NATURAL DRUGS

ANTIOXIDANT CAPACITY AND QUANTIFICATION ANALYSIS OF CHEMICAL CONSTITUENTS ISOLATED FROM *TETRASTIGMA HEMSLEYANUM* LEAVES

CHONG-LU SUN^{1#}, SHI-MIN LI^{1#}, SONG-LIN YU¹, YING-XIN YANG¹, YU-JIONG ZHANG¹,
SU-FANG HAN³, and XIN PENG^{1,2*}

¹Zhejiang Pharmaceutical College, Ningbo 315100, PR China

²Ningbo Research Institute of Zhejiang University, Ningbo 315100, PR China

³Zhejiang Academy of Forestry, Hangzhou 310023, PR China

Abstract: *Tetragastigma hemsleyanum* is an edible and medicinal plant in China. There are little researches on its leaves as the root is the conventionally used part. In this study, chemical constituents of *T. hemsleyanum* leaves (THL) were isolated and their antioxidant activities were studied. A total of fifteen flavonoids were obtained from this plant. They were kaempferol-3-O- β -rutinoside (1), quercetin (2), astragalin (3), quercitrin (4), isoquercitrin (5), rutin (6), vitexin (7), isovitexin (8), orientin (9), isoorientin (10), vicenin-2 (11), lucenin-2 (12), lucenin-1 (13), vicenin-1 (14), and vicenin-3 (15). Among them, compounds 12-15 were isolated from *T. hemsleyanum* for the first time. The results of antioxidant assays revealed that compounds 2, 4-6, 9-14 possess encouraging antioxidant activities. In addition to the antioxidant activity study, a sensitive and rapid analytical method was developed in this study to determine these antioxidants simultaneously by using ultra-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (UPLC-MS/MS). In conclusion, THL could be a new source for functional foods and a potential candidate for antioxidant agents.

Keywords: *Tetragastigma hemsleyanum*, antioxidant activity, chemical constituents, UPLC-MS/MS

Tetragastigma hemsleyanum Diels et Gilg (*T. hemsleyanum*) belongs to the family of Vitaceae and is a well-known edible and medicinal plant in China. It grows mainly in the area of the Yangtze River including Zhejiang, Fujian, Jiangxi, Guangxi, and Yunnan provinces in China (1). Previous pharmacology studies showed that the bulbous roots of *T. hemsleyanum* had multiple functions, such as anti-inflammation, anti-virus, and anti-tumor activities, and it can relieve cold and rheumatic pain, etc (2-4). The extracts of bulbous roots have been used as therapeutic agents in the Chinese herbal market. So far, most of the researches focused on the chemical composition and biological activities of the roots. But the phytochemical composition of *T. hemsleyanum* leaves (THL) is largely unknown, and little information is available on their healthful functions (5). However, extracts of the THL have been used for the treatment of hepatitis, rheumatoid arthritis, viral meningitis, and other diseases in folk medicine for a long time (6, 7). The results of our preliminary experiment

indicated that the n-butanol soluble fraction of THL might contain some antioxidants due to its significant free radical scavenging activity. Therefore, it is necessary to provide scientific evidence for the characterization of antioxidant substances in THL.

Many chronic diseases, including cardiovascular diseases, chronic inflammation, cancer, and Alzheimer's disease, are associated with the harmful effects induced by excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS). Some antioxidants may play a dominant role in the prevention of neurodegenerative diseases by scavenging free radicals and by relieving the oxidation of biomolecules (8, 9). In order to reduce the harm of reactive oxygen free radicals, more and more antioxidants have been explored, such as vitamin C, vitamin E, butylated hydroxyanisole (BHA), etc. With the enhancement of awareness of potential adverse effects, the use of these chemosynthetic antioxidants was limited by legislation (10). Compared with chemosynthetic antioxidants, natural antioxidants have the advantages of low toxicity and

[#] These authors contributed equally to this work

* Corresponding author: e-mail: pengx@nit.zju.edu.cn

high absorbance (11). Therefore, it seems to be a feasible approach to look for safe and effective antioxidant ingredients from traditional Chinese herbs (12).

In this study, 15 compounds were isolated and identified from THL. Then, their antioxidant activities were determined and ten of them were found to have strong antioxidant activities. In addition, an analytical method for simultaneous determination of the ten antioxidants was established by using ultra-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (UPLC-MS/MS) for the first time.

