韓藥作誌(Korean J. Medicinal Crop Sci.) 34(5) : 397 – 404 (2026)
ISSN(Print) 1225-9306
ISSN(Online) 2288-0186
http://www.medicinalcrop.org
http://dx.doi.org/10.7783/KJMCS.2026.34.5.397

^{1}H NMR 기반 하늘타리 뿌리의 일차대사체 프로파일링 및 암수 간 탐색적 비교

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^{1}H NMR-Based Primary Metabolite Profiling of *Trichosanthes kirilowii* Roots and Exploratory Comparison between Male and Female Plants

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Received: 2026 September 30
1st Revised: 2026 October 12
2nd Revised: 2026 October 16
3rd Revised: 2026 October 20
Accepted: 2026 October 20
Published: 2026 October 30

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ABSTRACT

Background: *Trichosanthes kirilowii* Maxim. is a dioecious plant whose roots are widely used in traditional medicine. Previous phytochemical studies have focused on secondary metabolites, whereas comprehensive profiling of primary metabolites in its roots, including possible sex-related differences, remains limited.

Methods and Results: Root samples from two female and two male plants were extracted in triplicate and analyzed using 600 MHz ^{1}H nuclear magnetic resonance spectroscopy. Forty-nine endogenous metabolites were identified and quantified, with citrulline, fructose, and glucose being the most abundant. Principal component analysis revealed clear differences among individual plants, whereas hierarchical cluster analysis grouped the samples by sex. Ten metabolites showed at least 1.5-fold differences in abundance between the sexes. Six were more abundant in males, including tryptophan, asparagine, and sarcosine, whereas four were more abundant in females: lactate, niacinamide, ornithine, and trehalose.

Conclusions: Despite the small number of plants analyzed per sex, this study provides a profile of primary metabolites in *T. kirilowii* roots and suggests sex-associated differences, particularly in amino acid and organic acid composition.

Key Words: *Trichosanthes kirilowii* Maxim., Dioecious Plant, Metabolomics, ^{1}H NMR, Primary Metabolites

INTRODUCTION

Trichosanthes kirilowii Maxim., a perennial climbing plant belonging to the family Cucurbitaceae, has long been used as a medicinal resource in East Asia. Various plant parts, including the root, fruit, and seed, have been utilized for medicinal purposes, and the root is traditionally used as Trichosanthis Radix (Ji *et al*., 2026). Previous phytochemical and metabolomic studies have demonstrated that *T. kirilowii* contains diverse primary and secondary metabolites, including amino acids, carbohydrates, nucleosides, flavonoids, phenolic compounds, and cucurbitane-type triterpenoids (Zhang *et al*., 2019). Recent multi-omics profiling has further demonstrated tissue-specific differences in the accumulation of both primary and secondary metabolites in *T. kirilowii*, with roots containing abundant amino acids, sugars, and several triterpenoids (Gao *et al*., 2026).

T. kirilowii is a dioecious species in which male and female flowers are produced on separate individuals. Accordingly, sex-

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related differences in this species have increasingly been investigated at morphological, physiological, transcriptomic, and post-transcriptional levels. Transcriptome analysis of male and female flower buds revealed extensive differential gene expression associated with reproductive development, hormone signaling, and carbohydrate metabolism (Hu *et al*., 2020), while subsequent miRNA profiling identified numerous sex-associated regulatory miRNAs and target genes (Hu *et al*., 2023). Moreover, chemical differences between female and male floral tissues have also been reported in the related species *Trichosanthes anguina*, in which volatile profiles differed significantly between sexes (Song *et al*., 2022). In other dioecious medicinal plants, sex-related differences have been reported not only in aerial tissues but also in roots. For example, root metabolomes and the accumulation of sinomenine differed between sexes in *Sinomenium acutum* (Luo *et al*., 2025). Despite this growing interest in sex-associated variation, whether such differences extend to the primary metabolite composition of *T. kirilowii* roots remains unclear.

