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Qualitative and Quantitative Analysis of Phenolics in *Tetrastigma hemsleyanum* and Their Antioxidant and Antiproliferative Activities

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ABSTRACT: The phenolic profiles of *Tetrastigma hemsleyanum* leaf extracts by different solvents (80% methanol, ethyl acetate and hexane) and their antioxidant and antiproliferative activities were investigated. Thirteen phenolic compounds (3-caffeoylquinic acid, 5-caffeoylquinic acid, 1-caffeoylquinic acid, 5-*p*-coumaroylquinic acid, isoorientin-2″-*O*-rhamnoside, isoorientin, orientin-2″-*O*-rhamnoside, orientin, 1-*p*-coumaroylquinic acid, vitexin-2″-*O*-rhamnoside, isovitexin-2″-*O*-rhamnoside, vitexin and isovitexin) were identified in *T. hemsleyanum* leaves for the first time, and six of them were quantified using a combination of LC−QTOF-MS and LC−QqQ-MS techniques. It was found that 80% methanol extract exhibited the highest antioxidant activities (DPPH, 3.32 mmol of Trolox/g DW; ABTS, 1.38 mmol of Trolox/g DW; FRAP, 1.85 mmol of $FeSO_4$/g DW), while the hexane extract had the lowest (1.23, 0.43 and 0.13, respectively). Total phenolic contents (TPC) of various extracts of *T. hemsleyanum* leaves ranged from 28.95 to 275.71 mg of GAE/g DW. Also, total antioxidant activities as evaluated by ABTS, FRAP and DPPH assays were correlated well with TPC. In addition, 80% methanol extract provided antiproliferative activity on HepG2 cells (IC_{50} = 524 μg/mL). This paper provides a complete picture of phenolics in *T. hemsleyanum* leaves and relates them to their antioxidant and antiproliferative activities.

KEYWORDS: *Tetrastigma hemsleyanum, phenolics, LC−QTOF-MS, LC−QqQ-MS, antioxidant activity, antiproliferative activity*

INTRODUCTION

Tetrastigma hemsleyanum Diels et Gilg, an herbaceous perennial species, is a family member of Vitaceae. It is distributed mainly in southern China, especially suited to grow in shady and moist hillsides and valleys. As an edible plant, the leaves of *T. hemsleyanum* have gained popularity within China, where it is consumed as a functional tea or dietary supplement for its health benefits, such as improving the immune system. The roots of *T. hemsleyanum* are widely used for the treatment of high fever, infantile febrile convulsion, pneumonia, asthma, hepatitis, rheumatism, menstrual disorders, sore throat and scrofula due to its anti-inflammatory, analgesic and antipyretic properties.[1,2] In addition, it has antiviral, hepatoprotective and antipyretic functions as well as improving the immune system for anticancer purpose.[3−6]

The roots of *T. hemsleyanum* have been reported to contain flavonoids, phenolic acids and polysaccharide.[7] Among them, the main active compounds in the roots were flavonoids, which have various bioactivities.[6] However, most of the research mainly focuses on the roots rather than its leaves. The leaves with higher phytochemical contents may have several advantages over roots.[8] This study is focused on investigating the chemical compositions and the functions of *T. hemsleyanum* leaves.

Many modern analytical techniques have been used for qualitative and quantitative analysis of phytochemicals in various plants. Among these techniques, LC−QTOF-MS (liquid chromatography orthogonal acceleration quadrupole time-of-flight mass spectrometry) has become a powerful tool for identifying the complicated compounds, while LC−QqQ-MS (liquid chromatography triple quadrupole tandem mass spectrometry) has appeared strong in quantitive analysis of the individual compounds.[9,10] LC−QTOF-MS technique with higher mass accuracy and resolution can be used to obtain the molecular formula of several unknown peaks from the isotope pattern and the accurate ion mass. Thus, this technique is particularly suitable for qualitative analysis. In addition, the LC−QqQ-MS technique could provide specific, sensitive and selective quantitative results by working in multiple reaction monitoring (MRM) mode. It is considered as one of the most efficient techniques for quantitative analysis. In the present study, a combination of LC−QTOF-MS and LC−QqQ-MS was applied to the analysis of phenolics in *T. hemsleyanum* leaves and 13 compounds were identified from this plant for the first time.

It has been demonstrated that the total phenolic contents (TPC) extracted from the same samples differed with solvent polarities. Razali et al. reported that methanol, ethyl acetate and hexane significantly affected the TPC of *Tamarindus indica*.[11] Hossain et al. also found that the methanol extract of *Tetrastigma* sp. leaves showed higher TPC than ethyl acetate, chloroform and hexane.[12] Moreover, the stabilities of diverse extracts from the same plant depend on the extraction solvent which is used to remove the phenolic compounds, and it is obvious that the extracts from the same plant may vary widely with respect to their antioxidant concentrations and activities.[13] Consequently, the *T. hemsleyanum* leaf extracts by different solvents may have different phytochemical compositions and bioactivities.

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Table 1. Extraction Yields, Total Phenolic Contents, Antioxidant and Antiproliferative Activities of Different *T. hemsleyanum* Leaf Extracts[a]

sample	extraction yield (% w/w)[b]	total phenolic content (mg of GAE/g DW)[c]	DPPH assay (mmol of Trolox/g DW)[d]	ABTS assay (mmol of Trolox/g DW)[d]	FRAP assay (mmol of $FeSO_4$/g DW)[e]	IC_{50} on HepG2 (μg/mL)
hexane extract	4.02 ± 0.25 a	28.95 ± 1.32 a	1.23 ± 0.11 a	0.43 ± 0.04 a	0.13 ± 0.02 a	nd
ethyl acetate extract	4.39 ± 1.09 a	91.26 ± 1.98 b	2.16 ± 0.12 b	0.80 ± 0.02 b	0.49 ± 0.05 b	705.3 ± 5.22 c
80% methanol extract	19.3 ± 2.26 b	275.71 ± 2.64 c	3.32 ± 0.19 c	1.38 ± 0.06 c	1.85 ± 0.09 c	524.9 ± 6.48 b
cisplatin						8.88 ± 0.10 a

[a]Results are expressed as mean ± standard deviation of three replicates. Values followed by the different letters (a, b, c) within the same column are significantly different ($P < 0.05$). [b]Extraction yield is shown as the % of extracts dry weight of plant material ± standard deviation. [c]Phenolic contents are expressed as mg of gallic acid equivalents (GAE) in 1 g of dry weight of extracts ± standard deviation. [d]DPPH and ABTS radical-scavenging activities of the samples were compared with a Trolox standard curve, and results were expressed as mmol of Trolox equivalents in 1 g of dry weight material ± standard deviation. [e]Antioxidant activity of FRAP assay was compared with a $FeSO_4$ standard curve, and results were expressed as mmol of $FeSO_4$ equivalents in 1 g of dry weight material ± standard deviation.