EXPERIMENTAL

Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl and 2,4,6-tripyr-
idyl triazine were purchased from Beijing Solarbio
Technology Co., Ltd. Acetonitrile was of HPLC
grade (Tedia, USA), and formic acid was of HPLC
grade (Merck, Germany). Deionized water (H₂O)
was purified by a super purification system (Eped
Technology Development, Nanjing, China). Other
reagent solutions were of analytical grades, such as
ethanol, CHCl₃, MeOH, and EtOAc, and were pur-
chased from Sinopharm Chemical Reagent Co., Ltd.

General experimental procedures

NMR spectra were determined using a Bruker
spectrometer at 400 MHz for ¹H and 100 Mz for ¹³C

in DMSO as solvent using tetramethylsilane (TMS)
as internal standard. HERSIMS spectra were mea-
sured using a Bruker MaXis ultra-high-resolution
TOF apparatus with electrospray ionization (ESI).
UV was recorded by a JASCO V-650 spectropho-
tometer. Prep-HPLC separation was performed on
the P3500 instrument (Dalian, China) with Shimadzu
column C₁₈ (250 × 10 cm, 5.0 μm). Pre-TLC was car-
ried out on precoated silica gel F254 plates (20 ×
20 cm, 1.0 mm layer thickness, Merck). Sephadex
LH-20 (25-100 μm, Solarbio, China) and silica gel
(200-300 mesh, Qingdao Haiyang) were used for col-
umn chromatography. TLC was performed on pre-
coated 0.25 mm thick Merck (Darmstadt, Germany)
silica gel F254 aluminum-backed plates. Fractions
were monitored by TLC with visualization under UV
light (254 and 365 nm) and visualized by spraying
with 5% H₂SO₄-ethanol reagent and heating at 120°C
for 3 min. The quantification analysis was performed
using the LC-20AD system (Shimadzu, Japan) cou-
pled with SCIEX QTRAP 4500 ESI-MS/MS (SCIEX,
USA) consisting of a triple quadrupole and ion source
of electrospray ionization (ESI). All other chemicals
and solvents were of analytical grade.

Plant material

The fresh *T. hemsleyanum* leaves were col-
lected at a different time from nine provinces, in-
cluding Zhejiang, Anhui, Guangxi, Guangdong,
Jiangxi, Chongqing, Fujian, Guizhou, and Yunnan.

Table 1. The sample information of *T. hemsleyanum* from different habitats.

Accession No.	Gathering areas	Latitude	Longitude	Collection time
S-1	Wuyi Zhejiang	N28°76'	E119°41'	2019.10
S-2	Suichang Zhejiang	N29°31'	E118°58'	2019.10
S-3	Lishui Zhejiang	N28°26'	E119°50'	2019.10
S-4	Longquan Zhejiang	N28°08'	E119°13'	2019.10
S-5	Quzhou Zhejiang	N28°66'	E118°91'	2019.11
S-6	Yongkang Zhejiang	N28°45'	E119°53'	2019.10
S-7	Ningbo Zhejiang	N29°46'	E121°31'	2018.11
S-8	Chongming Guangxi	N22°45'	E107°21'	2018.10
S-9	Guilin Guangxi	N24°78'	E110°87'	2019.10
S-10	Baile Guangxi	N24°79'	E114°56'	2018.10
S-11	Huangshan Anhui	N30°13'	E118°18'	2019.10
S-12	Qingyuan Guangdong	N23°81'	E115°78'	2019.01
S-13	Shangrao Jiangxi	N28°67'	E117°34'	2018.11
S-14	Sanhe Chongqing	N29°35'	E106°11'	2018.11
S-15	Chenggong Yunnan	N24°51'	E102°50'	2019.10
S-16	Sanming Fujian	N26°12'	E117°31'	2019.10
S-17	Xinyi Guizhou	N24°59'	E104°50'	2019.11

The detailed sample information was shown in Table 1. The specimens were identified and authenticated by Prof. Xin Peng, Zhejiang University. All voucher specimens were deposited at the Biotechnology Institute of Zhejiang Pharmaceutical College, Zhejiang province in China.

