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Research article

Flavonol profiles of mature leaves allow discriminating *Toona sinensis* Roem from different north-south geographical origins across China with varied antioxidant activities

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ABSTRACT

Toona sinensis (A. Juss.) Roem, a multipurpose economic tree, is widely cultivated across Asia, but its high-yielding mature leaves are largely overlooked. This study systematically analysed the flavonols in the mature leaves of *T. sinensis* from 44 different geographic locations across China, using HPLC-DAD and HPLC-ESI-MS^2 techniques. In total, 18 flavonols were detected, among which 6 (f1, f3, f7, f14, f15, and f17) were firstly identified in this plant. Significant variations in quality among different *T. sinensis* varieties were observed ($p < 0.01$). Through OPLS-DA analysis, all samples could be clearly categorised into two distinct geographical groups. The northern varieties (N1–N20) exhibited concise flavonol fingerprints with higher total flavonol content (TFC) (727.55 ± 22.79 mg/100 g fresh weight, FW), predominantly non-acylated flavonols (705.95 ± 21.65 mg/100 g FW), particularly quercetin glycosides (614.60 ± 22.76 mg/100 g FW). In contrast, the southern varieties (S1–S24) presented more intricate flavonol profiles with lower TFC (622.81 ± 21.82 mg/100 g FW) and balanced amounts of quercetin (344.75 ± 16.41 mg/100 g FW) and kaempferol glycosides (278.06 ± 12.29 mg/100 g FW). Notably, the southern samples possessed higher content of acylated flavonols (184.50 ± 12.87 mg/100 g FW), especially galloylated ones, which contributed to their heightened antioxidant activities. Quercetin 3-*O*-rhamnoside (f11′) and kaempferol 3-*O*-galloylglucoside (f11) were determined to be the crucial biomarkers for quality discrimination. Considering quality control of mature *T. sinensis* leaves as potential resources for natural flavonol extraction, this study suggested that their northern/southern geographic origins should be distinguished first. Additionally, the flavonol profiles allow for discriminating the origin and assessing the quality of *T. sinensis*.

1. Introduction

Flavonols, a subclass of flavonoids widely found in fruits and vegetables [1], have consistently fascinated researchers due to their significant roles in plant morphogenesis and evolution [2,3], as well as their potential therapeutic benefits for humans [4,5]. Flavonols are known to possess robust health-promoting properties, surpassing other types of flavonoids, including antioxidant, antimicrobial,

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antifungal, and antiviral activities [4,5]. In light of the growing mistrust in synthetic ingredients, there is an increasing focus on the extraction, isolation, and identification of natural flavonols from plant sources in the current century [6–8]. Over 900 flavonols have been characterised in the plant kingdom to date [4], and databases have been created to guide healthy dietary choices for the public [9]. With greater awareness of their physiological benefits, consumers have shown positive attitudes towards foods enriched with more flavonols/flavonoids [10]. Moreover, the rapid development of purified flavonol extracts is being pursued for the creation of healthcare products or drugs [11,12]. As concerns for maximising land resource utilisation efficiency and earning potential have grown, various tree leaves have been explored as woody resources for the industrial extraction of natural flavonols [13,14]. One exemplary case is *Ginkgo biloba*, whose leaf extract is rich in flavonol glycosides (≥24%, w/w) serving as the major bioactive components, contributing to an annual sales of $250 million in the USA [15].

Toona sinensis (A. Juss.) Roem (Meliaceae), is widely distributed throughout Asia, including China, Malaysia, India, Nepal, Burma, Thailand, and Java, and has many uses [16]. The wood (used for the production of boards) [17], seeds (used for oil production) [18], young buds (picked in early spring with length ≤20 cm, used as vegetables) [19], roots and bark (used for Traditional Chinese Medicines) [20] of *T. sinensis* have been commercially exploited for centuries. In China, the nutritious buds with distinct flavours are the mainstay of *T. sinensis* industry, with over 1.3×10^5 ha cultivated specifically for bud harvesting [21]. However, once the buds grow longer than 20 cm, the leaves are no longer edible as they are high in fibre and have reduced flavour [19]. Notably, the flavonoid content of *T. sinensis* leaves is among the highest level across hundreds of vegetables and fruits [9,22], and its accumulation is increased alongside leaf maturation [23,24]. The flavonoid content and antioxidant activity of mature leaves (grown for over three months) of *T. sinensis* have been reported to be even higher than that of *Ginkgo biloba* leaves [25]. Moreover, the estimated biomass of mature leaves from *T. sinensis* with a diameter breast height (DBH) of 10–19 cm was measured to be 8–11 kg/tree, and with a DBH of 20–45 cm, the level increased to 18–23 kg/tree [26]. Flavonoids in plant leaves are primarily flavonols, including *T. sinensis* [1,23,27]. Thus, mature leaves of *T. sinensis* hold great industrial potential to act as quality resources for the extraction and refining of natural flavonols.

However, the specific flavonol composition of mature *T. sinensis* leaves is rarely analysed, and only 10 glycosyl derivatives of quercetin and kaempferol have been identified [20,23,28,29]. Additionally, *T. sinensis* has a high genetic differentiation across populations [30,31], but little knowledge related to flavonol distribution within the species has been uncovered. A large number of data have demonstrated that the same plant arising from different origins [32], varieties [8,33] or developmental stages [23] may possess significant differences in flavonol compositions and functional activities. Therefore, to ensure the steady and orderly industrialisation and use of flavonols from *T. sinensis*, it is necessary to identify the critical phytochemicals impacting quality control. Thus, the present study aimed to comprehensively identify the flavonol profiles in mature *T. sinensis* leaves, evaluate their differences across varieties from different geographic origins, and clarify the critical phytochemicals for quality discrimination.

2. Materials and methods

2.1. Chemicals

Quercetin 3-*O*-rutinoside (rutin), quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-arabinoside, quercetin 3-*O*-rhamnoside, kaempferol 3-*O*-rhamnoside and gallic acid (GA) standards were obtained from Yuanye (Shanghai, China). 2,4,6-Tripyridyl-S-triazine (TPTZ), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{\cdot+}$) and 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH·) were purchased from Sigma-Aldrich (St. Louis, USA). HPLC-grade acetonitrile and methanol were acquired from Alltech Scientific (Beijing, China). Ultrapure water was produced using a Milli-Q System from Millipore (Billerica, USA). All other regents were analytical grade and were obtained from Beijing Chemical Works (Beijing, China).

2.2. Plant materials

Varieties of *T. sinensis* originating from a total of 44 different geographical locations were collected across China. For each variety, *T. sinensis* seeds were collected from trees older than 20 years from the major producing areas (including Beijing, BJ; Hebei province, HB; Shandong province, SD; Henan province, HEN; Shaanxi province, SX; Hunan province, HN; Guizhou province, GZ; Jiangxi province, JX; and Guangxi province, GX) to obtain representative samplings for this investigation. Thirty semi-sib seedlings from each variety were grown in blocks (50 cm × 50 cm) at the Golden farm (Beijing) under the same cultivation conditions. Every three trees (serving as replicates) of each variety presenting normal development during the entire growing season, containing no pests or diseases, and possessing similar basal diameters were randomly chosen and marked throughout the long-term field investigations. After the leaves had fallen, the selected trees were cut to 20 cm above the ground to ensure consistent growth. In the following year, a pair of mature pinnate leaves that had grown for three months after germination around the middle of the trunk of each selected tree was carefully hand-picked and marked. Subsequently, the collected leaves were stored at −20 °C for further analysis. The geographical distributions of the sampling sites were divided by the 30° latitude. Twenty samples from the north of the 30° latitude (BJ, HB, SD, HEN, and SX) were assigned as northern origins labeled N1–N20. Additionally, 24 samples from the south of the 30° latitude (HN, GZ, JX, and GX) were assigned as southern origins and labeled S1–S24. For more detailed sampling information, refer to Supplementary Fig. S1 and Table S1.

