worth noting that multiple environmental variables have

a greater impact on the metabolites of *A. villosum* fruit than a single environmental variable. Therefore, multiple environmental variables should be comprehensively considered to evaluate the habitat suitability and the introduction of high-quality *A. villosum*.

Yunnan-Mengla region: The prominent metabolites of *A. villosum* fruits from Laos were (S)-Norlaudanine and Syringic acid, which was significantly increased than in Myanmar region. The natural phenolic substance, syringic acid, exists in the majority of plant varieties and certain fungal species (Genger et al. 2008; Horvat et al. 2020). Syringic acid has the potential to elevate levels of antioxidants and peroxidation, serving as a remedy for oxidative stress (Cotoras et al. 2014). Syringic acid safeguards cells in plants, animals and humans from lipid and protein oxidation, inflammation, heart toxicity, protein carbonylation, osteoporosis, neurological harm and oxidative stress, while also serving as a preventative agent against diabetes and ischemia reperfusion (Tanaka et al. 2019).

Phongsali province-Laos region: The prominent metabolites of *A. villosum* fruits from Laos were Ciliatine, Methyl benzoate, 1,3-Benzenediol, (2'E,4'Z,8E)-Colneleic acid and beta-D-Fructose 6-phosphate, which were significantly increased in Laos region than in Yunnan-Mengla region. Polysaccharides are important quality markers in many medicinal plants and have significant pharmacological effects in immune regulation and anti-tumor (Zhou et al. 2022).