Metabolomics provides a useful approach for characterizing biochemical variation associated with biological and environmental factors, and has been applied to the classification and quality-related evaluation of medicinal crops (Yang *et al*., 2014; Jiang and Leem, 2016). Previous studies have also shown that developmental stage and cultivation environment can substantially influence amino acid and major metabolite composition in medicinal crops (Im *et al*., 2021; Jeong *et al*., 2025). ^{1}H nuclear magnetic resonance (NMR) spectroscopy is particularly suitable for comparative profiling of plant primary metabolism because of its high reproducibility, quantitative capability, and ability to detect a broad range of low-molecular-weight metabolites with minimal sample preparation (Schripsema, 2010; Mascellani Bergo *et al*., 2024). To our knowledge, comprehensive ^{1}H NMR-based comparison of male and female *T. kirilowii* roots has not been reported. Therefore, the present study aimed to profile the primary metabolites of *T. kirilowii* roots by ^{1}H NMR and to make an exploratory comparison of their concentrations between male and female plants.

MATERIALS AND METHODS

1. Plant materials

T. kirilowii Maxim. plants were cultivated in an open field at Hamyang, Gyeongsangnam-do, Korea, for one year under natural

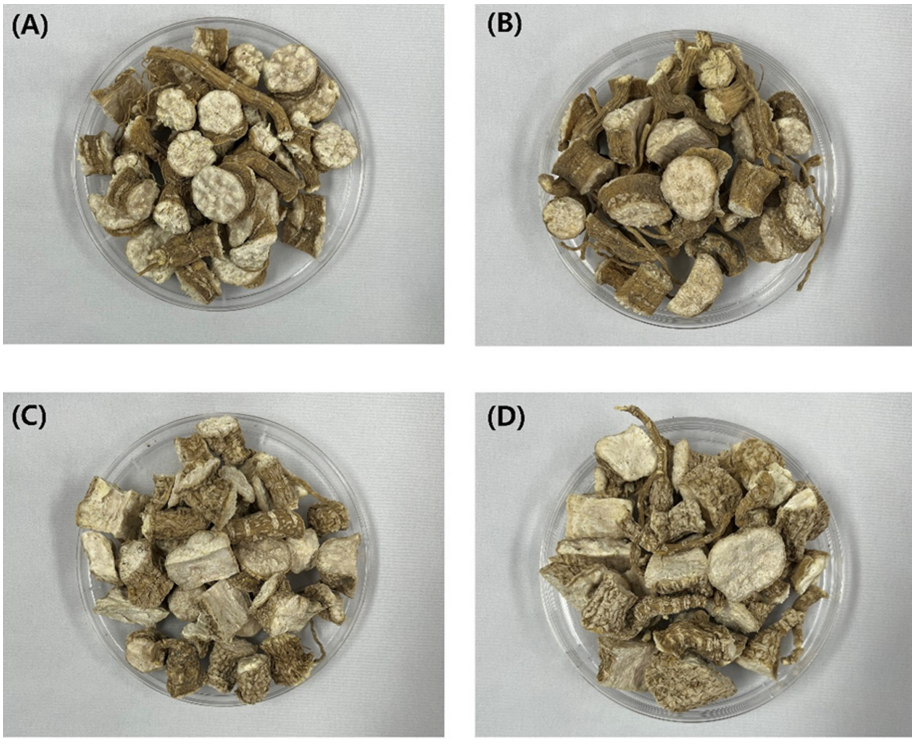


Fig. 1. Roots of *T. kirilowii*. (A, B) Female plant; (C, D) Male plant.

environmental conditions. Plant sex was determined based on floral characteristics after flowering, and roots were then collected from four individual plants, two females and two males (Fig. 1). Whole roots were dried, cut into pieces, and stored until analysis. Each root was homogenized using a Retsch MM400 mixer mill (Retsch GmbH, Haan, Germany) and three 100 mg aliquots of the powder were extracted and analyzed separately as technical replicates, giving a total of 12 samples for NMR analysis.