2.3. Extraction of flavonols

The mature leaves of different *T. sinensis* varieties were ground using liquid nitrogen and a Tissue Crusher (Hefan, China).

Ultrasound-assisted extraction of flavonols was conducted according to a previous report from Su et al. [27]. For each *T. sinensis* variety, 0.5 g ground powder was weighed, and 5 mL methanol containing 0.2% formic acid was added. The extract was evenly mixed and sonicated at 20 °C for 20 min. Then the supernatant was collected by centrifugation. The extraction was repeated until the residue was colorless. The collected supernatants from each *T. sinensis* variety were pooled. Ultrapure water, which accounted for 30% of the total volume of the extraction, was added, evenly mixed, and placed at −20 °C overnight. Then the extraction was centrifuged at 4 °C, 10,000 rpm for 10 min, and the supernatant was collected. The resultant samples were filtrated through 0.22 μm reinforced nylon membrane filters (Shanghai ANPEL, China) and retained at −20 °C until further analysis.

2.4. Identification and quantitation of flavonols

Qualitative and quantitative analyses of flavonols were based on methods provided by a prior study by Su et al. [27]. An Agilent 1200 series HPLC–DAD (Sunnyvale, USA) with a ZORBAX SB-C18 column (4.6 × 250 mm, 5 μm i.d.) protected with a matched C18 guard column was utilised for the analysis of flavonols using double distilled water including 0.05% formic acid (A) and 0.7% formic acid in acetonitrile (*v*/*v*) (B) as the mobile phases. The identification of flavonols was performed using HPLC (Agilent 1200 series) with a 6410 series of triple quadrupole mass spectrometer (HPLC–MS/MS) in both positive (PI) and negative ion (NI) modes of ESI sources and all-ion scanning (100–1000 *m*/*z*). Commercial standard of rutin ranging from 0.05625 to 1.0 mg/g was used to create a calibration curve using the same analytical method. Flavonols content in each sample was semi-quantitatively measured by comparing chromatographic peak areas to the calibration curve of rutin: Y (chromatographic peak area) = 24,981X (rutin content) + 252.84 (R^2 = 0.999). The results were expressed as mg of rutin equivalent per 100 g fresh weight (FW) of samples.

2.5. Antioxidant activity analysis

The antioxidant activity analysis was performed according to the methods of Wang et al. [34]. Gallic acid was used as the standard compound to create the calibration curve to quantify the antioxidant capabilities of samples. In the DPPH· scavenging assay, 10 μL of *T. sinensis* extraction was added to 0.2 mM DPPH· solution (990 μL) in the dark at room temperature for 30 min, and then the absorbance at 515 nm was recorded. The amount of GA equivalents (GAE) presenting in each sample was calculated by Y (scavenging ratio) = 0.1947X (GA content)+0.0226 (R^2 = 0.999). In the FRAP assay, 5 μL of sample was reacted with 995 μL FRAP reagent in the

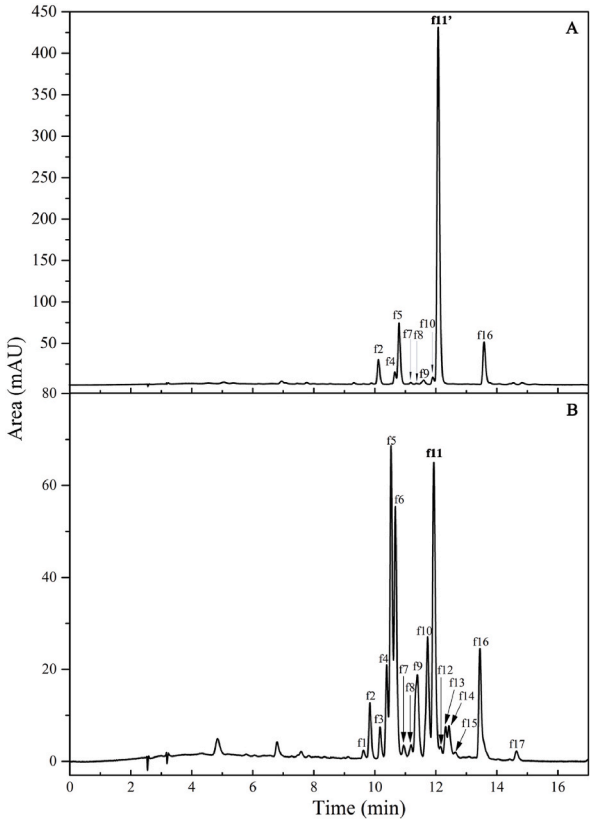


Fig. 1. Typical HPLC fingerprints of flavonols (f1-f17, f11′) detected at 350 nm of *T. sinensis* varieties from the northern (A) and southern (B) origins across China. Peak assignments are listed in Table 1.

dark at room temperature for 15 min, and then the absorbance at 593 nm was measured. The calibration curve used was Y (absorbance) = 0.5945X (GA content)+0.0279 (R^2 = 0.997). In the $ABTS^{\cdot+}$ scavenging assay, 5 μL of sample was added to 1495 μL diluted $ABTS^{\cdot+}$ solution in the dark at room temperature for 10 min, and then the absorbance at 732 nm was detected. The final value was calculated with the calibration curve as Y (scavenging ratio) = 0.1484X (GA content)+0.0091 (R^2 = 0.999). All results were expressed as mg of GAE per 100 g FW of sample.

2.6. Statistical analysis

All experiments were carried out in triplicates and the final results were presented as mean ± standard error (n = 3). Data was

Table 1
The UV–*vis* spectra and MS data used for the identification of flavonols in mature leaves of *T. sinensis*.