Extraction and isolation

T. hemsleyanum sample (S-7) was provided by Shengwang Biotechnology Corporation, and used for the extraction and separation of compounds in subsequent studies. The air-dried and powdered plant of THL (5000 g) was extracted with 20 L of 70% ethanol at room temperature for 3 h, repeat 3 times. The extract was filtered and evaporated to dryness under vacuum at 45°C. The obtained dry extract (800 g) was then suspended in hot MeOH-H₂O (1: 10, v/v) and successively partitioned with n-hexane, methylene chloride, ethyl acetate, n-butanol to yield the corresponding fractions. Each fraction was tested for the radical-scavenging effect by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Among these fractions, the n-butanol soluble fraction (150 g) showed the most significant free radical scavenging activity. The n-butanol soluble fraction was subjected to chromatography on a Sephadex LH-20 column (MeOH) to afford six fractions (Fr.1-Fr.6). Fr.1 was separated on silica gel column chromatography (EtOAc-MeOH, 5: 1) to give three subfractions (Subfr.1.1-Subfr.1.3). Fr.2 was subjected to silica gel column chromatography (CHCl₃/MeOH 10: 1) to yield compound 1 (21 mg) and compound 6 (22 mg), respectively. Compound 2 (18 mg) and compound 5 (22 mg) were crystallized from Fr.3 and Fr.4, respectively. Fr.5 was subjected to silica gel column chromatography (EtOAc/EtOH/H₂O 10:1:0.2) and prep-HPLC (ACN/H₂O) to obtain compound 4 (27 mg), compound 7 (30 mg), and compound 8 (29 mg). Fr.6 was purified by prep-TLC (EtOH/MeOH 2:1) and prep-HPLC (ACN/H₂O) to obtain compound 3 (27 mg), compound 9 (29 mg), and compound 10 (38 mg). Subfr.1.1 was purified by prep-HPLC using Shimadzu column (ACN/H₂O) to give compound 12 (63 mg). Subfr.1.2 was crystallized with MeOH to yield compound 13 (88 mg). Subfr.1.3 was purified by prep-TLC (EtOH/MeOH 1: 1) and prep-HPLC (ACN/H₂O) to obtain compound 11 (53 mg), compound 14 (69 mg), and compound 15 (86 mg).

Evaluation of antioxidant activity

DPPH free radical scavenging activity assay

The evaluation of DPPH free radical scavenging activity was carried out according to the previously reported method with some modifications

(13). 1.0 mL of methanol solutions at various concentrations (1-100 µg/L) was mixed with 2.0 mL of DPPH in ethanol (0.1 mM). The reaction mixture was shaken and incubated at room temperature. The absorbance of the reaction mixture was measured at 517 nm by UV-Vis spectrophotometer 30 min later. The inhibition percentage of DPPH free radicals was calculated as follows: inhibition% = $[(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$; A_{blank} was the absorbance of DPPH without sample, and A_{sample} was the absorbance of DPPH with a sample in it. The results were expressed by IC₅₀, which represented the sample concentration required to inhibit 50% DPPH free radicals. IC₅₀ was measured by the relationship curve between concentration and absorbance. The results presented are the mean of two experiments.

Scavenging ABTS free radical activity assay

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging activity was measured according to the previously reported method with some modifications (14). 7 mM ABTS solution prepared in water was mixed with 2.45 mM potassium persulphate solution and the mixture was kept at room temperature in dark for 12-16 h to produce ABTS⁺ free radicals. The absorbance of the ABTS⁺ solution was set at 0.700 ± 0.02 at 734 nm by diluting with ethanol. Then, 2 mL of ABTS⁺ solution was added to 1.0 mL of each sample solution at various concentrations (1-100 µg/L). The mixture was incubated for 4 min and the absorbance was measured at 734 nm. The antioxidant activity was calculated by the same equation as the DPPH method.

Ferric-reducing antioxidant power (FRAP) assay

FRAP assay was carried out using the method based on a previous study (15). The capability of reducing the tripyridyltriazine complex of Fe³⁺ i.e. (Fe³⁺/TPTZ) into blue-colored complex Fe²⁺/TPTZ was measured. 0.3 M acetate buffer (pH 3.6), 10 mmol of TPTZ solution in 40 mmol of HCl, and 20 mmol of ferric chloride hexahydrate solutions in the ratio of 10: 1: 1 (v/v/v) were mixed and heated at 37°C to prepare FRAP reagent. A series of standard solutions of ferrous ions (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol/L) were prepared by diluting the stock solution (10mmol/L) with water for the establishment of standard curves. 0.2 mL of the above standard solution was added into a 3mL FRAP reagent. The reaction mixture was incubated at room temperature for 15 min and the absorbance was measured at 595 nm. Antioxidant activity was calculated according to the standard curve (for example, the absorbance of 0.2 mmol/L sample is equivalent to

1 mmol/L FeSO₄ standard solution, the relative antioxidant ability of the sample is 5).