The main objective of this study was therefore to identify and quantify total and individual phenolics in *T. hemsleyanum* leaves by a combination of LC−QTOF-MS and LC−QqQ-MS technique, to investigate the antioxidant activities of *T. hemsleyanum* leaf extracts using DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)], FRAP (ferric reducing ability of plasma) assays, and their antiproliferative activities by cell-based models.

■ MATERIALS AND METHODS

Chemicals and Reagents. 5-caffeoylquinic acid, DPPH, DMSO and Folin−Ciocalteu reagent were purchased from sigma co. (MO, USA). Isoorientin, orientin, vitexin-2″-*O*-rhamnoside, vitexin and isovitexin were obtained from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). ABTS, Trolox and FRAP assay kits were purchased from Beyotime Institute of Biotechnology Co., Ltd. (Nantong, China). Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Formic acid was purchased from ACROS Organics (Morris, USA). Water was purified using a Milli-Q system, Millipore (Bedford, USA).

Plant Materials. *T. hemsleyanum* leaves were provided by Jiangxi Shangrao Red Sun Agricultural Development Co., Ltd. (Jiangxi, China). The plants were grown at *T. hemsleyanum* Seeding Base in China (Shangrao, Jiangxi, China, 28°53′N, 117°57′E) on red loam soil of Jiangxi in 2012. The leaves were dried at 50 °C with an electro-thermostatic blast oven (Cimo, China) and ground into fine powder. These materials were stored in polyethylene tubes at −80 °C prior to analysis.

Sample Extraction. The dried powder (5.0 g) was extracted with 100 mL of hexane, ethyl acetate and 80% methanol for 90 min at 60 °C by reflux extraction, respectively. The organic extracts were centrifuged at 5,000 rpm for 10 min with a Sorvall SL16 centrifuge (Thermo Scientific, USA). The extraction was repeated twice, and the supernatants were combined, and evaporated under reduced pressure at 45 °C using a rotary vacuum evaporator (Eyela N-100, Japan). Then, the concentrated solution was thoroughly dried by a vacuum drying oven (StableTemp XQ-05053-10, USA).

TPC. The TPC of various extracts were determined by the Folin−Ciocalteu method.[14] All samples were tested in triplicate.

Antioxidant Activity. *a. DPPH Radical Scavenging Activity.* The antiradical activity of the extracts was determined spectrophotometrically based on Blois's method with minor modifications.[15]

b. ABTS Assay. ABTS radical scavenging activity was determined using an assay kit (Beyotime Institute of Biotechnology, Nantong, China) according to Wu's method.[16]

c. FRAP Assay. The FRAP assay was carried out according to the method of Li et al.[17]

Antiproliferative Activity. *a. Cell Lines and Cell Culture.* Human hepatoma HepG2 cell line was purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). HepG2 cell lines were grown in 90% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 U/mL penicillin. Then the cells were put in a humidified atmosphere of 5% CO_2 and kept at 37 °C with medium replenishment every 3 days.

b. MTT Assay. Antiproliferative activity of *T. hemsleyanum* leaf extracts was evaluated by MTT (methyl thiazolyl tetrazolium) assay which was reported by Wu et al.[18] with minor modifications.

Qualitative Analysis by LC−ESI-QTOF-MS/MS. *a. LC Conditions.* The Agilent 1290 infinity series UPLC system consisted of a degasser, a binary pump Bin Pump SL, a thermostated HiP-ALS autosampler, a TCC SL column oven and a DAD detector (Agilent Technologies, USA). Separation was done in an Agilent Eclipse XDB-C_{18} column (4.6 mm × 250 mm, 5 μm) protected with an Agilent Eclipse XDB-C_{18} guard column (4.6 mm × 12.5 mm, 5 μm). The mobile phase consisted of 0.1% formic acid in deionized water (A) and acetonitrile (B). The solvent gradient was as follows: 0−10 min, 12−16% B; 10−35 min, 16−23% B; 35−38 min, 23−12% B. The mobile phase was returned to the initial conditions and the column was re-equilibrated during a 6 min postrun procedure. The column was thermostatically controlled at 40 °C, and the flow rate was set at 0.5 mL/min. The UV−visible absorbance of the peaks was detected between 190 and 400 nm. All samples and standards were dissolved in methanol, and the sample injection volume was 10 μL.

b. MS Conditions. An orthogonal acceleration quadrupole time-of-flight mass spectrometer (6538 Accurate-Mass QTOF LC/MS system; Agilent Technologies, USA) was equipped with an orthogonal electrospray ionization source (ESI). The ESI source was operated in the negative ion mode, and full scan mass spectral data were acquired over a range from m/z 100 to 1000. The optimum values of the source parameter were as follows: capillary voltage, +4.0 kV; drying gas flow, 10.0 L/min; drying gas temperature, 350 °C; nebulizing gas pressure, 40 psi. Moreover, the pseudomolecular ions $[M - H]^-$ were selected as precursor ions and subjected to MS/MS analysis. The collision energy was set at 20 eV, and the fragmentor voltage was set at 135 V, using nitrogen as collision gas.

Quantitative Analysis by LC−ESI-QqQ-MS/MS. *a. LC−MS Conditions.* A triple quadrupole mass spectrometer (6430 QqQ LC/MS system; Agilent Technologies, USA) coupled to an Agilent 1260 HPLC system was used for quantitative analysis. LC conditions for quantitative analysis were the same as those for qualitative analysis. The Agilent 6430 QqQ LC/MS system was also equipped with an orthogonal ESI, which was operated in the negative ion in multiple reaction monitoring (MRM) mode. The optimum values of the source parameters were as follows: capillary voltage, +4.0 kV; drying gas flow, 11.0 L/min; drying gas temperature, 300 °C; nebulizing gas pressure, 15 psi. The fragmentor voltages and collision energies were optimized individually for each transition.

b. Calibration and Quantification of Phenolics. In order to prepare a specific calibration plot, each compound (1.0 mg) was accurately weighted and dissolved in 1 mL of methanol. The resulting stock solution was diluted with methanol to obtain reference solutions

containing 10, 25, 50, 100, 150, and 200 μg/mL of external standard. The calibration curves were made by injecting each external standard solution in triplicate at each concentration level.