2. Sample preparation and extraction

An aliquot of 100 mg of each powdered sample was extracted with 30% methanol (v/v) by ultrasonication at room temperature for 30 min. The resulting suspensions were centrifuged at 13,000 × g for 10 min at 4°C, and the supernatants were collected and filtered through a 0.22 μm polyvinylidene fluoride (PVDF) syringe filter. The filtered extracts were dried using a centrifugal evaporator (CVE-3100, EYELA, Tokyo, Japan), followed by freeze-drying using a freeze dryer (FDU-1200, EYELA, Tokyo, Japan).

The dried residues were reconstituted in 0.1 M sodium phosphate buffer (pH 7.5) prepared in deuterium oxide (D_2O) containing 2 mM 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4). TSP-d_4 was used as an internal standard for chemical shift calibration (δ 0.00 ppm) and metabolite quantification. The resulting solutions were transferred into 5 mm NMR tubes for NMR measurements.

3. ^{1}H NMR analysis

^{1}H NMR spectra were acquired using a Bruker Avance 600 spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a cryogenic triple-resonance probe, operating at a ^{1}H frequency of 600.20 MHz and a temperature of 298 K. One-dimensional (1D) spectra were recorded using a nuclear Overhauser effect spectroscopy (NOESY)-presaturation pulse sequence (noesygppr1d) with 64 scans and 32,768 acquired data points over a spectral width of 12,500 Hz. The acquisition time and relaxation delay were set to 2.6 and 4.0 s, respectively.

Manual phase and baseline corrections were applied using TOPSPIN software (version 4.1.0; Bruker Biospin). Metabolite identification and quantification were performed using Chenomx NMR Suite 8.4 Professional (Chenomx Inc., Edmonton, AB, Canada). A metabolite was assigned only when all of its characteristic resonances in the library matched the observed signals in chemical shift, multiplicity, and coupling pattern. Each identified metabolite was quantified by fitting its reference spectral profile to the corresponding signals, and its absolute concentration (mM) was calculated relative to the TSP-d_4 (2 mM). Metabolite concentrations obtained by Chenomx profiling were averaged over the three replicates of each plant.

4. Data analysis

For multivariate statistical analysis, ^{1}H NMR spectra were divided into 0.04 ppm spectral bins over the range of 0.5–10.0 ppm, excluding the residual water region. The binned spectral data were normalized to the total spectral area and Pareto-scaled prior to analysis.

Principal component analysis (PCA) and hierarchical cluster analysis (HCA, Ward's method) were performed as unsupervised, exploratory approaches using SIMCA version 15 (Sartorius Stedim Data Analytics AB, Umeå, Sweden) to visualize overall patterns of variation among the plants and their replicates.

For each metabolite, the $\log_2$ fold change ($\log_2$FC) was calculated as $\log_2$-transformed ratio of the mean concentration of the two female plants to that of the two male plants (positive, higher in females; negative, higher in males). Metabolites with a fold change of $\geq$ 1.5 in either direction (i.e., $|\log_2 FC| \geq 0.585$), in which both plants of one sex were higher than both plants of the other sex, were regarded as notably different between the sexes. Because only two independent biological replicates (plants) were available per sex, no statistical tests were applied.

RESULTS

1. ^{1}H NMR-based primary metabolite profiling of *T. kirilowii* roots

Representative ^{1}H NMR spectra of *T. kirilowii* root extracts are shown in Fig. 2. The spectra exhibited numerous resonances across the aliphatic, carbohydrate, and aromatic regions, reflecting the diverse primary metabolite composition of the roots. A total of 49 endogenous metabolites were identified, including 14 amino acids, nine amino acid-related metabolites, nine organic acids, eight sugars and sugar-related metabolites, four nucleosides and related compounds, two nitrogen-containing compounds, and three other metabolites (Table 1).