UPLC conditions

UPLC analysis was performed on a Shimadzu LC-20 AD system (Shimadzu Corp., Tokyo, Japan) equipped with an autosampler, a binary solvent delivery pump, and Analyst Software 1.6.3. The separation was performed on the Kinetex XB-C₁₈ column (50 mm × 2.1 mm, 2.6 μm), with the mobile phase consisting of aqueous formic acid (0.1% in water, solvent A) and acetonitrile (solvent B) in gradient elution mode. The gradient for the negative electrospray ionization (ESI) mode was started and held at 10% B for 1 min, raised linearly from 10 to 60% B during the next 6 min, and then maintained at 60% B for 2 min. Then, the mobile phase returned to 10% B within 2 min and the system was equilibrated for 5 min before the next run. The flow rate was 0.1 mL/min, the injection volume was 1.0 μL and the column temperature was 20°C.

Mass Spectrometric Conditions

An AB-SCIEX 4500 Triple-Quad mass spectrometer was connected to the Shimadzu LC-20 AD Ultra Performance Liquid Chromatograph via ESI interface for analysis. The MultiQuant software packages were used for data acquisition and processing. The determination for all analytes was carried out on tandem mass spectrometry in the negative ion ESI mode and multiple reaction monitoring (MRM) mode. The parameters in the negative ion mode were as follows: ion source temperature, 550°C; ion spray voltage, 4.5 kV; air curtain gas, 40 psi; spray gas, 50 psi; auxiliary heating gas, 50 psi. The collision gas (N₂) flow was set at medium.

Preparation of standard solutions

A mixed standard stock solution containing compounds 2, 4-6, 9-14 was prepared in methanol. Then it was diluted with methanol to obtain a series concentrations of working standard solutions within ranges: compound 2, 5-500 ng/mL; compound 4, 5-500 ng/mL; compound 5, 5-500 ng/mL; compound 6, 5-1000 ng/mL; compound 9, 5-1000 ng/mL; compound 10, 5-2000 ng/mL; compound 11, 5-2000 ng/mL; compound 12, 5-2000 ng/mL; compound 13, 5-1000 ng/mL; compound 14, 5-2000 ng/mL. All solutions were stored at -20°C and filtered through a 0.22 μm membrane prior to injection.

Preparation of sample solutions

Seventeen batches of 300 mg dried and powdered THL were soaked with 50 mL of 75% methanol for 30 minutes, then ultrasonicated twice for

30 minutes, and finally made up to a volume of 50 mL with 75% methanol. The solution was filtered through a 0.22 μm membrane and the filtrates were diluted ten-fold with methanol. Then 1.0 μL of aliquot was injected into the UPLC-MS/MS system for analysis. All the experiments were repeated three times.

Method validation

Various concentrations of standard solutions were prepared for the establishment of calibration curves. It was obtained by linear regression between the peak area (Y) and analyte concentration (X), the corresponding linear range and correlation coefficient were also gained. The limits of detection (LOD) and limits of quantification (LOQ) were determined when the signal-to-noise ratio was 10 and 3, respectively. They were determined by serial dilution of standard solution using the above-mentioned UPLC-MS/MS conditions. The repeatability, intra-day, and inter-day precisions were expressed as percentage relative standard deviations (RSD). The mixture standard solution was measured six times during a single day and six consecutive days for evaluation for intra-day and inter-day based on changes in the peak area. Repeatability was confirmed with six independent samples from the same batch and the variations were expressed by RSD. Recovery was used to evaluate the accuracy of this method. It was determined by comparing the average peak areas of extracted samples at low, medium, and high concentrations with the average peak areas of spike-after-extraction samples.

RESULTS AND DISCUSSION

Identification of compounds

The n-butanol fraction was subjected to separation by various sessions of chromatography to produce 15 compounds (Figure 1). Based on the analysis of chemical property and spectral data, compounds 1-15 were identified as: kaempferol-3-O-β-rutinoside (1); quercetin (2); astragalin (3); quercitrin (4); isoquercitrin (5); rutin (6); vitexin (7); isovitexin (8); orientin (9); isoorientin (10); vicienin-2 (11); lucenin-2 (12); lucenin-1 (13); vicienin-1 (14); vicienin-3 (15), respectively. Among them, compounds 12-15 were isolated from *T. hemsleyanum* for the first time.