Statistical Analysis. Results were expressed as means ± standard deviations. The LC–MS data were acquired and analyzed by the software MassHunter Acquisition B.03.01, Qualitative Analysis B.03.01 and Quantitative Analysis B.03.02. Other data were analyzed by the SPSS statistical software, version 18.0 (SPSS Inc., USA). Statistical analysis was performed using the ANOVA (analysis of variance) followed by Duncan's multiple range tests to determine statistically different values on the level of significance at $P < 0.05$. Regression analysis was used to determine the correlation of two data sets.

RESULTS AND DISCUSSION

Extraction Yields and TPC of Various Extracts. The extraction yields of *T. hemsleyanum* leaves obtained by three

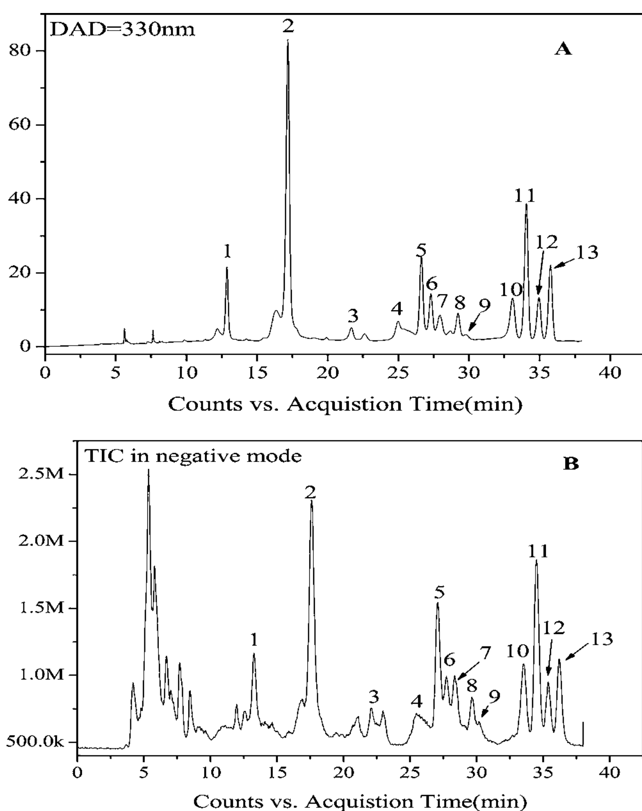

Figure 1. (A) DAD chromatogram in 80% methanol extract from *T. hemsleyanum* leaves detected at 330 nm. (B) Total ion chromatogram (TIC) in negative mode. Peaks: 3-caffeoylquinic acid (1); 5-caffeoylquinic acid (2); 1-caffeoylquinic acid (3); 5-*p*-coumaroylquinic acid (4); isoorientin-2″-*O*-rhamnoside (5); isoorientin (6); orientin-2″-*O*-rhamnoside (7); orientin (8); 1-*p*-coumaroylquinic acid (9); vitexin-2″-*O*-rhamnoside (10); isovitexin-2″-*O*-rhamnoside (11); vitexin (12) and isovitexin (13).

solvents (Table 1), ranging from 4.02% to 19.3%, were in the following order from the highest to the lowest: 80% methanol > ethyl acetate > hexane. As shown in Table 1, extraction with 80% methanol yielded the greatest quantity of total phenolics (275.71 mg of GAE/g DW), followed by ethyl acetate (91.26 mg of GAE/g DW) and hexane (28.95 mg of GAE/g DW). The results showed that 80% methanol was a more suitable solvent for extracting phenolics in *T. hemsleyanum* leaves compared to ethyl acetate and hexane. Methanol was also used to extract the phenolic compounds from leaves of *Salvia miltiorrhiza* and found to be more effective than acetone.[19]

The TPC (173.44–386.22 mg of GAE/g DW) extracted from the leaves of another kind of *Tetrastigma* (*Tetrastigma* sp.) were higher than that of *T. hemsleyanum.*[12] However, the TPC of leaves in many other plants were much lower. The TPC in the methanol extracts of *M. malabathricum* and *S. miltiorrhiza* leaves were reported to be 30.6 and 54.3 mg of GAE/g DW, respectively.[19,20] These results suggested that *T. hemsleyanum* leaves were rich in phenolic compounds.

Antioxidant Activities. *a. DPPH Radical Scavenging Activity.* DPPH assay was widely used for determining the antioxidant activity in plants because it can accommodate many samples in a short period and it was sensitive enough to detect active ingredients at low concentrations.[16,21] All of the extracts from *T. hemsleyanum* leaves showed high DPPH radical scavenging activities, with the values of 3.32, 2.16, and 1.23 mmol of Trolox/g DW for 80% methanol, ethyl acetate and hexane extracts, respectively (Table 1). These results suggested that 80% methanol extract had the highest DPPH radical scavenging activity. A similar result was also found in the leaves of *Tetrastigma* sp., which was the same family with *T. hemsleyanum.*[12]

b. ABTS Radical Scavenging Activity. As shown in Table 1, the antioxidant activities as measured by the ABTS assay were 1.85 mmol of Trolox/g DW (80% methanol), 0.49 mmol of Trolox/g DW (ethyl acetate) and 0.13 mmol of Trolox/g DW (hexane). Similar to the DPPH values, the 80% methanol extract had the highest ABTS radical scavenging activity among these extracts.