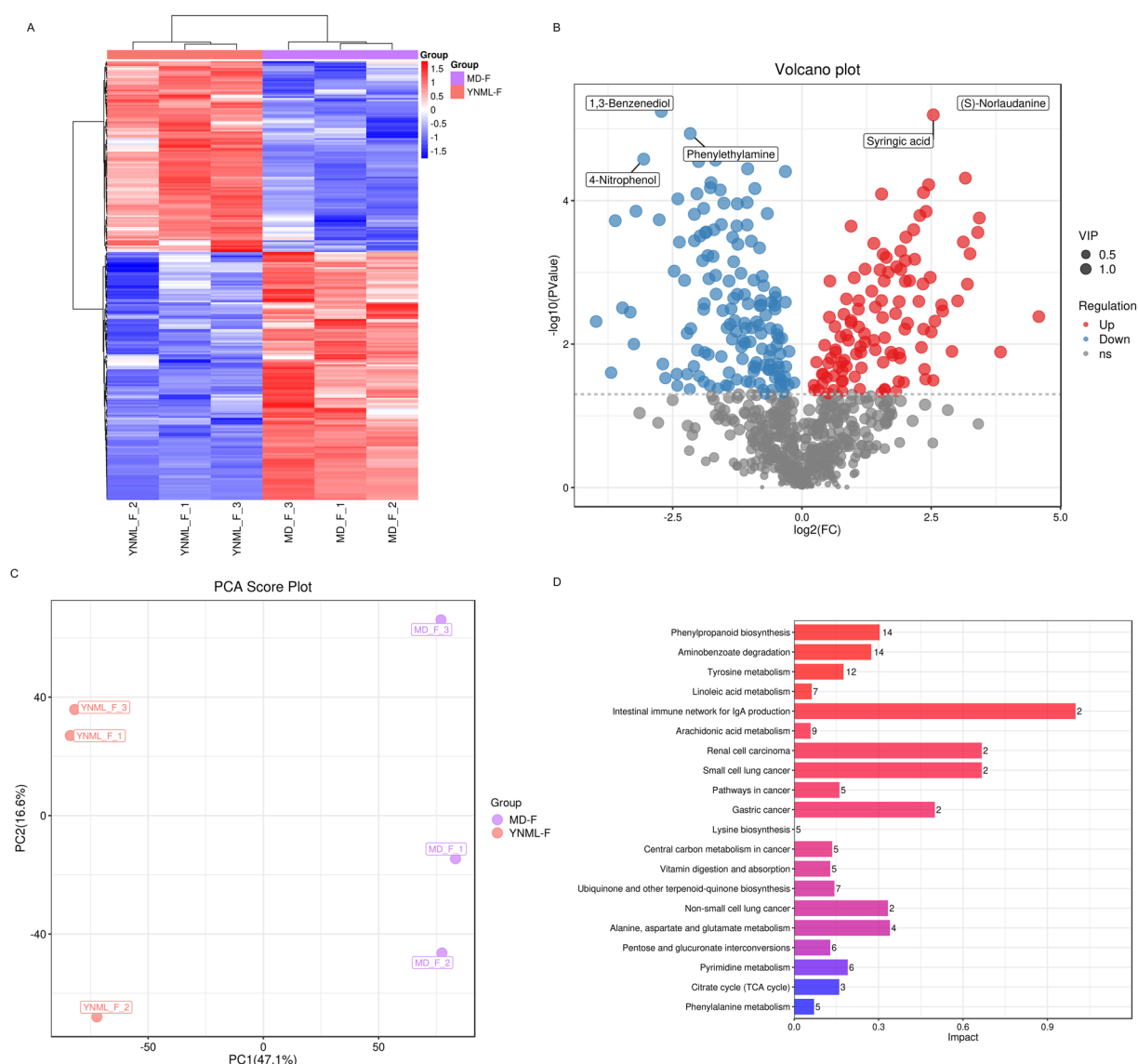


Fig. 8 Integrative metabolomic phenotypes showing cultivation-region specificity in YNML-F versus MD-F. **A** The heatmap of differentially abundant metabolites in YNML-F versus MD-F. **B** Metabolite profiles are visualized using volcano plots. Red dots represent differential metabolites whose relative contents were significantly higher in Mengla than Myanmar region. Blue dots represent differential metabolites with relative contents that were significantly lower in Mengla than Myanmar region. **C** PCA analysis of all metabolites identified in YNML-F versus MD-F. **D** Results of KEGG pathways analysis (top 20 terms)

Myanmar-Mongla region: The prominent metabolites of *A. villosum* fruits from Myanmar region were 1,3-Benzenediol, phenylethylamine, 4-Nitrophenol and geranyl acetate content than those in the Yunnan-Mengla region. Phenylethylamine can enhance plant tolerance to various adversities (such as drought and salinity), thereby improving plant viability and adaptability (Guo et al. 2018). Although the specific mechanism is not completely clear, studies have shown that phenylethylamine may promote plant growth and development by affecting the synthesis and signaling of plant hormones. This helps plants cope better

with environmental changes and increase their yield and quality (Horbowicz et al. 2015). Phenylethylamine may enhance the immune system of plants, help plants resist the invasion of pathogens, reduce the occurrence of diseases and protect the healthy growth of plants (Niehl et al. 2006).

However, this study has potential limitations. Firstly, fruit samples of *A. villosum* were collected over a year. Environmental changes affect the productivity of plants (Borghi et al. 2019). Due to climate change, there may be differences in the metabolite profile of *A. villosum* fruits harvested in different years. To draw a clear conclusion, a