Peak	**Rt (min)**	**UV–*vis* (nm)**	**MS PI mode (*m/z*)**	**MS NI mode (*m/z*)**	Identification	Reference
f1[a]	9.626	252,360	633.1 $[M+Na]^+$ (44%)	609.1 $[M-H]^-$ (66%), 336.9 $[Y_0+Cl]^-$ (41%)	Quercetin 3-*O*-neohesperidose	Zhao et al., 1990; Jang et al., 2018; Devkota et al., 2019
f2	9.838	254,356	633.1 $[M+Na]^+$ (77%)	609.0 $[M-H]^-$ (100%), 336.9 $[Y_0+Cl]^-$ 23%)	Quercetin 3-*O*-rutinoside	Standard; Shen et al., 2018; Su et al., 2020
f3[a]	10.169	266,356	785.0 $[M+Na]^+$ (24%)	761.1 $[M-H]^-$ (100%), 336.8 $[Y_0+Cl]^-$ (53%)	Quercetin 3-*O*-galloylrutinoside	Pascale et al., 2020
f4	10.388	266,356	487.0 $[M+Na]^+$ (85%)	463.0 $[M-H]^-$ (100%), 336.8 $[Y_0+Cl]^-$ (41%)	Quercetin 3-*O*-galactoside	Standard; Shen et al., 2018; Su et al., 2020
f5	10.531	256,356	487.0 $[M+Na]^+$ (100%)	463.0 $[M-H]^-$ (100%), 336.9 $[Y_0+Cl]^-$ (9%)	Quercetin 3-*O*-glucoside	Standard; Shen et al., 2018; Su et al., 2020
f6	10.673	268,356	639.0 $[M+Na]^+$ (53%) 486.9 $[M+Na-152]^+$ (7%)	615.0 $[M-H]^-$ (100%), 336.8 $[Y_0+Cl]^-$ (18%)	Quercetin 3-*O*-galloylglucoside	El-Toumy et al., 2018; Cheng et al., 2019; Pascale et al., 2020
f7[a]	10.942	266,340	769.1 $[M+Na]^+$ (15%)	745.0 $[M-H]^-$ (91%), 336.8 $[Y_0+Cl]^-$ (40%)	Quercetin galloyl-di-rhamnoside	Pascale et al., 2020
f8	11.184	268,350	471.0 $[M+Na]^+$ (100%), 286.9 $[Y_0]^+$ (20%)	447.0 $[M-H]^-$ (100%)	Kaempferol 3-*O*-galactoside	Su et al., 2020
Peak	Rt (min)	UV–*vis* (nm)	MS PI mode (*m/z*)	MS NI mode (*m/z*)	Identification	Reference
f9	11.393	254,352	457.0 $[M+Na]^+$ (41%)	433.0 $[M-H]^-$ (100%), 336.8 $[Y_0+Cl]^-$ (22%)	Quercetin 3-*O*-arabinoside	Standard; Shen et al., 2018; Su et al., 2020
f10	11.730	268,346	471.0 $[M+Na]^+$ (100%) 287.0 $[Y_0]^+$ (28%)	447.0 $[M-H]^-$ (100%)	Kaempferol 3-*O*-glucoside	Shen et al., 2018; Su et al., 2020
f11′	11.933	266,348	471.0 $[M+Na]^+$ (97%) 449.0 $[M+H]^+$ (21%) 303.0 $[Y_0]^+$ (100%)	447.0 $[M-H]^-$ (100%)	Quercetin 3-*O*-rhamnoide	Standard; Shen et al., 2018; Su et al., 2020
f11	11.933	262,348	623.0 $[M+Na]^+$ (100%), 287.0 $[Y_0]^+$ (14%)	599.0 $[M-H]^-$ (100%)	Kaempferol 3-*O*-galloyglucoside	Kawakami et al., 2011; Kakumu et al., 2014; Si et al., 2016; El-Toumy et al., 2018
f12	12.154	236,358		335 $[M\text{-}2H+Cl]^-$ (86%), 336.9 $[M\text{-}H+Cl]^-$ (14%)	Quercetin	Jiang et al., 2019
f13	12.321	254,356	441.0$[M+Na]^+$ (52%), 287.0 $[Y_0]^+$ (35%)	417.0 $[M-H]^-$ (100%)	Kaempferol 3-*O*-arabinoside	Wang et al., 2007; Su et al., 2020
f14[a]	12.429	266,350	775.0 $[M+Na]^+$ (25%)	751.0$[M-H]^-$ (76%), 336.8 $[Y_0+Cl]^-$ (15%)	Quercetin di-galloyl-rhamnoside	Pascale et al., 2020
f15[a]	12.636	274,358	742.6$[M+Na]^+$ (6%)	718.5$[M-H]^-$ (28%), 336.9 $[Y_0+Cl]$ (46%)	Quercetin galloyl-benzoyl-hexoside	Pascale et al., 2020
f16	13.447	262,344	455.0 $[M+Na]^+$ (100%) 287.0 $[Y_0]^+$ (77%)	431.0 $[M-H]^-$ (100%)	Kaempferol 3-*O*-rhamnoside	Standard; Shen et al., 2018; Su et al., 2020
f17[a]	14.647	252,350	633.0 $[M+Na]^+$ (11%)	609.1 $[M-H]^-$ (100%), 336.9 $[Y_0+Cl]^-$ (48%)	Quercetin monoacyl glycoside	Pascale et al., 2020

[a] **First report in *T. sinensis*.**

analysed using SPSS 20.0 (IBM). The significant difference was determined at $p < 0.05$ through a one-way ANOVA followed by Duncan's least significant difference test. Thereafter, SIMCA 14.1 (Umetrics, Malmo, Sweden) was employed for orthogonal partial least squares discriminant analysis (OPLS-DA), and the S-plot was drawn to determine the significance of each composition to the whole phytochemical characteristics of various samples.

3. Results and discussion

3.1. Flavonols identification

A total of 18 flavonols (f1-f17 and f11′) were separated and identified from *T. sinensis* varieties (Fig. 1, Table 1) through analysis of HPLC retention time (Rt), elution order, UV–*vis* spectroscopy, and MS data, and comparing to commercial standards and prior reports. Nine flavonols from the mature leaves of *T. sinensis* carried the same spectral and MS characteristics as those identified in young buds [27], and were identified as quercetin 3-*O*-rutinoside (f2), quercetin 3-*O*-galactoside (f4), quercetin 3-*O*-glucoside (f5), kaempferol 3-*O*-galactoside (f8), quercetin 3-*O*-arabinoside (f9), kaempferol 3-*O*-glucoside (f10), quercetin 3-*O*-rhamnoside (f11′), kaempferol 3-*O*-arabinoside (f13), and kaempferol 3-*O*-rhamnoside (f16). Among these, f2, f4, f5, f9, f11′, and f16 were further confirmed by co-elution with authentic standards. Peak f12 was proved to be quercetin aglycone due to the sharp and strong ion identified around *m/z* 355.0 $[M\text{-}2H + Cl]^-$ and 336.9 $[M\text{-}H + Cl]^-$ in NI mode, consistent with the previous studies [19,20].

The remaining compounds of f1, f3, f6, f7, f14, f15, and f17 all exhibited mass spectrometry fragments $[Y_0{+}Cl]^-$ around *m/z* 336.8 in NI mode (Table 1), indicating they were quercetin derivatives.

Peak f1 with $[M{+}Na]^+$ and $[M - H]^-$ ions at *m/z* 633.1 and 609.1, respectively, was determined to be the isomer of f2 quercetin 3-*O*-rutinoside. Considering that neohesperidosides are usually eluted prior to rutinosides with the same aglycone from the C18 column depending on their polarity based on previous reports [27,35], f1 was tentatively identified as quercetin 3-*O*-neohesperidoside. It has been found in the fruits of *Prunus mume* [35], the pollen of *Typhae angustifolia* L. [36], and the leaves of *Aphananthe aspera* (Thunb.) Planch [37]. This was the first characterization of quercetin 3-*O*-neohesperidoside in *T. sinensis*.