Kaempferol-3-O-β-rutinoside (1): ESI-MS m/z: 595[M+H]⁺. ¹H-NMR (400 MHz, DMSO-d₆) δ: 12.55 (1H, s, 5-OH), 10.85 (1H, s, 7-OH), 10.13 (1H, s, 4'-OH), 7.97 (2H, d, J = 8.8 Hz, H-2', 6'), 6.86 (2H, d, J = 8.8 Hz, H-3', 5'), 6.40 (1H, d, J = 1.6 Hz, H-8), 6.20 (1H, d, J = 1.6 Hz, H-6), 5.32 (1H, d, J = 8.0 Hz, Glu-H-1), 4.38 (H, s, Rha-H-1), 0.98

Figure 1. Structures of compound 1-15.

(3H, d, $J = 3.2$ Hz, Rha-CH₃); ¹³C-NMR (100 MHz, DMSO-d₆, δ_c) See Table 2. These data were in consistent with those of kaempferol-3-O- β -rutinoside (16).

Quercetin (2): ESI-MS m/z : 303[M+H]⁺. ¹H-NMR (400 MHz, DMSO-d₆, δ): 12.48 (1H, s, 5-OH), 9.59 (1H, s, 7-OH), 9.32 (2H, s, 3', 4'-OH), 7.68 (1H, d, $J = 2.0$ Hz, H-2'), 7.54 (1H, dd, $J = 8.8$, 2.2 Hz, H-6'), 6.88 (1H, d, $J = 8.8$ Hz, H-5'), 6.40 (1H, d, $J = 2.0$ Hz, H-8), 6.18 (1H, d, $J = 2.0$ Hz, H-6); ¹³C-NMR (100 MHz, DMSO-d₆, δ_c) See Table 2. These data were in consistent with those of quercetin (17).

Astragalin (3): ESI-MS m/z : 449[M+H]⁺. ¹H-NMR (400 MHz, DMSO-d₆, δ): 12.61 (1H, s, 5-OH), 10.22 (1H, s, 7-OH), 8.05 (2H, d, $J = 9.2$ Hz, H-2', 6'), 6.88 (2H, d, $J = 9.2$ Hz, H-3', 5'), 6.43 (1H, d, $J = 2.0$ Hz, H-8), 6.21 (1H, d, $J = 2.1$ Hz, H-6), 5.45 (1H, d, $J = 7.2$ Hz, Glu-H-1), 2.90~3.57 (6H, m, Glu-H); ¹³C-NMR (100 MHz, DMSO-d₆, δ_c) See Table 2. These data were in consistent with those of astragalin (18).

Quercitrin (4): ESI-MS m/z : 449[M+H]⁺. ¹H-NMR (400 MHz, DMSO-d₆, δ): 12.65 (1H, s, OH-5), 10.86 (1H, s, OH-7), 9.69 (1H, s, OH-4'), 9.36 (1H, s, OH-3'), 7.30 (1H, d, $J = 2.0$ Hz, H-2'), 7.26 (1H, dd, $J = 8.4$, 2.4 Hz, H-6'), 6.86 (1H, d, $J = 8.4$ Hz, H-5'), 6.39 (1H, d, $J = 2.0$ Hz, H-8), 6.20 (1H, d, $J = 2.0$ Hz, H-6), 5.25 (1H, d, $J = 1.2$ Hz, Rha-H-1), 0.82 (3H, d, $J = 7.0$ Hz, Rha-H-6); ¹³C-NMR (100 MHz, DMSO-d₆, δ_c) See Table 2. These data were in consistent with those of quercitrin (19).

Isoquercitrin (5): ESI-MS m/z : 465[M+H]⁺. ¹H-NMR (400 MHz, DMSO-d₆, δ): 12.62 (1H, s, 5-OH), 10.89 (1H, s, 7-OH), 9.75 (1H, s, 4'-OH), 9.22 (1H, s, 3'-OH), 7.68 (1H, d, $J = 1.6$ Hz, H-2'), 7.54 (1H, dd, $J = 2.0$, 8.5 Hz, H-6'), 6.82 (1H, d, $J = 8.4$ Hz, H-5'), 6.41 (1H, d, $J = 2.0$ Hz, H-8), 6.20 (1H, d, $J = 2.0$ Hz, H-6), 5.38 (1H, d, $J = 7.6$ Hz, Glu-H-1); ¹³C-NMR (100 MHz, DMSO-d₆, δ_c) See Table 2. These data were in consistent with those of isoquercitrin (20-21).