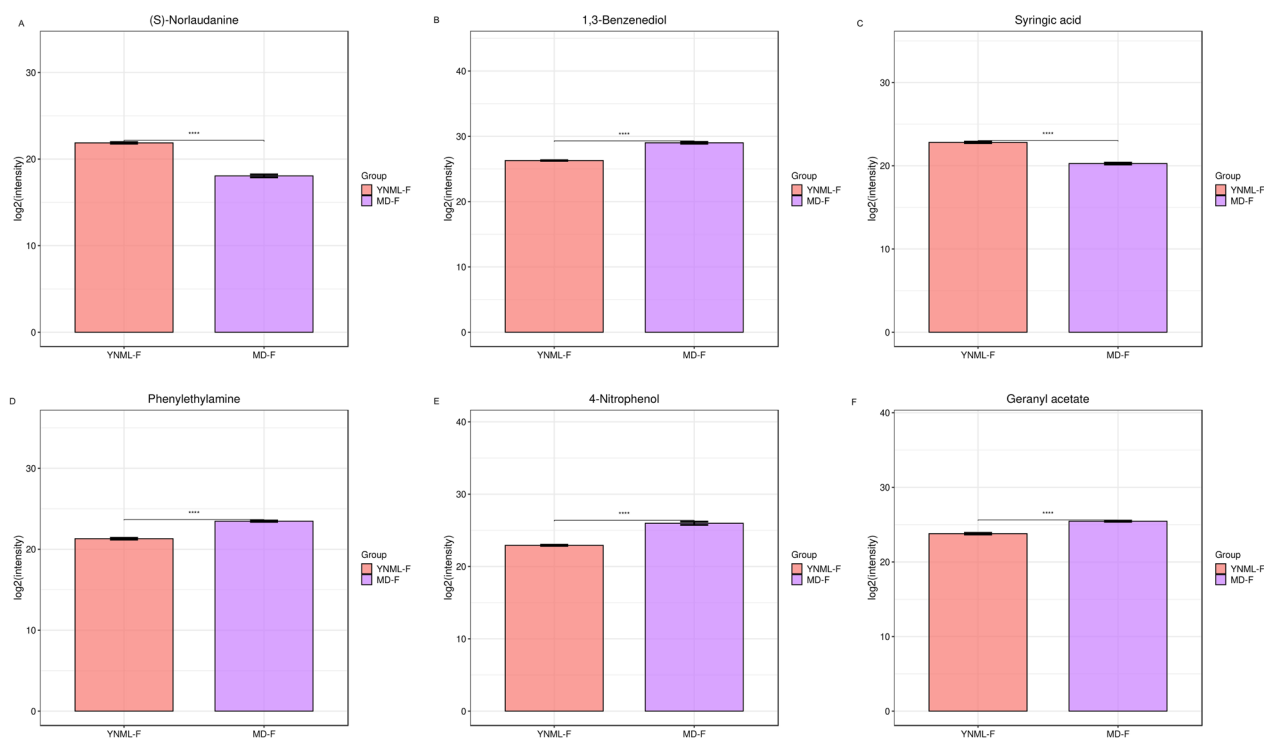


Fig. 9 Identified a biomarker cluster to distinguish samples from Yunnan-Mengla and Myanmar region. **A** (S)-Norlaudanine, **B** 1,3-Benzenediol, **C** syringic acid, **D** 1,3-Benzenediol, **E** 4-Nitrophenol, **F** geranyl acetate

long-term investigation is necessary. Secondly, although we investigated representative regions of China and foreign countries producing *A. villosum* (Guangdong, Yunnan, Laos and Myanmar), the number of sampling points is relatively small. Future research should use more sampling points to validate the results of this study.

Conclusion

This study examined variations in the metabolites of *A. villosum* samples from four distinct regions and discovered unique metabolites specific to each region through the use of LC–MS/MS and multivariate statistical methods. As a result, we have identified a group of biomarkers that can be used to differentiate samples from various regions, including 9,12,13-TriHOME, 2-Ketobutyric acid, alpha-linolenic acid, nobiletin, sodium deoxycholate, gluconolactone and adipic acid, mifepristone, Qing Hau Sau and p-Coumaroylquinic acid, coumarin, ciliatine, methyl benzoate, 1,3-Benzenediol, (2'E,4'Z,8E)-Colneleic acid and beta-D-Fructose 6-phosphate, phenylethylamine, 4-Nitrophenol and geranyl acetate. The research uncovered metabolic variances in *A. villosum* fruits across various countries' geo-authentic (Yangchun) and non-authentic production zones, providing a scientific foundation for evaluating habitat appropriateness and introducing superior *A. villosum*.

Materials and methods

Sample collection

Amomum villosum Lour. fruit samples were collected from Guangdong (Yangchun County), Yunnan (Mengla County), Laos (Phongsali province) and Myanmar (Mongla) in 2023 (March) (Fig. 1). Samples were authenticated by Professor ZHANG Lixia (Yunnan Branch, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, China). Detailed information of all samples collection regions is listed in Table 1.

Metabolome analysis

A broadly applied metabolomics technique was utilized to analyze the metabolites in both the control and treatment cohorts. Metabolite extraction, detection, identification and quantification were carried out in the Wuhan MetWare Biotechnology Co., Ltd. based on the methods described by Chen et al. (2013). In short, a 100 mg tissue sample was pulverized with liquid nitrogen and then extracted using 70% methanol solution. The specimen samples underwent analysis with an LC–ESI–MS/MS system.