The other compounds were rapidly identified via mass spectrometric comparison to the dedicated quercetin-derivatives-identification databases (QUEdb) (Table S2) developed by Pascale et al. [38], in which 5016 quercetin glycosylated and acylated derivatives were deposited. Peak f3 containing the $[M{+}Na]^+$ and $[M - H]^-$ ions at *m/z* 785.0 and 761.1, respectively, represented five types of possible compounds identified by QUEdb with MW around 762, including quercetin + glucose/galactose + rhamnose + galloyl, quercetin + glucose/galactose + galloyl + coumaroyl, quercetin + rhamnose + galloyl + feruloyl, quercetin + xylose/arabinose/apiose + galloyl + feruloyl, and quercetin + xylose/arabinose/apiose + glucuronic acid + galloyl. Ten acyl substituents have been identified among quercetin derivatives, including acetyl, malonyl, benzoyl, galloyl, caffeoyl, feruloyl, coumaroyl, succinyl, sinapoyl, and 1-hydroxy-propionyl [38]. Among them, the final three substituents have been rarely identified, while acetyl, malonyl, coumaroyl, caffeoyl, and galloyl are the most common acyl substituents [38,39]. In *T. sinensis*, gallic acid is reported to be the dominant phenolic acid found in the leaves [40], and galloylated quercetin and kaempferol are the only acylated flavonols which have been detected in it [20,28,29]. Additionally, it has been suggested that glycosylation could reduce the retention time, while acylations could increase the retention time within the same aglycone class in reversed-phase chromatography [35,38]. Therefore, f3 was hypothesized to be quercetin + glucose/galactose + rhamnose + galloyl. Considering the structure of the former peak f2 (quercetin 3-*O*-rutinoside), f3 was quercetin 3-*O*-galloylrutinoside. This is the first characterization of this compound in *T. sinensis*. With a similar strategy, peaks f6 ($[M - H]^-$ 615.0 *m/z*), f7 ($[M - H]^-$ 745.0 *m/z*), f14 ($[M - H]^-$ 751.0 *m/z*), f15 ($[M - H]^-$ 718.5 *m/z*), and f17 ($[M - H]^-$ 609.1 *m/z*) were temporarily speculated to be quercetin 3-*O*-galloylglucoside, quercetin galloyl-di-rhamnoside, quercetin di-galloyl-rhamnoside, quercetin galloyl-benzoyl-hexoside, and quercetin monoacyl-glycoside, respectively. Detailed comparisons are outlined in Supplementary Table S2. Among them, only compound f6 was previously identified from *T. sinensis* leaves [28].

Peak f11 containing the $[M{+}Na]^+$ and $[M - H]^-$ ions at *m/z* 623.0 and 599.0, respectively, showed mass spectrometry fragment $[Y_0]^+$ around 287 *m/z* in PI mode (Table 1) indicating it was kaempferol derivative. The ion at *m/z* 447.0 indicated a loss of 152u from $[M - H]^-$ which suggested a loss of galloyl moiety. As f10 had been identified as kaempferol 3-*O*-glucoside, f11 was hypothesized to be kaempferol 3-*O*-galloyglucoside. This compound had been purified from *T. sinensis*, and the galloylation was confirmed to have a strong influence on its antileukemic activity [29]. This compound has also been detected in *Morus alba* [41], *Sophora japonica* L. [42], and *Diospyros kaki* Thunb. (persimmon) [39]. Additionally, it was noticed that there were two types of MS spectral signatures of the peak surrounding 12 min. Peak f11 was determined to be highly abundant in southern samples (Fig. 1B), while f11' ($[M - H]^-$ 447.0, $[Y_0]^+$ 303.0, quercetin 3-*O*-rhamnoside) was dominant in northern samples (Fig. 1A). Quercetin 3-*O*-rhamnoside was also the most abundant flavonol found in young buds of *T. sinensis* [27].

3.2. Flavonols composition

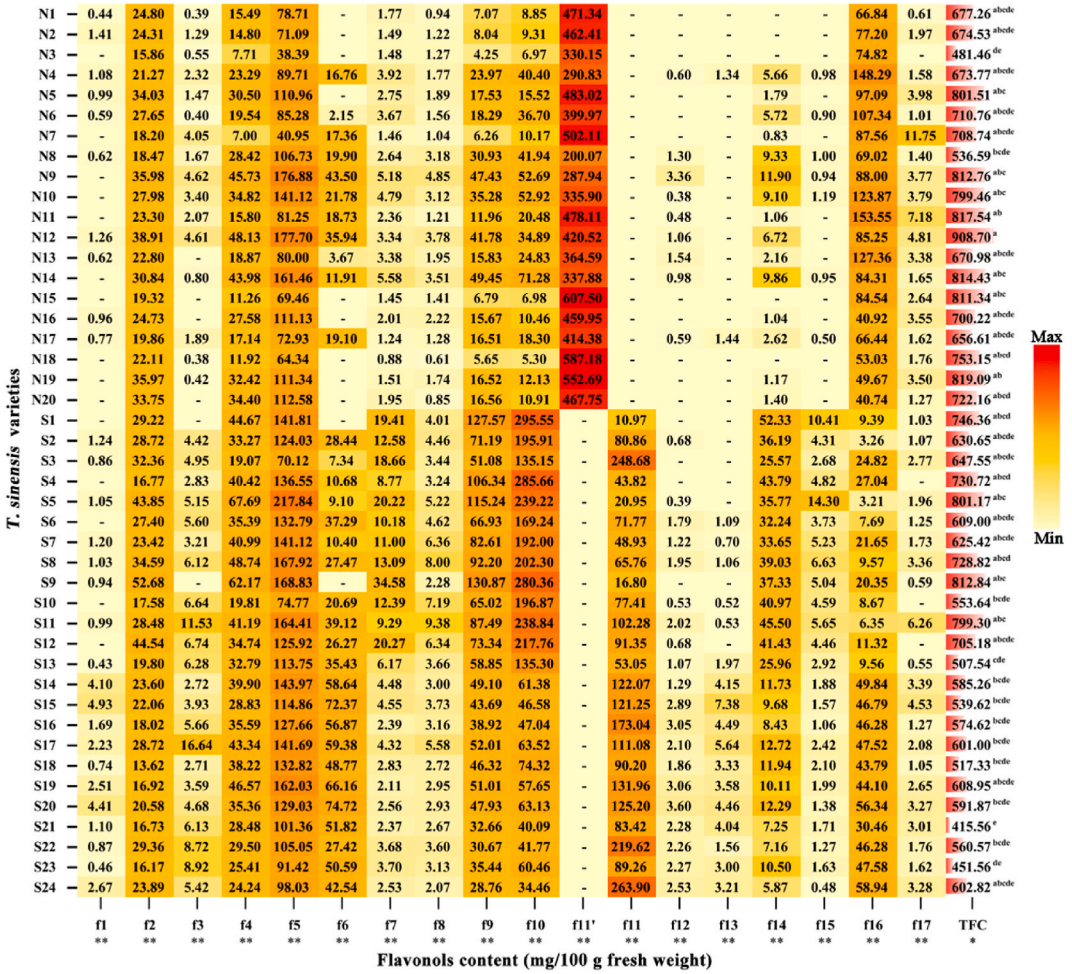
When comparing the HPLC fingerprints (Fig. 1), mature *T. sinensis* leaves contained more complex profiles with increased acylated flavonol levels than young buds [27]. Eleven mono-glycosylated quercetin and kaempferol derivates were detected in the young buds of *T. sinensis*, while 18 flavonols were identified in the mature leaves, with 7 of them modified through both glycosylation and acylation. This finding was aligned with Kawakami et al. [39], who investigated the seasonal compositional changes in the leaves of persimmon, in which an increase in the number of flavonol constituents from 4 to 8 was observed due to the accumulation of galloylated flavonols over the course of leaf development. Additionally, the galloylated flavonols presented approximately two-fold stronger antioxidant activity than the non-galloylated ones. Liang et al. [43] demonstrated that enhancing flavonoid biosynthesis

and activating the antioxidant capacity of kiwifruit leaves could delay leaf senescence. Combined with the consideration of energy conservation in metabolic processes of plants, it was hypothesized that *T. sinensis* could make use of structure-activity relationships in its secondary metabolites, such as flavonols, to improve its stress resistance. For example, coping with leaf senescence by altering the structures of flavonols. However, the precise physiological mechanisms of this hypothesis require further investigation.