c. Ferric Reducing Activity. In the FRAP assay, 80% methanol extract exhibited the highest FRAP value (1.85 mmol of $FeSO_4$/g DW), while the hexane extract had the lowest (0.13 mmol of $FeSO_4$/g DW). The results indicated that all of the *T. hemsleyanum* leaf extracts showed strong antioxidant activities measured by FRAP, DPPH and ABTS assays (Table 1). Moreover, 80% methanol extract exhibited the highest antioxidant activities (DPPH, 3.32 mmol of Trolox/g DW; ABTS, 1.38 mmol of Trolox/g DW; FRAP, 1.85 mmol of $FeSO_4$/g DW), followed by ethyl acetate extract (2.16, 0.80 and 0.49) and hexane extract (1.23, 0.43 and 0.13). It showed that 80% methanol is the best solvent for extracting the antioxidant compounds from *T. hemsleyanum* leaves. Therefore, we chose 80% methanol as the extractant for investigating the phenolics.

d. Relationship between Phenolic Content and Antioxidant Activity. Regression analysis indicated a positive correlation between TPC and ABTS values (R^2 = 0.9979), FRAP values (R^2 = 0.9772) or DPPH values (R^2 = 0.9545). In general, extracts with higher TPC also had higher antioxidant activity, suggesting that the phenolics of *T. hemsleyanum* leaf extracts were the main contributors to the antioxidant activities. The same positive correlation between the TPC and the antioxidant activities in other plants has been reported before.[22−24]

Antiproliferative Activity. Cancer-related death can be caused by oxidative stress, and chemoprevention by natural or synthetic antioxidants may prevent cancer formation or progression.[25] Due to the strong in vitro chemical-based antioxidant activities, the antiproliferative activities of *T. hemsleyanum* leaf extracts were investigated. The cytotoxicity of extracts against HepG2 cell line is shown in Table 1. Among these extracts, 80% methanol and ethyl acetate extracts showed effects against HepG2 cells with IC_{50} values 524.9 ± 6.48 and

Table 2. Phenolic Compounds Determined by UPLC−QTOF-MS/MS in *T. hemsleyanum* Leaf Extract (80% Methanol Extract)

peak no.	t_R (min)	λ_{max} (nm)	$[M - H]^-$ (m/z) exptl	$[M - H]^-$ (m/z) calcd	formula	error (ppm)	MS/MS fragments (m/z)	proposed compd [39]
1	13.26	324, 298	353.0879	353.0878	$C_{16}H_{18}O_9$	−0.35	191.0557, 179.0752, 135.0452	3-caffeoylquinic acid[a,b]
2	17.79	324, 298	353.0879	353.0878	$C_{16}H_{18}O_9$	−0.29	191.0559	5-caffeoylquinic acid[a,b,c]
3	22.06	324, 298	353.0881	353.0878	$C_{16}H_{18}O_9$	−0.92	191.0569, 127.0196	1-caffeoylquinic acid[a,b]
4	25.42	313	337.0930	337.0929	$C_{16}H_{18}O_8$	−0.25	191.0565, 163.0425	5-*p*-coumaroylquinic acid[a,b]
5	27.08	350, 250	593.1509	593.1512	$C_{27}H_{30}O_{15}$	0.53	473.1128, 447.0907, 429.0866, 357.0632 327.0512, 298.0478	isoorientin-2″-*O*-rhamnoside[a,b]
6	27.76	350, 270	447.0930	447.0933	$C_{21}H_{20}O_{11}$	0.6	429.0814, 369.0624, 357.0611, 327.0530	isoorientin[a,b,c]
7	28.40	350, 250	593.1508	593.1512	$C_{27}H_{30}O_{15}$	0.69	473.1135, 447.0892, 429.0814, 357.0632 327.0511, 309.0395	orientin-2″-*O*-rhamnoside[b]
8	29.68	350, 270	447.0934	447.0933	$C_{21}H_{20}O_{11}$	−0.29	357.0568, 339.0520, 327.0507, 297.0381	orientin[a,b,c]
9	30.18	313	337.0927	337.0929	$C_{16}H_{18}O_8$	0.72	191.0568	1-*p*-coumaroylquinic acid[a,b]
10	33.53	338, 268	577.1566	577.1563	$C_{27}H_{30}O_{14}$	−0.62	457.1159, 431.0840, 413.0881, 311.0575	vitexin-2″-*O*-rhamnoside[a,b,c]
11	34.48	338, 270	577.1572	577.1563	$C_{27}H_{30}O_{14}$	−1.5	457.1150, 431.0977, 413.0884, 353.0667	isovitexin-2″-*O*-rhamnoside[a,b]
12	35.35	338, 268	431.0988	431.0984	$C_{21}H_{20}O_{10}$	−1.12	341.0672, 323.0623, 311.0543, 283.0620	vitexin[a,b,c]
13	36.26	336, 270	431.0987	431.0984	$C_{21}H_{20}O_{10}$	−0.75	413.0860, 341.0666, 323.0582, 311.0572	isovitexin[a,b,c]

[a]Compared with the literature. [b]Compared with MS^n data and characteristic UV spectra. [c]Compared with an authentic standard.