Among the identified metabolites, citrulline was one of the most abundant components in both female and male roots, together with fructose and glucose. Arginine and citrate were also present at relatively high levels in both groups. Other

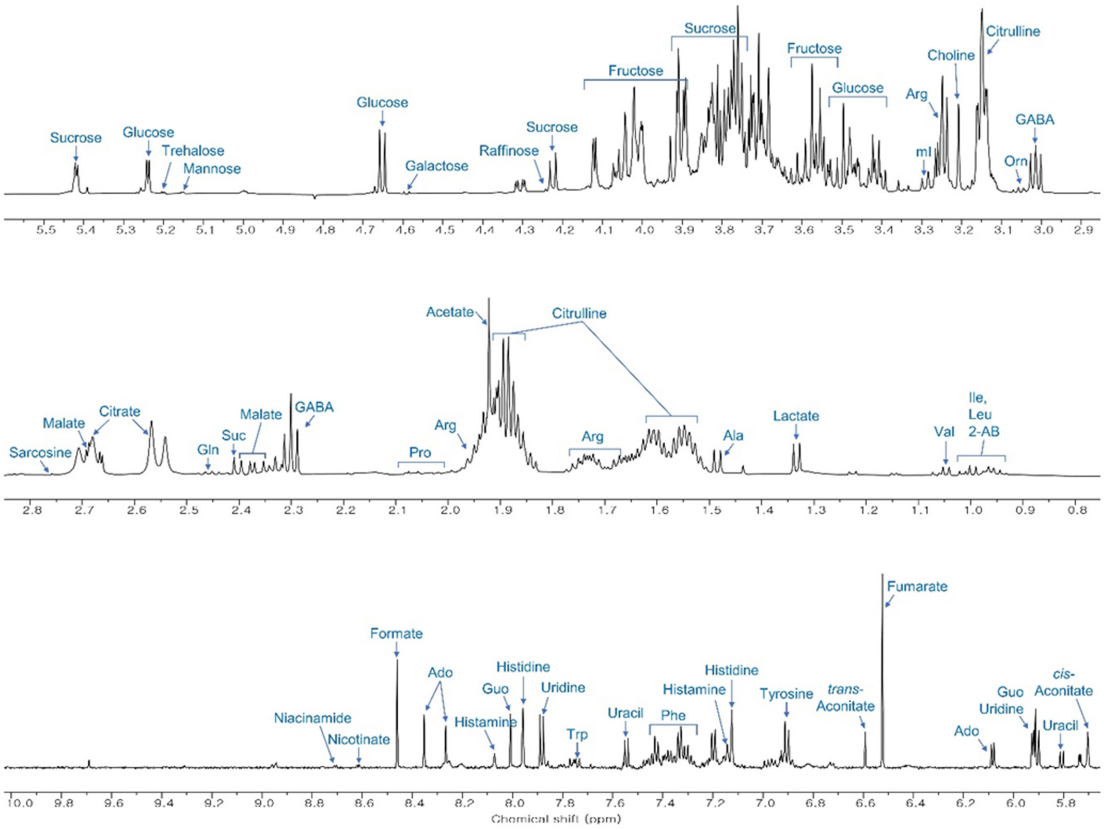


Fig. 2. Representative ^{1}H NMR spectrum of *T. kirilowii* root extract with assignments of major metabolites. 2-AB, 2-aminobutyrate; Ala, alanine; Ado, adenosine; Arg, arginine; GABA, 4-aminobutyrate; Gln, glutamine; Guo, guanosine; Ile, isoleucine; Leu, leucine; mI, myo-inositol; Orn, ornithine; Pro, proline; Suc, succinate; Trp, tryptophan.