Moreover, the distribution of flavonols in mature leaves differed among different *T. sinensis* varieties (Fig. 2). Most southern samples commonly contained 17 flavonols while the northern samples had an average of around 13 flavonols. The southern samples contained relatively more kinds of acylated flavonols than the northern samples, such as quercetin 3-*O*-galloylglucoside (f6), kaempferol 3-*O*-galloyglucoside (f11), quercetin di-galloyl-rhamnoside (f14), and quercetin galloyl-benzoyl-hexoside (f15). Moreover, the f11′ quercetin 3-*O*-rhamnoside accumulated in northern samples, while f11 kaempferol 3-*O*-galloyglucoside was uniquely detected in southern varieties. These two flavonols may serve as key phytochemicals to distinguish the northern and southern origins of *T. sinensis* varieties. It is well-established that flavonol metabolic regulation in plants is influenced by environmental conditions, as flavonols play primary defence roles against abiotic stresses [44]. Acylation increases the solubility, thermostability, and light-resistivity of flavonols [45] that may assist in protecting plants from photothermal sensitivities and UV radiation. Long-term differences in the growth environment can result in the adaptive evolution of plants. Therefore, the differences in flavonol distributions of *T. sinensis* were determined to be due to its adaptive evolution.

3.3. Flavonols content

Significant variations in flavonols content across different *T. sinensis* varieties were detected ($p < 0.01$) (Fig. 2, Table S1). Except for the eight flavonols that had average proportions in total flavonol content (TFC) below 1%, the remaining 10 flavonols, f2 (average content 25.75 ± 1.01 mg/100 g FW, average proportion in TFC 3.90 ± 0.12%), f4 (31.47 ± 1.60 mg/100 g FW, 4.78 ± 0.21%), f5



	f1	f2	f3	f4	f5	f6	f7	f8	f9	f10	f11′	f11	f12	f13	f14	f15	f16	f17	TFC
N1	0.44	24.80	0.39	15.49	78.71	-	1.77	0.94	7.07	8.85	471.34	-	-	-	-	-	66.84	0.61	677.26 abcde
N2	1.41	24.31	1.29	14.80	71.09	-	1.49	1.22	8.04	9.31	462.41	-	-	-	-	-	77.20	1.97	674.53 abcde
N3	-	15.86	0.55	7.71	38.39	-	1.48	1.27	4.25	6.97	330.15	-	-	-	-	-	74.82	-	481.46 de
N4	1.08	21.27	2.32	23.29	89.71	16.76	3.92	1.77	23.97	40.40	290.83	-	0.60	1.34	5.66	0.98	148.29	1.58	673.77 abcde
N5	0.99	34.03	1.47	30.50	110.96	-	2.75	1.89	17.53	15.52	483.02	-	-	-	1.79	-	97.09	3.98	801.51 abc
N6	0.59	27.65	0.40	19.54	85.28	2.15	3.67	1.56	18.29	36.70	399.97	-	-	-	5.72	0.90	107.34	1.01	710.76 abcde
N7	-	18.20	4.05	7.00	40.95	17.36	1.46	1.04	6.26	10.17	502.11	-	-	-	0.83	-	87.56	11.75	708.74 abcde
N8	0.62	18.47	1.67	28.42	106.73	19.90	2.64	3.18	30.93	41.94	200.07	-	1.30	-	9.33	1.00	69.02	1.40	536.59 bcde
N9	-	35.98	4.62	45.73	176.88	43.50	5.18	4.85	47.43	52.69	287.94	-	3.36	-	11.90	0.94	88.00	3.77	812.76 abc
N10	-	27.98	3.40	34.82	141.12	21.78	4.79	3.12	35.28	52.92	335.90	-	0.38	-	9.10	1.19	123.87	3.79	799.46 abc
N11	-	23.30	2.07	15.80	81.25	18.73	2.36	1.21	11.96	20.48	478.11	-	0.48	-	1.06	-	153.55	7.18	817.54 ab
N12	1.26	38.91	4.61	48.13	177.70	35.94	3.34	3.78	41.78	34.89	420.52	-	1.06	-	6.72	-	85.25	4.81	908.70 a
N13	0.62	22.80	-	18.87	80.00	3.67	3.38	1.95	15.83	24.83	364.59	-	1.54	-	2.16	-	127.36	3.38	670.98 abcde
N14	-	30.84	0.80	43.98	161.46	11.91	5.58	3.51	49.45	71.28	337.88	-	0.98	-	9.86	0.95	84.31	1.65	814.43 abc
N15	-	19.32	-	11.26	69.46	-	1.45	1.41	6.79	6.98	607.50	-	-	-	-	-	84.54	2.64	811.34 abc
N16	0.96	24.73	-	27.58	111.13	-	2.01	2.22	15.67	10.46	459.95	-	-	-	1.04	-	40.92	3.55	700.22 abcde
N17	0.77	19.86	1.89	17.14	72.93	19.10	1.24	1.28	16.51	18.30	414.38	-	0.59	1.44	2.62	0.50	66.44	1.62	656.61 abcde
N18	-	22.11	0.38	11.92	64.34	-	0.88	0.61	5.65	5.30	587.18	-	-	-	-	-	53.03	1.76	753.15 abcd
N19	-	35.97	0.42	32.42	111.34	-	1.51	1.74	16.52	12.13	552.69	-	-	-	1.17	-	49.67	3.50	819.09 ab
N20	-	33.75	-	34.40	112.58	-	1.95	0.85	16.56	10.91	467.75	-	-	-	1.40	-	40.74	1.27	722.16 abcd
S1	-	29.22	-	44.67	141.81	-	19.41	4.01	127.57	295.55	-	10.97	-	-	52.33	10.41	9.39	1.03	746.36 abcd
S2	1.24	28.72	4.42	33.27	124.03	28.44	12.58	4.46	71.19	195.91	-	80.86	0.68	-	36.19	4.31	3.26	1.07	630.65 abcde
S3	0.86	32.36	4.95	19.07	70.12	7.34	18.66	3.44	51.08	135.15	-	248.68	-	-	25.57	2.68	24.82	2.77	647.55 abcde
S4	-	16.77	2.83	40.42	136.55	10.68	8.77	3.24	106.34	285.66	-	43.82	-	-	43.79	4.82	27.04	-	730.72 abcd
S5	1.05	43.85	5.15	67.69	217.84	9.10	20.22	5.22	115.24	239.22	-	20.95	0.39	-	35.77	14.30	3.21	1.96	801.17 abc
S6	-	27.40	5.60	35.39	132.79	37.29	10.18	4.62	66.93	169.24	-	71.77	1.79	1.09	32.24	3.73	7.69	1.25	609.00 abcde
S7	1.20	23.42	3.21	40.99	141.12	10.40	11.00	6.36	82.61	192.00	-	48.93	1.22	0.70	33.65	5.23	21.65	1.73	625.42 abcde
S8	1.03	34.59	6.12	48.74	167.92	27.47	13.09	8.00	92.20	202.30	-	65.76	1.95	1.06	39.03	6.63	9.57	3.36	728.82 abcd
S9	0.94	52.68	-	62.17	168.83	-	34.58	2.28	130.87	280.36	-	16.80	-	-	37.33	5.04	20.35	0.59	812.84 abc
S10	-	17.58	6.64	19.81	74.77	20.69	12.39	7.19	65.02	196.87	-	77.41	0.53	0.52	40.97	4.59	8.67	-	553.64 bcde
S11	0.99	28.48	11.53	41.19	164.41	39.12	9.29	9.38	87.49	238.84	-	102.28	2.02	0.53	45.50	5.65	6.35	6.26	799.30 abc
S12	-	44.54	6.74	34.74	125.92	26.27	20.27	6.34	73.34	217.76	-	91.35	0.68	-	41.43	4.46	11.32	-	705.18 abcde
S13	0.43	19.80	6.28	32.79	113.75	35.43	6.17	3.66	58.85	135.30	-	53.05	1.07	1.97	25.96	2.92	9.56	0.55	507.54 cde
S14	4.10	23.60	2.72	39.90	143.97	58.64	4.48	3.00	49.10	61.38	-	122.07	1.29	4.15	11.73	1.88	49.84	3.39	585.26 bcde
S15	4.93	22.06	3.93	28.83	114.86	72.37	4.55	3.73	43.69	46.58	-	121.25	2.89	7.38	9.68	1.57	46.79	4.53	539.62 bcde
S16	1.69	18.02	5.66	35.59	127.66	56.87	2.39	3.16	38.92	47.04	-	173.04	3.05	4.49	8.43	1.06	46.28	1.27	574.62 bcde
S17	2.23	28.72	16.64	43.34	141.69	59.38	4.32	5.58	52.01	63.52	-	111.08	2.10	5.64	12.72	2.42	47.52	2.08	601.00 bcde
S18	0.74	13.62	2.71	38.22	132.82	48.77	2.83	2.72	46.32	74.32	-	90.20	1.86	3.33	11.94	2.10	43.79	1.05	517.33 bcde
S19	2.51	16.92	3.59	46.57	162.03	66.16	2.11	2.95	51.01	57.65	-	131.96	3.06	3.58	10.11	1.99	44.10	2.65	608.95 abcde
S20	4.41	20.58	4.68	35.36	129.03	74.72	2.56	2.93	47.93	63.13	-	125.20	3.60	4.46	12.29	1.38	56.34	3.27	591.87 bcde
S21	1.10	16.73	6.13	28.48	101.36	51.82	2.37	2.67	32.66	40.09	-	83.42	2.28	4.04	7.25	1.71	30.46	3.01	415.56 e
S22	0.87	29.36	8.72	29.50	105.05	27.42	3.68	3.60	30.67	41.77	-	219.62	2.26	1.56	7.16	1.27	46.28	1.76	560.57 bcde
S23	0.46	16.17	8.92	25.41	91.42	50.59	3.70	3.13	35.44	60.46	-	89.26	2.27	3.00	10.50	1.63	47.58	1.62	451.56 de
S24	2.67	23.89	5.42	24.24	98.03	42.54	2.53	2.07	28.76	34.46	-	263.90	2.53	3.21	5.87	0.48	58.94	3.28	602.82 abcde