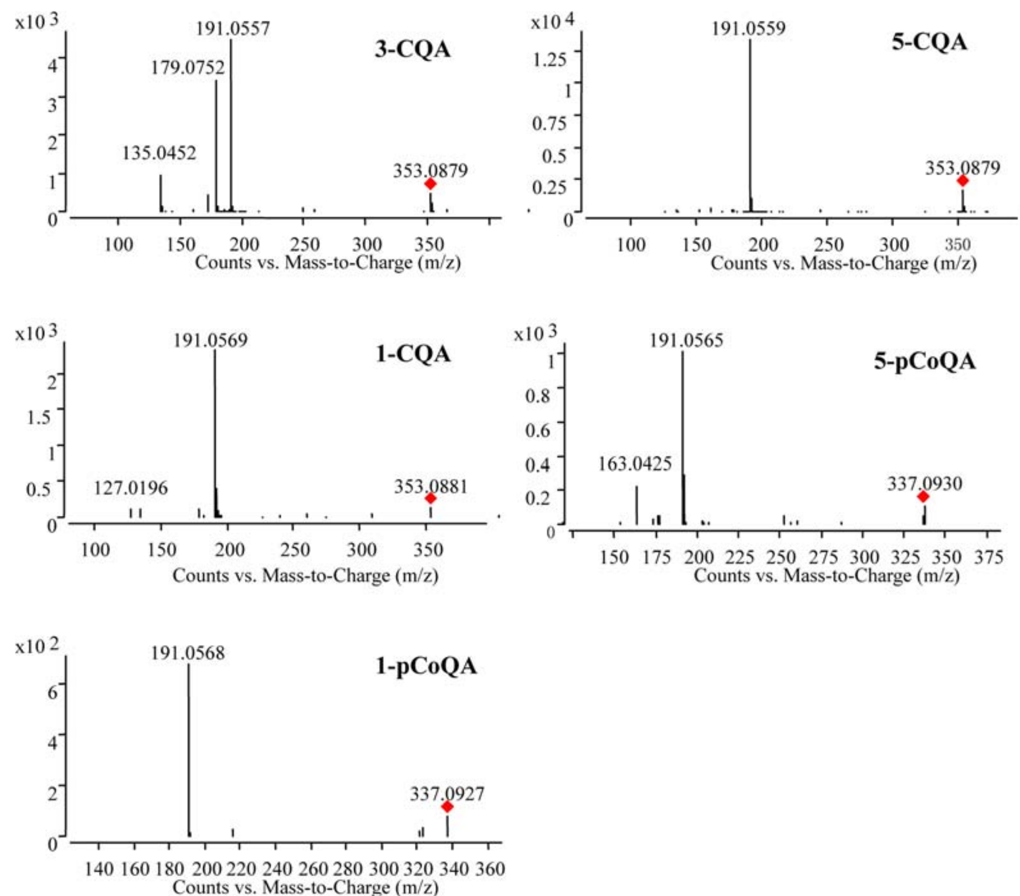

Figure 2. MS/MS spectra of chlorogenic acids in *T. hemsleyanum* leaf extracts.

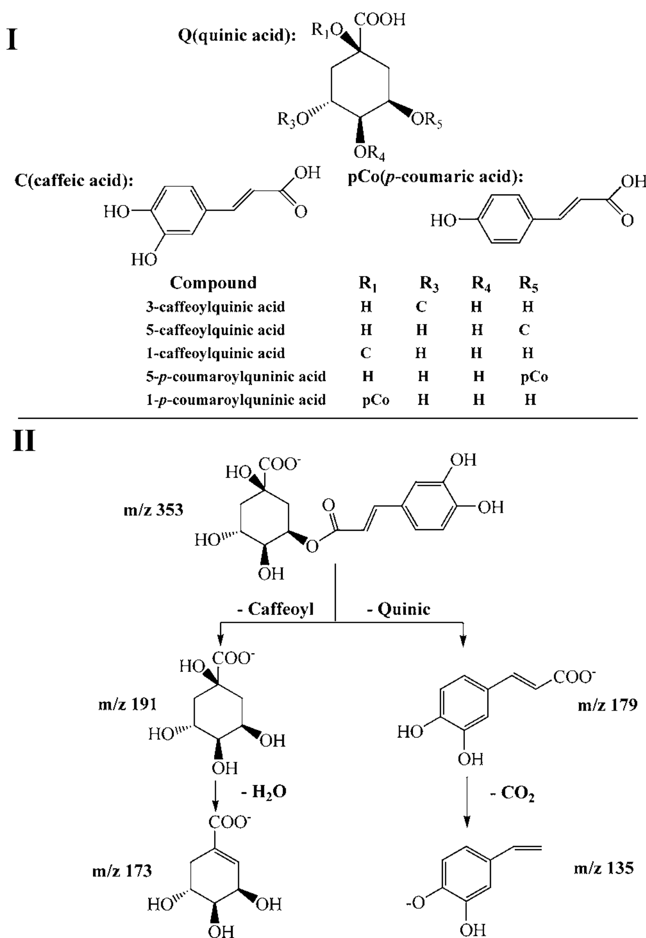

Figure 3. Structures (I) and fragmentation patterns (II, taking 5-caffeoylquinic acid for example) of chlorogenic acids in *T. hemsleyanum* leaf extracts.

705.3 ± 5.22 μg/mL, respectively, indicating that *T. hemsleyanum* leaves had an influence on HepG2 growth. However, the activities were much lower than that of positive control, cisplatin (8.88 ± 0.10 μg/mL). The antiproliferative activities of *T. hemsleyanum* root extracts have been reported before. Ding et al.[4] found that ethyl acetate fraction (1×10^{-3} g/L) of *T. hemsleyanum* root extracts exhibited a growth inhibition rate of 45% on HepG2, while Cheng and Lu[26] reported that the IC_{50} value of *T. hemsleyanum* root extracts on lung carcinoma cell line A549 was 1×10^{-3} g/L. Although the extracts of *T. hemsleyanum* roots may have stronger antiproliferative activities than those of leaves, it is still worth identifying the cytotoxic compounds in *T. hemsleyanum* leaves.

Qualitative Analysis. In this study, 80% methanol extract was selected for qualitative analysis because of the higher TPC and antioxidant activities. UPLC−ESI-QTOF-MS/MS analysis of phenolics in *T. hemsleyanum* leaf extracts (80% methanol extract) was performed to provide the retention time, fragmentation pattern and characteristic UV spectra for every peak and to identify HPLC peaks. Figure 1 showed the DAD chromatogram at 330 nm and the total ion chromatogram (TIC) in the negative ion mode. These peaks are summarized along with their retention times, characteristic UV spectra, observed and calculated *m/z*, error (ppm), molecular formula and MS/MS fragments (Table 2). Besides, the MS/MS spectra and proposed fragmentation patterns of the identified peaks are shown in Figures 2−5. In this study, thirteen peaks were detected and identified based on the MS^n data and by comparison with the literature and/or authentic standards: 3-caffeoylquinic acid (1), 5-caffeoylquinic acid (2), 1-caffeoylquinic acid (3), 5-*p*-coumaroylquinic acid (4), isoorientin-2″-*O*-rhamnoside (5), isoorientin (6), orientin-2″-*O*-rhamnoside (7), orientin (8), 1-*p*-coumaroylquinic acid (9), vitexin-2″-*O*-rhamnoside (10), isovitexin-2″-*O*-rhamnoside (11), vitexin (12) and isovitexin (13).