Table 2. NMR Spectroscopic data of compound 1-15.

Compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
position	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c	δ_c
2	156.5	146.8	156.4	156.5	156.3	156.7	164.0	163.5	164.1	163.6	164.2	164.3	164.3	164.2	164.0
3	133.2	135.7	133.2	134.2	133.6	133.6	102.5	102.8	102.4	102.8	102.5	102.8	102.5	102.5	102.2
4	177.4	175.8	177.5	177.8	177.5	177.4	182.1	181.9	182.0	181.8	182.4	182.3	182.3	182.4	182.4
5	161.2	156.1	161.2	161.3	161.3	161.3	160.4	161.2	160.4	160.6	158.7	158.6	158.3	158.3	158.2
6	93.7	93.3	98.7	98.7	98.7	98.7	98.2	108.9	98.2	108.8	107.6	107.5	108.1	108.2	108.5
7	164.1	163.9	164.1	164.2	164.2	164.1	162.6	163.2	162.7	163.2	161.3	160.9	161.3	161.3	161.3
8	98.2	98.2	93.7	93.6	93.6	93.6	104.6	93.6	104.0	93.5	103.3	103.3	103.9	105.2	104.3
9	156.8	160.7	156.3	157.3	156.4	156.5	156.0	156.2	156.0	156.2	155.2	155.1	155.2	155.2	154.4
10	104.0	103.0	104.0	104.1	104.0	104.0	104.1	103.4	104.6	103.4	102.9	102.8	102.8	103.8	103.1
1'	120.9	122.0	120.9	121.1	121.2	121.6	121.6	121.1	122.0	121.4	121.5	121.9	121.6	121.6	121.5
2'	130.9	115.1	130.9	115.7	115.2	115.3	128.9	128.5	114.0	113.3	129.1	114.3	114.3	129.2	128.8
3'	115.1	145.0	115.1	145.2	144.9	144.8	115.8	116.0	145.8	145.7	115.9	145.9	145.9	115.9	115.8
4'	159.9	147.7	159.9	148.4	148.5	148.4	161.1	160.6	150.0	149.7	160.9	149.8	149.9	161.1	161.3
5'	115.1	115.6	115.1	115.5	116.0	116.3	115.8	116.0	115.7	116.0	115.7	115.9	116.4	115.7	115.9
6'	130.9	120.0	130.9	121.1	122.2	121.2	128.9	128.5	119.4	119.0	128.8	119.5	119.7	129.2	128.8
1''	101.4		100.9	101.9	101.9	101.2	78.7	73.1	73.4	73.0	74.2	74.1	74.2	74.3	73.9
2''	74.2		74.2	70.4	71.3	74.1	73.4	70.6	73.4	70.6	72.1	70.8	70.1	68.4	71.3
3''	76.4		77.5	70.6	73.3	76.5	70.9	78.9	78.8	78.9	78.9	78.9	73.8	73.9	78.9
4''	69.9		69.9	71.2	68.0	70.6	70.6	70.3	70.7	70.2	70.6	70.7	68.4	69.9	70.9
5''	75.7		76.4	70.1	75.9	76.0	81.8	81.5	82.0	81.5	82.0	80.9	69.5	70.6	81.3
6''	66.9		60.9	17.5	60.2	67.0	61.3	61.5	61.7	61.5	61.4	61.4			60.8
1'''	100.7					100.8					73.5	73.5	73.2	73.4	75.0
2'''	70.3					70.1					71.2	71.9	71.8	71.2	70.1
3'''	70.6					70.4					77.9	77.9	78.9	78.9	73.9
4'''	71.8					71.9					69.1	70.8	70.8	70.8	70.1
5'''	68.2					68.3					81.0	80.9	82.0	81.9	70.9
6'''	17.7					17.7					60.0	61.7	61.5	61.2	

Note: ¹³C-NMR data(δ) were measured in DMSO-d₆ at 100 MHz.