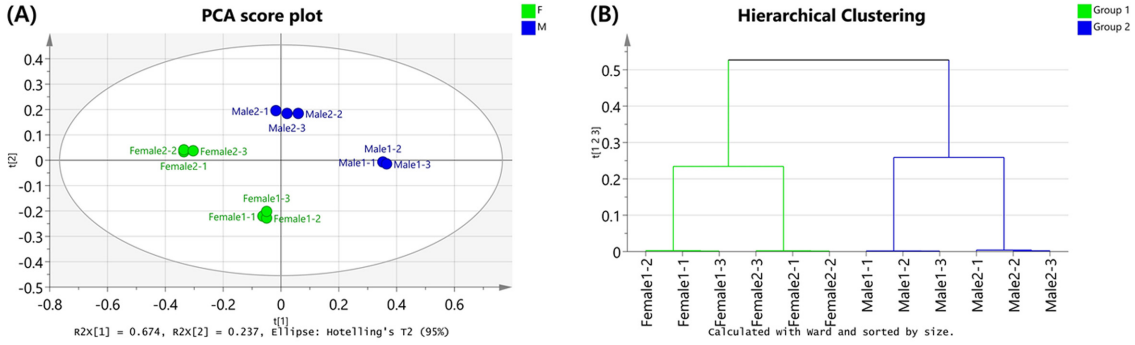


Fig. 3. Multivariate statistical analysis of NMR spectral data from *T. kirilowii* roots. (A) PCA score plot; (B) Hierarchical clustering analysis; F, female; M, male.

comparatively abundant metabolites included sucrose, 4-aminobutyrate (GABA), malate, and acetate. Overall, *T. kirilowii* roots were characterized by relatively high levels of citrulline and soluble sugars, together with substantial amounts of amino acids and organic acids.

2. Multivariate statistical analysis of root metabolomes

PCA was performed to evaluate overall variation in the ^{1}H NMR spectral profiles of *T. kirilowii* roots (Fig. 3A). PC1 and PC2 accounted for 67.4% and 23.7% of the total variance, respectively. The three replicates of each plant clustered closely together, whereas the four plants were clearly separated from

Table 1. Concentrations of the 49 metabolites identified in the roots of *T. kirilowii*.

Metabolites (mM)	Female 1	Female 2	Male 1	Male 2	log_2FC	≥1.5-fold difference
Amino acid-related metabolites						
2-Aminobutyrate	0.040	0.052	0.067	0.076	–0.64	M > F
4-Aminobutyrate	3.084	2.685	2.263	2.436	0.30	–
Citrulline	16.434	19.394	11.830	15.965	0.37	–
Histamine	0.059	0.076	0.082	0.125	–0.61	M > F
N-Phenylacetylglycine	0.029	0.021	0.028	0.027	–0.12	–
O-Acetylcarnitine	0.029	0.040	0.045	0.047	–0.41	–
Ornithine	0.298	0.415	0.193	0.278	0.60	F > M
Pyroglutamate	0.120	0.234	0.244	0.307	–0.64	M > F
Sarcosine	0.024	0.043	0.055	0.074	–0.95	M > F
Amino acids						
Alanine	0.594	1.118	0.763	1.091	–0.12	–
Arginine	4.561	4.429	3.680	5.138	0.03	–
Asparagine	0.151	0.147	0.216	0.558	–1.38	M > F
Aspartate	0.127	0.250	0.185	0.331	–0.45	–
Glutamate	0.147	0.147	0.156	0.157	–0.09	–
Glutamine	0.353	0.451	0.453	0.588	–0.37	–
Histidine	0.172	0.137	0.204	0.257	–0.58	–
Isoleucine	0.143	0.119	0.137	0.116	0.05	–
Leucine	0.191	0.158	0.204	0.169	–0.10	–
Phenylalanine	0.081	0.092	0.114	0.111	–0.37	–
Proline	0.567	0.518	0.523	0.498	0.09	–
Tryptophan	0.037	0.044	0.118	0.134	–1.64	M > F
Tyrosine	0.100	0.083	0.120	0.107	–0.31	–
Valine	0.266	0.233	0.229	0.203	0.21	–
Organic acids						
Acetate	1.778	1.495	1.395	1.418	0.22	–
Citrate	2.537	4.023	2.881	3.631	0.01	–
Formate	0.263	0.231	0.216	0.177	0.33	–
Fumarate	0.181	0.152	0.166	0.102	0.31	–
Lactate	0.627	0.217	0.202	0.179	1.15	F > M
Malate	2.418	2.580	2.067	1.563	0.46	–
Succinate	0.169	0.188	0.144	0.154	0.26	–
cis-Aconitate	0.104	0.079	0.108	0.097	–0.16	–
trans-Aconitate	0.090	0.140	0.098	0.122	0.07	–
Sugars & sugar related metabolites						
Fructose	11.896	13.317	13.044	13.849	–0.09	–
Galactose	0.442	0.489	0.517	0.404	0.02	–
Glucose	8.733	7.179	8.720	6.841	0.03	–
Mannose	0.133	0.244	0.166	0.195	0.07	–
myo-Inositol	1.051	1.228	1.040	0.953	0.19	–
Raffinose	1.657	0.756	1.075	0.628	0.50	–
Sucrose	2.699	1.055	2.964	2.227	–0.47	–
Trehalose	0.124	0.137	0.089	0.085	0.59	F > M

Table 1. Continued.