Fig. 2. Heat map of flavonols (f1-f17, f11′) content in the studied *T. sinensis* varieties. Flavonol assignments are listed in Table 1. Significant differences at the 0.05 and 0.01 confidence level were marked with * and **, respectively. Samples with northern origins were labeled N1–N20, while samples with southern origins were labeled S1–S24. TFC refers to the total flavonol content, and different letters indicate significance ($p < 0.05$).

(116.34 ± 4.67 mg/100 g FW, 17.60 ± 0.60%), f6 (40.48 ± 2.98 mg/100 g FW, 6.90 ± 0.55%), f9 (44.65 ± 3.15 mg/100 g FW, 6.85 ± 0.43%), f10 (87.06 ± 8.15 mg/100 g FW, 13.33 ± 1.14%), f11' (424.28 ± 22.34 mg/100 g FW, 57.11 ± 2.39%), f11 (103.84 ± 13.00 mg/100 g FW, 16.97 ± 1.71%), f14 (20.62 ± 1.65 mg/100 g FW, 3.20 ± 0.23%), and f16 (54.95 ± 4.17 mg/100 g FW, 8.36 ± 0.60%), made up 93.31% (in S17) - 99.51% (in N18) of the TFC were the major flavonols of *T. sinensis*. Among them, f5 (quercetin 3-*O*-glucoside), f10 (kaempferol 3-*O*-glucoside), f11' (quercetin 3-*O*-rhamnoside), and f11 (kaempferol 3-*O*-galloyglucoside) were the dominant with respect to their average proportions in TFC, with all persisting at levels above 10%. Generally, quercetin 3-*O*-rhamnoside (f11′), quercetin 3-*O*-glucoside (f5), and kaempferol 3-*O*-rhamnoside (f16) were the top 3 flavonols in terms of content in northern samples, while kaempferol 3-*O*-glucoside (f10), quercetin 3-*O*-glucoside (f5), and kaempferol 3-*O*-galloyglucoside (f11) were the most abundant in southern samples.

The TFC of mature *T. sinensis* leaves from different geographic origins ranged from 415.56 mg/100 g FW (S21) to 908.70 mg/100 g FW (N12) with an average value of 671.83 ± 14.68 mg/100 g FW (Fig. 2). The TFC was much higher than commonly consumed vegetables and fruits (often less than 300 mg/100 g FW) [9], and was comparable to the green leaves of *G. biloba* (about 755 mg/100 g FW) [46]. Considering the fast-growing nature and large biomass of *T. sinensis* leaves [26], the mature leaves of *T. sinensis* are quality potential resources for the extraction and refining of natural flavonols. However, significant regional characteristics were also uncovered in the TFC of *T. sinensis* ($p < 0.05$). It was determined that the TFC of northern samples (481.46 ± 99.01 mg/100 g FW - 908.70 ± 66.60 mg/100 g FW) were much higher than that of southern samples (415.56 ± 103.25 mg/100 g FW - 812.84 ± 58.45 mg/100 g FW). Previous reports indicated that flavonol accumulation in plants is often elevated in environments with lower temperatures and/or higher radiation [1,2]. The northern samples in this study originated from sites at latitudes above 30°N that were much colder than the southern samples. These results indicated that the outstanding heterogeneity in flavonols content of *T. sinensis* varieties may be due to their geographic original differences.

3.4. Antioxidant activity

The results of antioxidant activity are outlined in Fig. 3, and significant differences were detected across different samples ($p < 0.01$). The DPPH· scavenging activity varied from 88.76 ± 13.82 mg GAE/100 g FW (N20) to 216.28 ± 11.91 mg GAE/100 g FW (S15). FRAP value varied from 24.19 ± 0.24 mg GAE/100 g FW (S1) to 60.90 ± 3.09 mg GAE/100 g FW (S15), while the $ABTS^{\cdot+}$ scavenging capacity varied from 66.98 ± 10.33 mg GAE/100 g FW (N9) to 246.65 ± 28.96 mg GAE/100 g FW (S15). S15 exhibited the highest antioxidant activity, followed by S14, S18, and S20. The southern samples generally had higher antioxidant activity than the northern samples, as evidenced by the DPPH· and $ABTS^{\cdot+}$ scavenging assays shown in Fig. 2. According to prior reports [38,39,47], this result may be due to the higher accumulation of acylated flavonols in southern samples than in the northern ones (Fig. 2). Typically, acylation, especially galloylation, reinforces the biological activity, such as the antioxidant activity of flavonols.

In summary, due to the significant differences in flavonols composition, content and antioxidant activities, the quality of mature *T. sinensis* leaves prepared for flavonol extraction or processing should be differentiated and controlled through flavonol fingerprinting. Combined with the significant regional pattern outlined in the analytical results obtained, the geographical origin of *T. sinensis* may assist in quality control.