The roots of *T. hemsleyanum* were reported to contain phenolics such as kaempferol, quercetin and kaempferol-3-*O*-neohesperidoside.[7] Other phytochemicals such as apigenin-6-α-L-rhamnopyranosyl(1−4)-α-L-arabinopyranoside, apigenin-8-α-L-rhamnopyranosyl(1−4)-α-L-arabinopyranoside, kaempferol-7-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-glucopyranoside, apigenin-6,8-di-β-D-glucopyranoside, salicylic acid, succinic acid, lacceroic acid and ethyl gallate were found in the ethanol extract of whole herb in *T. hemsleyanum*.[1,27] Three phenolic acids (gallic acid, ethyl gallate and palmitic acid) were determined in the ethanol extract of whole herb from *Tetrastigma hypoglaucum Planch. ex Franch* (another family member of *Tetrastigma*).[28] Different from these phenolics, the *T. hemsleyanum* leaves contained chlorogenic acids, *C*-glycosidic flavonoids and *O*,*C*-diglycosidic flavonoids, which were identified for the first time in this study. The specific characteristics, used for the identification of peaks 1−13, are described in greater details as follows.

a. Chlorogenic Acids. Peaks 1, 2, and 3 with retention time (t_R) of 13.26, 17.79, and 22.06 min showed the same pseudomolecular ions $[M - H]^-$ at *m/z* 353 with maximum UV absorption at 298 and 324 nm. The MS^2 spectra of peak 2 gave fragment ions at *m/z* 191 $[\text{quinic acid} - H]^-$ (Figure 2). The t_R and UV/vis spectra of peak 2 were matched with those of the authentic standard (5-caffeoylquinic acid), and similarly the molecular ion and fragmentation pattern of the mass spectrum of the peaks were confirmed by LC−QTOF-MS.[29] As a result, peaks 1 and 3, with major fragment ions at *m/z* 191 $[\text{quinic acid} - H]^-$, *m/z* 179 $[\text{caffeoyl} - H]^-$ (peak 1) and *m/z* 191 $[\text{quinic acid} - H]^-$ (peak 3), were identified as 3-caffeoylquinic acid and 1-caffeoylquinic acid (Figure 3).[30,31] These peaks can also be distinguished by considering the elution order of chlorogenic acids.[32] 3-caffeoylquinic acid appeared first on a C18 HPLC column, followed by 5-caffeoylquinic acid and 1-caffeoylquinic acid.

Peaks 4 and 9 (t_R 7.08 and 30.18 min) showed the same $[M - H]^-$ ions at *m/z* 337. In MS^2 spectra (Figure 2), peak 4 showed the fragment ions at *m/z* 191 $[\text{quinic acid} - H]^-$ and 163 $[\text{coumaric acid} - H]^-$, while peak 9 had a major fragment ion at *m/z* 191 $[\text{quinic acid} - H]^-$. The MS data combined with the UV−vis spectra and the elusion order of the peaks in LC led to the tentative identification of peaks 4 and 9 to be 5-*p*-coumaroylquinic acid and 1-*p*-coumaroylquinic acid (Figure 3).[30,31]

b. C-Glycosidic Flavonoids. Peaks 6 and 8 with t_R of 27.76 and 29.68 min exhibited the similar maximum UV absorption (λ_{max} 270) and gave pseudomolecular ions *m/z* at 447. Peaks 6 and 8 were identified as isoorientin and orientin according to the eluting order, MS^n data (Figure 4), fragmentation patterns (Figure 5) and the authentic standards.[33,34] Peak 6 showed the fragment ions at *m/z* 429 ($[M - H - H_2O]^-$), 411 ($[M - H - 2H_2O]^-$), 369 ($^{0,4}X^- - H_2O$), 357 ($^{0,3}X^-$), 327 ($^{0,2}X^-$), 311 ($^{0,2}A^-$), 297 ($^{0,1}X^-$) and 285(Y^-), respectively. Peak 8 was associated with the fragment ions at *m/z* 357 ($^{0,3}X^-$), 339 ($^{0,3}X^- - H_2O^-$), 327 ($^{0,2}X^-$), 311 ($^{0,2}A^-$), 297 ($^{0,1}X^-$) and 285