Rutin (6): ESI-MS m/z: 611[M+H]⁺. ¹H-NMR (400 MHz, DMSO-d₆) δ : 12.59 (1H, s, OH-5), 10.85 (1H, s, OH-7), 9.69 (1H, s, OH-4'), 9.20 (1H, s, OH-3'), 7.54 (1H, d, *J* = 2.0 Hz, H-2'), 7.53 (1H, s, H-6'), 6.83 (1H, d, *J* = 8.4 Hz, H-5'), 6.38 (1H, d, *J* = 1.2 Hz, H-8), 6.20 (1H, d, *J* = 1.2 Hz, H-6), 5.34 (1H, d, *J* = 6.8 Hz, Glu-H-1), 4.38 (1H, s, Rha-H-1), 0.99 (3H, d, *J* = 6.0 Hz, Rha-H-6); ¹³C-NMR (100 MHz, DMSO-d₆, δ_c) See Table 2. These data were in consistent with those of rutin (22).

Vitexin (7): ESI-MS m/z: 433[M+H]⁺. ¹H-NMR (400 MHz, DMSO-d₆) δ : 13.17 (1H, s, 5-OH), 10.85 (1H, s, 7-OH), 10.37 (1H, s, 4'-OH), 8.03 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.89 (2H, d, *J* = 8.4 Hz, H-3', 5'), 6.78 (1H, s, H-3), 6.27 (1H, s, H-6), 4.68 (1H, d, *J* = 10.0 Hz, Glu-H-1); ¹³C-NMR (100 MHz,

DMSO-d₆, δ_c) See Table 2. These data were in consistent with those of vitexin (23).

Isovitexin (8): ESI-MS m/z: 433[M+H]⁺. ¹H-NMR (400 MHz, DMSO-d₆) δ : 13.55 (1H, s, 5-OH), 10.38 (1H, s, 4'-OH), 7.93 (2H, d, *J* = 8.8 Hz, H-2', 6'), 6.91 (2H, d, *J* = 8.8 Hz, H-3', 5'), 6.78 (1H, s, H-3), 6.52 (1H, s, H-8), 4.60 (1H, d, *J* = 9.6 Hz, Glu-H-1); ¹³C-NMR (100 MHz, DMSO-d₆, δ_c) See Table 2. These data were in consistent with those of isovitexin (24).

Orientin (9): ESI-MS m/z: 449[M+H]⁺. ¹H-NMR (400 MHz, DMSO-d₆) δ : 13.17 (1H, s, 5-OH), 10.56 (1H, s, 7-OH), 10.04 (1H, s, 3'-OH), 9.22 (1H, s, 4'-OH), 7.53 (1H, d, *J* = 8.4 Hz, H-6'), 7.48 (H, brs, H-2'), 6.86 (1H, d, *J* = 8.4 Hz, H-5'), 6.64 (1H, s, H-3), 6.26 (1H, s, H-6), 4.68 (1H, d, *J* = 10.0 Hz, Glu-H-1);

^{13}C -NMR (100 MHz, DMSO- d_6 , δ_c) See Table 2. These data were in consistent with those of orientin (25).

Isoorientin (10): ESI-MS m/z : 449 $[M+H]^+$. ^1H -NMR (400 MHz, DMSO- d_6) δ : 13.56 (1H, s, 5-OH), 10.53 (1H, s, 7-OH), 9.65 (1H, s, 3'-OH), 9.47 (1H, s, 4'-OH), 7.42 (1H, d, $J = 8.4$ Hz, H-6'), 7.40 (1H, brs, H-2'), 6.89 (1H, d, $J = 8.0$ Hz, H-5'), 6.68 (1H, s, H-3), 6.48 (1H, s, H-8), 4.58 (1H, d, $J = 10.0$ Hz, Glu-H-1); ^{13}C -NMR (100 MHz, DMSO- d_6 , δ_c) See Table 2. These data were in consistent with those of isoorientin (26).

Vicenin-2 (11): ESI-MS m/z : 596 $[M+H]^+$. ^1H -NMR (400 MHz, DMSO- d_6) δ : 13.71 (1H, s, OH-5), 10.39 (1H, s, OH-7), 9.33 (1H, s, OH-4'), 8.05 (2H, d, $J = 8.8$ Hz, 2H, H-2', 6'), 6.98 (d, $J = 8.8$ Hz, 2H, H-3', 5'), 6.85 (1H, s, H-3); ^{13}C -NMR (100 MHz, DMSO- d_6 , δ_c) See Table 2. These data were in consistent with those of vicenin-2 (27).