3.5. OPLS-DA analysis

To fully verify the above presumptions, OPLS-DA, a widely used pattern recognition method [48], was employed to further identify

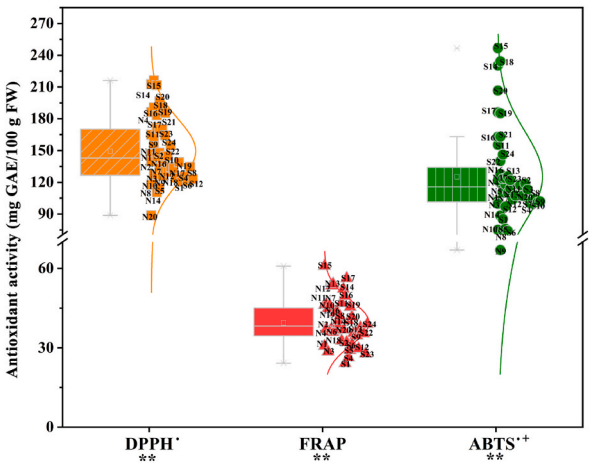


Fig. 3. Antioxidant activities of *T. sinensis* varieties obtained from various geographical origins detected by FRAP, DPPH·, and $ABTS^{\cdot+}$ scavenging assays. Significant differences at the 0.01 confidence level were marked with **. FW: fresh weight. GAE: gallic acid equivalents. Samples with northern origins were labeled N1–N20, while samples with southern origins were labeled S1–S24.

the quality characteristics of *T. sinensis*. Discrimination and aggregation of *T. sinensis* varieties were observed using the OPLS-DA model with Q^2 and R^2X values of 0.949 and 0.808, respectively, which indicating an acceptable model with respect to goodness of fit and prediction [49] (Fig. 4). As illustrated in the biplot (Fig. 4A), all samples were clearly differentiated into two groups with obvious geographical dependence: the category on the left quadrant included *T. sinensis* from the northern geographic origins (N1–N20), while the category on the right quadrant included the southern varieties (S1–S24). Based on the distribution range of samples, the characteristics of the southern samples were relatively more divergent than that of the northern samples. This result suggested that the composition of flavonols in the southern samples was more complex than in the northern samples.

Combined with Fig. 5A, outside of f2 and f17, other flavonols were significantly different in content between northern and southern

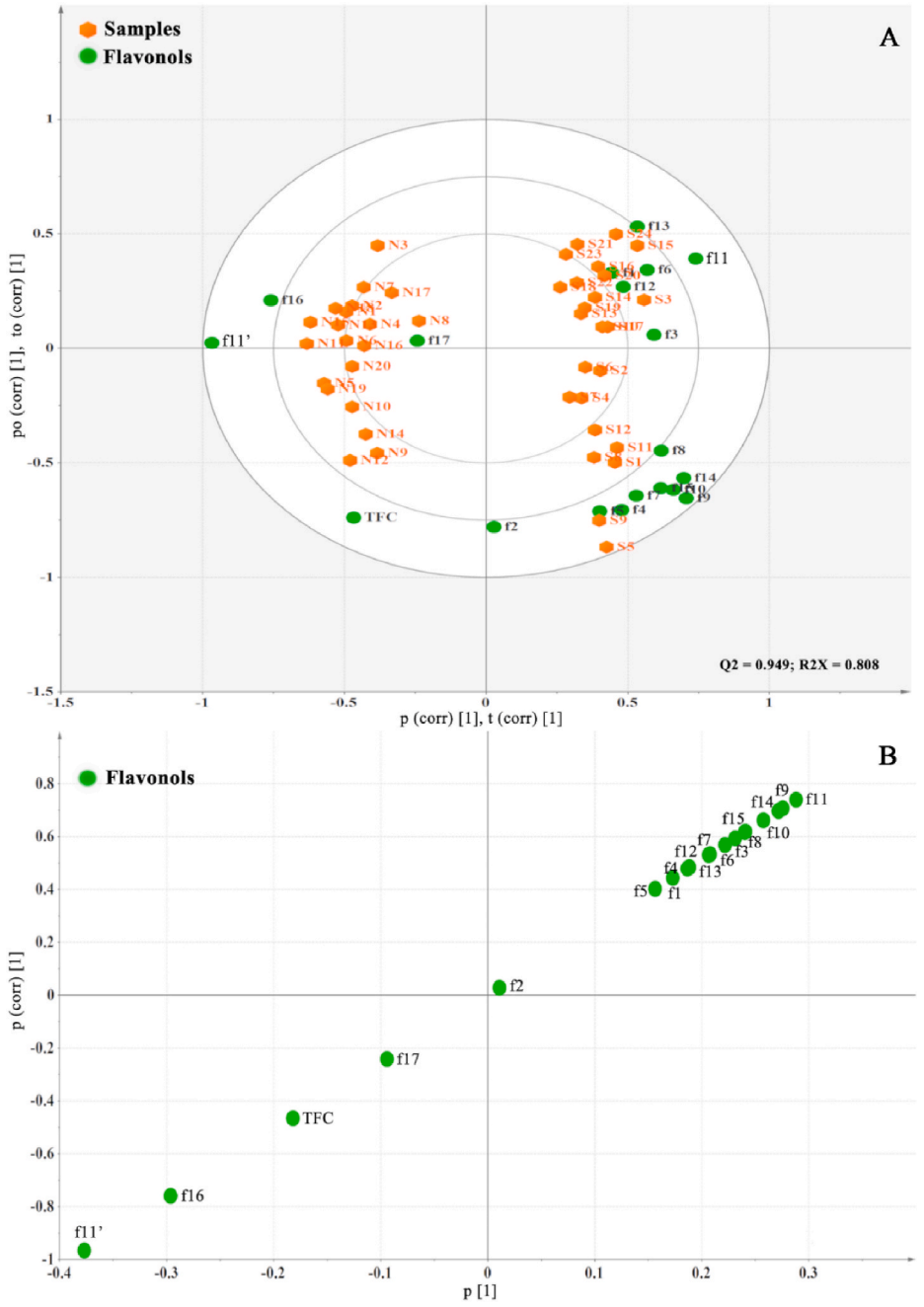


Fig. 4. Results of orthogonal projections to latent structures discriminant analysis (OPLS-DA). A: Biplot of OPLS-DA result on flavonol profiles of *T. sinensis* varieties based on their geographical origins. B: The S-plot of OPLS-DA result on flavonol profiles from *T. sinensis* varieties. Samples with northern origins were labeled N1–N20, and samples with southern origins were labeled S1–S24. Flavonol (f1-f17, f11′) assignments are listed in Table 1. TFC refers to the total flavonol content.

samples ($p < 0.01$). Additionally, only flavonols of f11′, f16, and f17 were elevated in northern samples and were categorised on the left quadrant, while the other flavonols were increased in southern samples and were categorised on the right quadrant (Fig. 4A). Interestingly, the northern samples possessed higher TFC (average of 727.55 ± 22.79 mg/100 g FW), and higher content of non-acylated flavonols (705.95 ± 21.65 mg/100 g FW, the proportion of TFC as 97.09 ± 0.57%), predominantly quercetin glycosides (614.60 ± 22.76 mg/100 g FW, 84.34 ± 1.35%). In contrast, the southern samples possessed lower TFC (622.81 ± 21.82 mg/100 g FW) with relatively balanced quercetin (344.75 ± 16.41 mg/100 g FW, 55.24 ± 1.45%) and kaempferol glycosides (278.06 ± 12.29 mg/100 g FW, 44.76 ± 1.45%), and with higher content of acylated flavonols (184.50 ± 12.87 mg/100 g FW, 30.78 ± 2.37%). It was also demonstrated in Fig. 5B that while the differences in FRAP activity were not significant, the antioxidant activities assigned by DPPH· and $ABTS^{\cdot+}$ scavenging assays were significantly ($p < 0.01$) higher in southern samples (average DPPH· scavenging value 162.50 ± 6.39 mg GAE/100 g FW, average $ABTS^{\cdot+}$ scavenging value 140.88 ± 10.46 mg GAE/100 g FW) than those in northern samples (133.84 ± 4.57 mg GAE/100 g FW, 106.48 ± 3.94 mg GAE/100 g FW). The results of OPLS-DA were consistent with previous analyses.