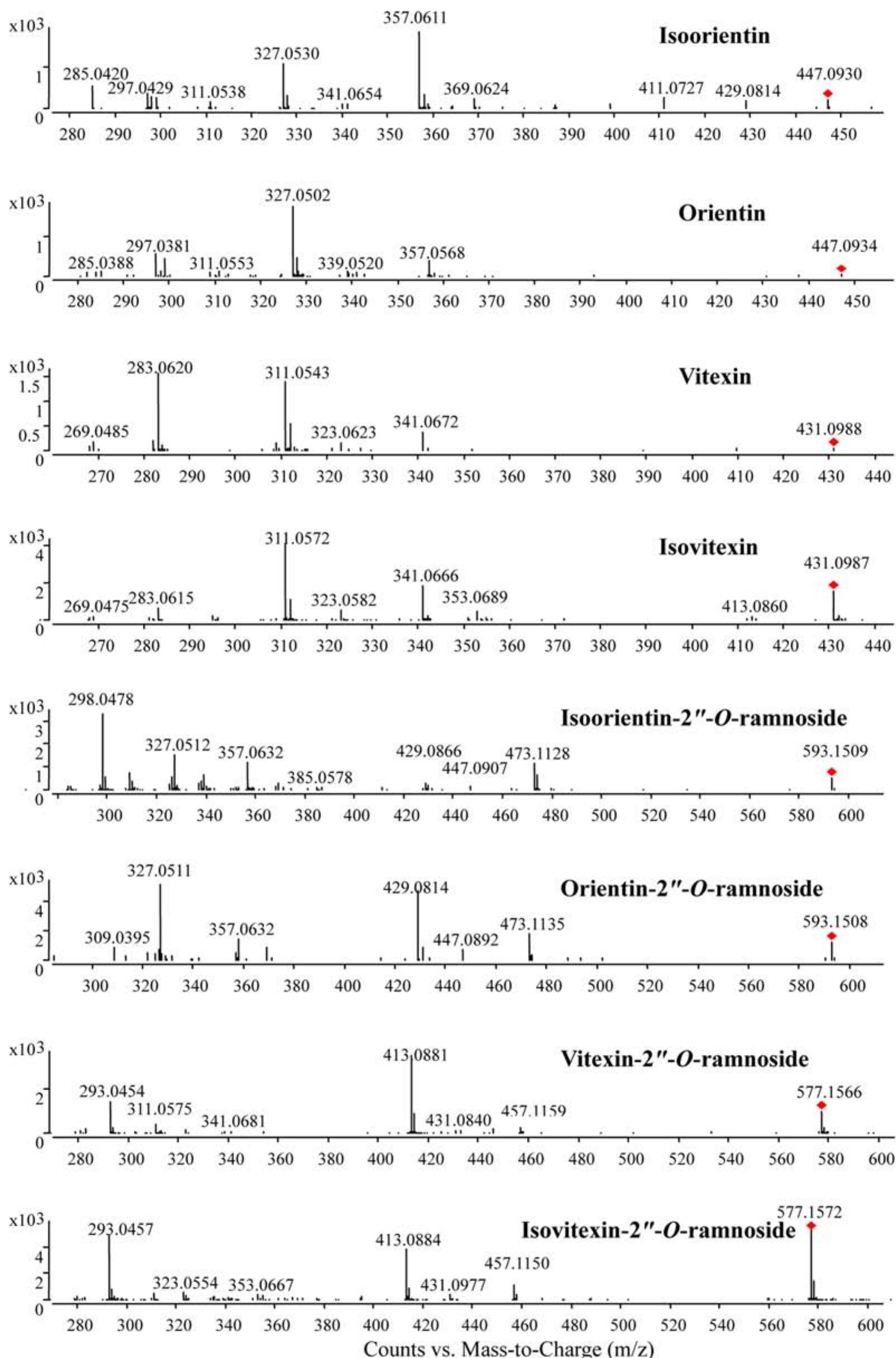

Figure 4. MS/MS spectra of *C*-glycosidic flavonoids and *O*,*C*-diglycosidic flavonoids in *T. hemsleyanum* leaf extracts.

(Y^-). Among these fragment ions, *m*/*z* 429 ($[M - H - H_2O]^-$) was only found in MS^2 of isoorientin, while it was not detected in orientin or its relative abundance was too low to be detected.[35] Hence, the fragment ion $[M - H - H_2O]^-$ can be used to distinguish 6-*C*-glycosidic flavonoid from 8-*C*-glycosidic flavonoid.

Peaks 12 (t_R 35.35 min) and 13 (t_R 36.26 min) had the same pseudomolecular ions at *m*/*z* 431 $[M - H]^-$. In MS^2 spectra (Figure 4), peak 12 produced fragment ions at *m*/*z* 413 ($[M - H - H_2O]^-$), 353 ($^{0,4}X^- - H_2O$), 341 ($^{0,3}X^-$), 323 ($^{0,3}X^- - H_2O$), 311 ($^{0,2}X^-$), 283 ($^{0,2}X^-$+2H•) and 269 (Y^-), while peak 13 gave fragment ions at *m*/*z* 341 ($^{0,3}X^-$), 323 ($^{0,3}X^- - H_2O$), 311 ($^{0,2}X^-$), 283 ($^{0,2}X^-$+2H•) and 269 (Y^-), respectively.

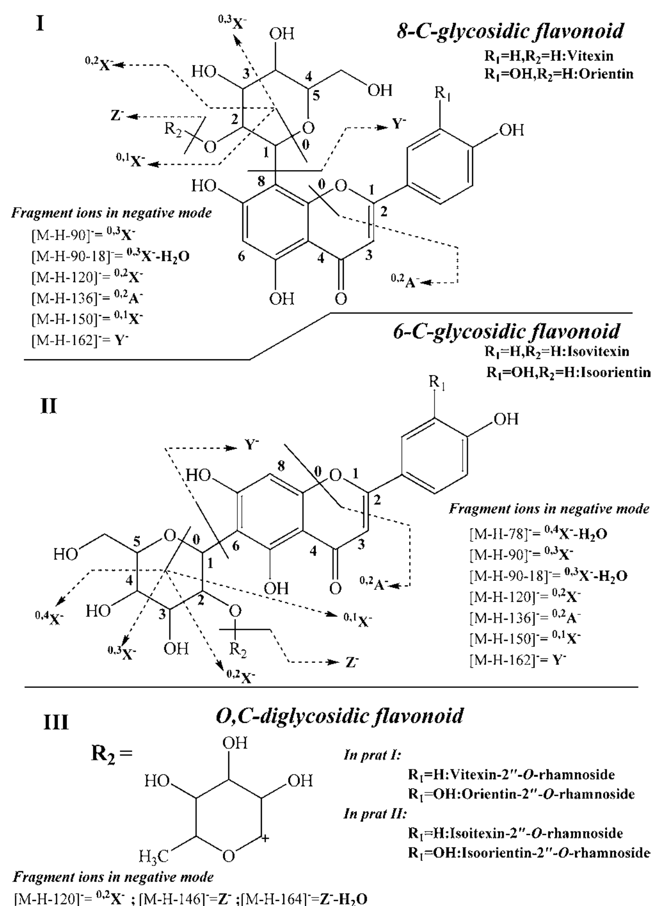

Figure 5. Structures and fragmentation patterns of *C*-glycosidic flavonoids (I, II) and *O,C*-diglycosidic flavonoids (III) in *T. hemsleyanum* leaf extracts.

These were in agreement with typical fragments of vitexin and isovitexin (Figure 5).[34,35] However, the fragmentation patterns of isovitexin and vitexin were the same as those of isoorientin and orientin. The fragment ion 413 $[M - H - H_2O]^-$ was found in peak 12, which was suggested to be 6-*C*-glycosidic flavonoid. Therefore, peaks 12 and 13 were identified as vitexin and isovitexin by comparing the fragment pattern and real standards.

c. O,C-Diglycosidic Flavonoids. For peaks 5 and 7 (t_R 27.08 and 28.40 min), with pseudomolecular ions $[M - H]^-$ at m/z 593, the fragment ions at m/z 473 $[M - H - 120]^-$, 447 $[M - H - \text{rhamnose}]^-$, 429 $[M - H - \text{rhamnose} - H_2O]^-$ and 327 $[M - H - 284]^-$ were found in the MS^2 spectra (Figure 4). Among these fragment ions, m/z 447 $[M - H - \text{rhamnose}]^-$ can be explained by the loss of a rhamnose moiety and then further fragmented to an ion at m/z 429 $[M - H - \text{rhamnose} - H_2O]^-$, corresponding to the loss of a water molecule. Besides, m/z 457 $[M - H - 120]^-$ resulted from a cross-ring cleavage of the *C*-glycosidic moiety and m/z 293 $[M - H - 284]^-$ was because of the combination of both fragmentations (Figure 5). The difference between peaks 5 and 7 was the relative abundance of m/z 327, which was also the difference between isoorientin and orientin. In addition, the collision energy can be another factor to distinguish this pair of isomers. Because the structure of isoorientin-2″-*O*-rhamnoside is more stable, it needs higher collision energy to be broken. Peaks 5 and 7 were therefore tentatively identified as isoorientin-2″-*O*-rhamnoside and orientin-2″-*O*-rhamnoside.[36]