The ESI source was set with a source temperature of 500 °C, Ion spray voltage of 5500 V in positive ion mode and –4500 V in negative ion mode, gas I (GSI) at 50 psi, gas II (GSII) at 60 psi, curtain gas (CUR) at 25 psi and high collision activation dissociation (CAD). The QQQ scan was a medium collision gas (nitrogen) MRM

experiment. Maximize DP (grouping capability) and CE (impact energy) for a singular MRM shift. Track a particular group of MRM changes for every interval using the metabolites that were separated during that time frame.

Data preprocessing

Initially, the unprocessed data were transformed into mzXML format using MSConvert within the ProteoWizard software package (v3.0.8789) (Rasmussen et al. 2022). Subsequently, the data were analyzed for feature detection, retention time correction and alignment using R XCMS (v3.12.0) (Navarro-Reig et al. 2015), retention time correction and alignment. Key parameters settings were set as follows: ppm=15, peakwidth=c (5, 30), mzdif=0.01, method=centWave. Eliminating the batch effect involved data correction using QC samples. Metabolites with a relative standard deviation greater than 30% in quality control samples were excluded before being utilized for further data analysis.

Metabolites were detected using accurate mass and MS/MS data, then compared with various databases including HMDB (<http://www.hmdb.ca>) (Wishart et al. 2007), MassBank (<http://www.massbank.jp/>) (Horai et al. 2010), KEGG (<https://www.genome.jp/kegg/>) (Kanehisa and Goto 2000), LIPID MAPS (<http://www.lipidmaps.org>) (Sud et al. 2007), mzCloud (<https://www.mzcloud.org>) (Abdelrazig et al. 2020) and a metabolite database created by Panomix Biomedical Tech Co., Ltd. in Suzhou, China. Metabolite molecular weights were calculated based on the mass-to-charge ratio (m/z) of the precursor ions in mass spectrometry data. The molecular formula was determined using ppm and adduct ion, and subsequently compared to the database for metabolite identification through MS. Simultaneously, the MS/MS data from a quantitative table were compared with the fragment ions and additional information of each metabolite in the database in order to achieve the identification of metabolites through MS/MS (Table 2).

Pathway analysis

MetaboAnalyst was used to analyze differential metabolites through a combination of pathway enrichment analysis and pathway topology analysis (Xia and Wishart 2011). The metabolites that were recognized in metabolomics were subsequently correlated with the KEGG pathway to analyze the biological implications of broader systemic functions. The KEGG Mapper tool was utilized to visualize the metabolites and their associated pathways.

Data analysis

Two distinct multivariate statistical analysis techniques, unsupervised and supervised, were utilized to differentiate the groups using the R ropls (v1.22.0) package (Thévenot et al. 2015). The statistical test between

groups yielded the statistical significance of the *P*-value. Ultimately, biomarker metabolites were identified by combining *P*-value, VIP (importance of variables in OPLS-DA) and FC (fold change between groups). Metabolites were deemed to have significant differential expression if the *P*-value was less than 0.05 and the VIP value exceeded 1 by default.

Acknowledgements

None.

Author contributions

Z.D. C.G. and X.L. designed the experiments. J.Y. and P.S. performed all experiments. D.N. and Y.C. analyzed the data and wrote the manuscript. X.Y. and Y.C. revised the manuscript. All authors reviewed the manuscript.

Funding

West Yunnan University of Applied Sciences University-level Engineering Research Center projects :Characteristic Dai-Medicine Resource ERC of West Yunnan University of Applied Science (2022KYPT0004). National Natural Science Foundation of China: Study on the symbiotic system of Sharen (*Fructus Amomi*) and weevil pollination and its "push-pull" pollination mechanism (82260736).

Availability of data and materials

Data are provided within the manuscript or supplementary information files.

Declarations

Competing interests

The authors declare that they have no conflict of interest.

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Received: 25 December 2024 Accepted: 11 March 2025

Published: 2 April 2025

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