The above geographical phytochemistry differences were consistent with the reported result of the genetic division of *T. sinensis* across China, in which the northern and southern varieties were clearly separated by their variants in random amplified polymorphic DNA, inter-simple sequence repeat, and sequence-related amplified polymorphisms [30,31]. The mentioned references indicated that the evolutionary migratory route of *T. sinensis* in China generally occurred in two different directions, from West-South to East-North. The present study suggested that their evolutionary strategies at the phytochemical level also differed: the northern varieties of *T. sinensis* increased the total accumulation of flavonols to enhance their adaptation to environmental adversity, while the southern varieties underwent structural modifications, such as acylation, to increase their flavonol bioactivity to improve stress resistance. The underlying molecular mechanisms require further study. In terms of the quality control of mature *T. sinensis* leaves as resources for flavonol extraction, the northern or southern geographic origin of raw materials should first be determined.

To further identify the specific critical flavonol composition for the quality/geographic origin discrimination of *T. sinensis*, the S-plot was drawn after OPLS-DA analysis. As shown in Fig. 4B, f11′ and f11 were distributed at the extreme ends of the S-curve, meaning they exhibit the strongest explanatory potential for discrimination, followed by f16, f9, f14, and f10. Combined with previous findings related to flavonol content and antioxidant analysis, a higher content of f11' (average of 424.28 ± 22.34 mg/100 g FW) referred to the

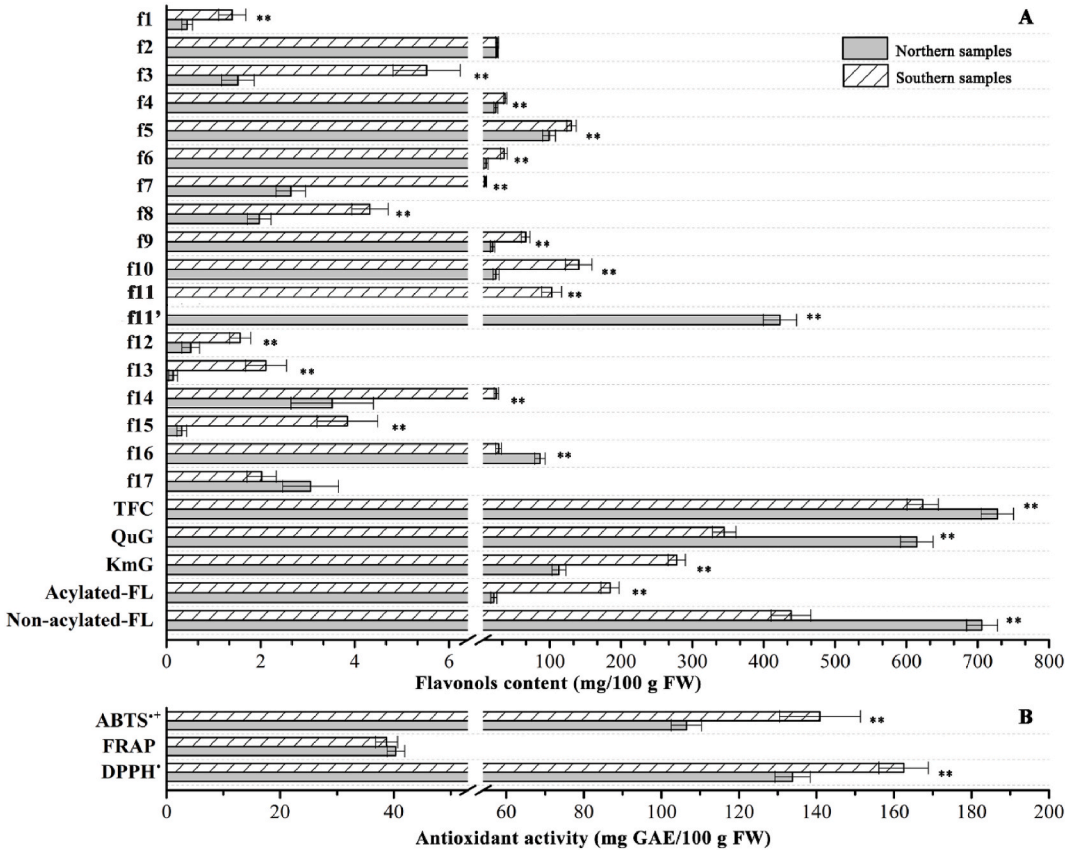


Fig. 5. The flavonols compositions (A) and antioxidant activities (B) of *T. sinensis* varietis from both northern and southern origins. Flavonols (f1-f17, f11′) assignments are listed in Table 1. TFC: total flavonol content; QuG: quercetin glycoside; KmG: kaempferol glycoside; Acylated-FL: acylated flavonols; Non-acylated-FL: non-acylated flavonols; FW: fresh weight.

northern origins with higher content of TFC and non-acylated quercetin glycosides. Meanwhile, a higher content of f11 (average of 103.84 ± 13.00 mg/100 g FW) indicated the southern origins which possessed elevated acylated flavonol content and higher antioxidant activity. Diverse compositions of ingredients may alter the rating of raw materials, just as both the TFC content and proportion of quercetin/kaempferol of raw materials were limited in the ginkgo extraction industry [15]. This result suggested that the content of f11′ quercetin 3-*O*-rhamnoside and f11 kaempferol 3-*O*-galloyglucoside in *T. sinensis* were the most important determinants for judging its north-south origin and were also critical biomarkers for quality control.

4. Conclusions

Flavonols in mature *T. sinensis* leaves from 44 diverse geographic origins in China were systematically analysed through HPLC-DAD and HPLC-ESI-MS^2. In total, 18 flavonols were detected, with 6 (f1, f3, f7, f14, f15, and f17) represented the first characterization in this plant. Significant variations were detected in qualitative compositions among different samples ($p < 0.01$), and all samples could be clearly separated into two geographical groups by OPLS-DA analysis. The northern varieties (N1–N20) contained higher TFC (727.55 ± 22.79 mg/100 g FW), and generally higher content of non-acylated flavonols (705.95 ± 21.65 mg/100 g FW) dominated by quercetin glycosides (614.60 ± 22.76 mg/100 g FW). In contrast, the southern varieties contained lower TFC (622.81 ± 21.82 mg/100 g FW), but higher acylated flavonols content (184.50 ± 12.87 mg/100 g FW) and relatively higher antioxidant activity. The composition of quercetin 3-*O*-rhamnoside (f11′) and kaempferol 3-*O*-galloyglucoside (f11) were determined to be the most important biomarkers for the quality discrimination of *T. sinensis*. With respect to the quality control of mature *T. sinensis* leaves as resources for flavonol extraction, the present study demonstrated that, at a minimum, the northern or southern geographic origin should be distinguished, which could be accomplished based on their flavonol profiles.

Data availability statement

Date will be made available on request.

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CRediT authorship contribution statement

Shang Su: Data curation, Funding acquisition, Investigation, Methodology, Resources, Visualization, Writing – review & editing. **Lijin Wang:** Data curation, Methodology, Writing – review & editing. **Yonghang Geng:** Investigation, Resources. **Junhui Wang:** Funding acquisition, Supervision, Writing – review & editing.

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.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27040.

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