Metabolites (mM)	Female 1	Female 2	Male 1	Male 2	log_2FC	≥1.5-fold difference
Nucleosides & related compounds						
Adenosine	0.069	0.039	0.054	0.027	0.41	–
Guanosine	0.093	0.067	0.092	0.090	–0.19	–
Uracil	0.075	0.083	0.101	0.092	–0.28	–
Uridine	0.128	0.109	0.117	0.115	0.03	–
Nitrogen-containing compounds						
Choline	0.521	0.646	0.591	0.701	–0.15	–
O-Phosphocholine	0.047	0.057	0.047	0.053	0.06	–
Others						
Niacinamide	0.020	0.023	0.010	0.012	0.94	F > M
Nicotinate	0.023	0.024	0.021	0.023	0.11	–
Pantothenate	0.015	0.016	0.018	0.016	–0.14	–

Values are the mean concentrations (mM) of three technical replicates of each plant. F > M or M > F indicates a fold change of at least 1.5 ($|log_2FC| \geq 0.585$) with the higher concentration in female or male roots, respectively.

one another, indicating pronounced inter-individual differences in root metabolite profiles. Along PC1, the female samples were located on the negative side and the male samples on the positive side, showing a tendency toward separation by sex. Hierarchical cluster analysis separated the 12 samples into two clusters, one containing all female and the other all male samples, and within each cluster the replicates grouped according to individual plant (Fig. 3B). Thus, although the plants differed from one another, the overall spectral profiles clustered by sex.

3. Differences in metabolites between male and female roots

Ten metabolites differed by at least 1.5-fold between the sexes, with six higher in male roots and four higher in female roots (Table 1). Because only two independent plants were available per sex, no significance tests were applied, and these differences should be regarded as exploratory.

Among the metabolites higher in male roots, tryptophan showed the largest difference (3.1-fold; $log_2FC = -1.64$), followed by asparagine (2.6-fold; −1.38), sarcosine (1.9-fold; −0.95), and pyroglutamate, 2-aminobutyrate, and histamine (1.5–1.6-fold; −0.64 to −0.61). In females, lactate (2.2-fold; $log_2FC = 1.15$), niacinamide (1.9-fold; 0.94), ornithine, and trehalose (each about 1.5-fold; 0.60 and 0.59) were higher; the lactate difference was due mainly to one female plant.

Several other metabolites differed in the same direction in both plants of a sex but by smaller fold changes (1.1–1.5-fold): phenylalanine, tyrosine, histidine, and glutamine in males, and citrulline, GABA, malate, succinate, formate, and valine in females. Citrulline, one of the most abundant metabolites, was on average 1.29-fold higher in female roots than in male roots ($log_2FC = 0.37$), but this difference was similar in magnitude to that between the two plants within each sex.

The major sugars, glucose and fructose, showed little difference between the sexes (fold change within 1.1; $log_2FC = 0.03$ and −0.09).

DISCUSSION

In this study, 49 primary metabolites were identified and quantified in *T. kirilowii* roots by ^{1}H NMR. ^{1}H NMR allows simultaneous, derivatization-free detection of amino acids, sugars, and organic acids in a single measurement (Schripsema, 2010; Mascellani Bergo *et al*., 2024), and because signal intensity is proportional to the number of contributing protons, metabolites can be quantified against an internal standard (TSP-d_4) without compound-specific calibration curves. Although identification was not confirmed with authentic standards or 2D NMR, metabolites were assigned only when all characteristic resonances matched the library, and those that could not be reliably fitted because of signal overlap or limited sensitivity, particularly low-abundance ones, were not assigned. The absolute concentrations can therefore serve as baseline data for future studies (Table 1).