Similarly, peaks 10 and 11, with t_R of 33.53 and 34.48 min, showed similar UV spectra. In full scan, the same pseudomolecular ions $[M - H]^-$ at m/z 577 were found in these peaks, suggesting that they were vitexin-2″-*O*-rhamnoside and isovitexin-2″-*O*-rhamnoside.[37] Their MS^2 ions at m/z 457 $[M - H - 120]^-$, 431 $[M - H - \text{rhamnose}]^-$, 413 $[M - H - \text{rhamnose} - H_2O]^-$ and 293 $[M - H - 284]^-$ corresponded to the same fragmentation patterns (Figure 5) of peaks 5 and 7. The MS^2 spectra are shown in Figure 4. Moreover, it was possible to accurately distinguish these peaks by comparison with the authentic standard (only vitexin-2″-*O*-rhamnoside was commercially available).

Quantitative Analysis. *a. Quantitative HPLC−ESI-QqQ-MS/MS Analysis.* The contents of individual phenolic compounds in *T. hemsleyanum* leaf extracts were determined by HPLC−ESI-QqQ-MS/MS. Since it is important to find a special fragment ion for each compound, quantitative analysis was recorded for the authentic standards. Due to the lack of standards, only six of the phenolics were quantitatively analyzed in this study.

In order to monitor vitexin-2″-*O*-rhamnoside by MRM, the special fragment ion at m/z 413.0 was selected. In addition, isovitexin and vitexin were monitored by the transition from the molecular ions at m/z 431.1 and 431.1 to the corresponding fragment ions at m/z 311.0 and 283.0, while isoorientin and orientin were monitored by the transition from the molecular ions at m/z 447.0 and 447.0 to the corresponding fragment

Table 3. Calibration Curve Data and Quantitative Results by HPLC−ESI-QqQ-MS/MS for the Phenolics in *T. hemsleyanum* Leaf Extracts[a]

compound	t_R (min)	calibration curve equation	R^2	LOD (ng/mL)	LOQ (ng/mL)	80% methanol extract (mg/g DW)[b]	ethyl acetate extract (mg/g DW)[b]	hexane extract (mg/g DW)[b]
5-caffeoylquinic acid	17.79	$y = 2.9388x - 10298.9144$	0.9996	1.24	13.26	38.47 ± 2.49	11.28 ± 1.12	nd
isoorientin	27.76	$y = 0.0944x + 36.6801$	0.9993	1.78	11.58	10.17 ± 1.52	1.56 ± 0.45	nd
orientin	29.68	$y = 0.0454x + 31.2403$	0.9999	1.32	9.63	11.30 ± 1.97	1.91 ± 0.33	nd
vitexin-2″-*O*-rhamnoside	33.53	$y = 0.1341x - 163.5998$	0.9970	1.47	10.42	10.21 ± 0.94	1.58 ± 1.32	nd
vitexin	35.35	$y = 0.0909x + 135.7908$	0.9964	1.62	12.13	8.28 ± 2.03	2.12 ± 0.55	nd
isovitexin	36.26	$y = 0.1393x - 140.2163$	0.9992	1.71	10.38	10.94 ± 2.71	2.43 ± 0.94	nd

[a]Results are expressed as mean ± standard deviation of three replicates. [b]DW, dry weight of extract.

ions at m/z 357.0 and 327.0. Besides, 5-caffeoylquinic acid was monitored by using a special fragment ion at m/z 191.0.

The quantitative results of extracts from *T. hemsleyanum* leaves are summarized in Table 3. For 80% methanol extract, the main phenolics was 5-caffeoylquinic acid, followed by orientin, isovitexin, vitexin-2″-*O*-rhamnoside, isoorientin and vitexin. Interestingly, ethyl acetate extract contained these compounds in a different order: 5-caffeoylquinic acid, isovitexin, vitexin, orientin, vitexin-2″-*O*-rhamnoside and isoorientin. However, these compounds were not found in hexane extract. The results suggested that the 80% methanol extract had higher contents of these compounds (8.28 to 38.47 mg/g DW) than ethyl acetate extract (1.56 to 11.28 mg/g DW), and 5-caffeoylquinic acid was the main phenolic compound in *T. hemsleyanum* leaves.

b. Validation of the Method. According to the European Medicines Agency (EMEA) guidelines[38] in terms of the validation of analytic methods, the HPLC−ESI-QqQ-MS/MS method was validated. The intraday precision and accuracy were calculated by analyzing three samples of each compound at three different concentration levels (25, 50, and 300 μg/mL) on the same day. Interday evaluation was executed during three consecutive days. The standard margin of error was <5%. The calibration curves obtained by plotting the ratio of the analyte signal to the external standard signal as a function of the analyte concentration were linear in the range of 10−200 μg/mL. The limit of detection (LOD) and limit of quantification (LOQ) values calculated for the six compounds were less than 2 and 14 ng/mL, respectively. The validation data of the method, which was developed for quantitative analysis of these compounds, are shown in Table 3. The analytical method was found to be reliable with a good reproducibility, as evidenced by the low values of standard deviation.

In conclusion, the qualitative and quantitative analysis of phenolics in *T. hemsleyanum* leaves were first reported. A total of thirteen phenolics were identified by LC−QTOF-MS, and six of them were further quantified by LC−QqQ-MS. Moreover, this study suggested that the higher TPC of *T. hemsleyanum* leaf extracts resulted in the higher antioxidant activity and antiproliferative activity. The results indicated that *T. hemsleyanum* leaves higher in TPC were a good source of antioxidants. This study may provide fundamental information for the development of *T. hemsleyanum* leaves as nutraceuticals and functional foods.

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Notes

The authors declare no competing financial interest.

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