Citrulline, fructose, and glucose were the dominant constituents, together accounting for approximately 59% of the total quantified concentration, followed by arginine, citrate, GABA, sucrose,

and malate. The prominence of citrulline and arginine, which together accounted for about one-third of the quantified metabolites, is consistent with the high accumulation of citrulline reported in Cucurbitaceae and its role as a nitrogen-rich storage compound (Winter *et al*., 2015; Joshi and Fernie, 2017; Joshi *et al*., 2019). Citrulline has also been reported as a major amino acid-related constituent of *T. kirilowii* roots (Zhang *et al*., 2019), and it serves as the reference compound for the identification of Trichosanthis Radix in the Korean and Chinese pharmacopoeias (MFDS, 2024; Chinese Pharmacopoeia Commission, 2025). The abundance of soluble sugars (fructose, glucose, sucrose, raffinose, and trehalose) and organic acids (citrate and malate) suggests that the root is a storage organ rich in both carbohydrates and nitrogen reserves.

Root metabolite profiles of *T. kirilowii* differed considerably among individual plants, although sex-related patterns were also observed. Sex-related differences in metabolite composition have been reported in several dioecious medicinal plants, including *Sinomenium acutum*, *Eucommia ulmoides,* and *Morus alba* (Wu *et al*., 2022; Luo *et al*., 2025; Wang *et al*., 2025). In the present study, however, only two plants per sex were available, so no valid statistical test could be applied and the comparison was limited to differences in mean concentration. Ten metabolites differed by at least 1.5-fold between the sexes, with both plants of one sex higher than both plants of the other sex. Tryptophan (3.1-fold), asparagine (2.6-fold), sarcosine (1.9-fold), pyroglutamate, 2-aminobutyrate, and histamine (1.5–1.6-fold) were higher in males, whereas lactate (2.2-fold), niacinamide (1.9-fold), ornithine, and trehalose (1.5-fold) were higher in females. For ornithine, pyroglutamate, histamine, and lactate, however, some individual female–male pairs differed only slightly, and the lactate difference was driven mainly by one female plant.

Phenylalanine and tyrosine also tended to be higher in males, although by smaller margins (1.2–1.3-fold). Together with tryptophan, these aromatic amino acids are closely associated with the shikimate pathway at the interface of primary and secondary metabolism (Maeda and Dudareva, 2012). This suggests that secondary metabolites may also differ by sex. In females, citrulline and GABA (1.2–1.3-fold) and malate and succinate (1.2–1.4-fold) tended to be higher, along with ornithine. Citrulline and ornithine are intermediates of arginine metabolism, which contributes to nitrogen storage and remobilization in plants (Winter *et al*., 2015; Joshi and Fernie, 2017), and GABA catabolism through the GABA shunt generates succinate, linking amino acid metabolism with the tricarboxylic acid cycle (Fait *et al*., 2008; Michaeli *et al*., 2011; Michaeli and Fromm, 2015). These patterns may reflect sex-related differences in nitrogen and carbon metabolic pools, possibly related to reproductive investment after flowering, although this remains to be confirmed. Notably, citrulline, the pharmacopoeial identification marker of Trichosanthis Radix, tended to be higher in females (1.29-fold), and whether such differences, together with possibly linked secondary metabolites, affect the quality and efficacy of this medicinal material should be verified in a larger number of plants.

This study has several limitations. First, only two plants per sex were analyzed, so no inferential statistics could be applied and the sex-related differences remain tentative. Second, the plants came from a single cultivation site and sampling time. Future studies with a larger number of plants should therefore confirm whether these primary metabolite differences are reproducible. In addition, secondary metabolite analysis by LC-MS or GC-MS, integrated with the primary metabolite analysis, would help determine whether sex-related differences extend to pharmacologically relevant constituents and to the quality of Trichosanthis Radix.

ACKNOWLEDGEMENTS

This work was carried out with the support of “Cooperative Research Program for Agriculture Science and Technology Development (Project No. RS-2026-25518677)” Rural Development Administration, Republic of Korea.

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