Lucenin-2 (12): ESI-MS m/z : 611 $[M+H]^+$. ^1H -NMR (400 MHz, DMSO- d_6) δ : 13.71 (1H, s, OH-5), 9.29 (2H, s, 3', 4'-OH), 7.54 (1H, d, $J = 8.4$ Hz, H-6'), 7.48 (1H, s, H-2'), 6.88 (1H, d, $J = 8.0$ Hz, H-5'), 6.69 (1H, s, H-3), 4.81 (1H, d, $J = 9.6$ Hz, 6-Glu-H-1), 4.76 (1H, d, $J = 9.6$ Hz, 8-Glu-H-1); ^{13}C -NMR (100 MHz, DMSO- d_6 , δ_c) See Table 2. These data were in consistent with those of lucenin-2 (28).

Lucenin-1 (13): ESI-MS m/z : 581 $[M+H]^+$. ^1H -NMR (400 MHz, DMSO- d_6) δ : 13.71 (1H, s, OH-5), 9.29 (2H, s, 3', 4'-OH), 7.52 (1H, d, $J = 8.0$ Hz, H-6'), 7.47 (1H, d, $J = 2.0$ Hz, H-2'), 6.87 (1H, d, $J = 8.0$ Hz, H-5'), 6.66 (1H, s, H-3), 4.75 (1H, d,

$J = 10.0$ Hz, Glu-H-1), 4.69 (1H, d, $J = 9.6$ Hz, Xyl-H-1); ^{13}C -NMR (100 MHz, DMSO- d_6 , δ_c) See Table 2. These data were in consistent with those of lucenin-1 (29-30).

Vicenin-1 (14): ESI-MS m/z : 565 $[M+H]^+$. ^1H -NMR (400 MHz, DMSO- d_6) δ : 13.66 (1H, s, OH-5), 8.02 (2H, d, $J = 8.4$ Hz, H-2', 6'), 6.89 (2H, d, $J = 8.6$ Hz, H-3', 5'), 6.81 (1H, s, H-3), 4.81 (1H, d, $J = 10.0$ Hz, Glu-H-1), 4.73 (1H, d, $J = 10.0$ Hz, Xyl-H-1); ^{13}C -NMR (100 MHz, DMSO- d_6 , δ_c) See Table 2. These data were in consistent with those of vicenin-1 (31).

Vicenin-3 (15): ESI-MS m/z : 565 $[M+H]^+$. ^1H -NMR (400 MHz, DMSO- d_6) δ : 13.66 (1H, s, OH-5), 8.01 (2H, d, $J = 9.6$ Hz, H-2', 6'), 6.91 (2H, d, $J = 8.8$ Hz, H-3', 5'), 6.81 (1H, s, H-3), 4.82 (1H, d, $J = 9.6$ Hz, Glu-H-1), 4.71 (1H, d, $J = 10.0$ Hz, Xyl-H-1); ^{13}C -NMR (100 MHz, DMSO- d_6 , δ_c) See Table 2. These data were in consistent with those of vicenin-3 (32).

Determination of antioxidant activity *in vitro*

Flavonoids showed various biological functions with low toxicity. The diverse biological functions were considered to be related with their strong antioxidant activities (33-35). Therefore, the antioxidant activities of the flavone compounds 1-15 were measured by DPPH, ABTS and FRAP assays, respectively, while ascorbic acid was used as the positive control. Most compounds exhibited strong antioxidant activities, the IC_{50} and FRAP values were given in Table 3. The results showed that compounds 2,

Table 3. Antioxidant activities of compound 1-15.

Compound	DPPH $\text{IC}_{50}/(\mu\text{g/L})$	ABTS $^{++}$ $\text{IC}_{50}/(\mu\text{g/L})$	FRAP
1	>100	>100	1.02
2	6.96	5.42	6.84
3	>100	>100	0.70
4	11.64	8.01	6.99
5	11.67	7.23	5.57
6	13.79	9.65	8.31
7	>100	>100	0.77
8	>100	>100	0.91
9	19.85	4.22	9.80
10	10.27	9.63	4.25
11	87.91	29.44	3.89
12	25.08	12.68	4.69
13	25.43	10.43	5.32
14	98.83	89.43	2.46
15	>100	98.17	1.84
Ascorbic acid	12.43	5.89	3.21

Table 4. The condition of MS optimization for MRM mode.

Compound	Retention time (min)	[M-H] ⁻ (m/z)	Quantitative ions (m/z)	Declustering potential eV	Collision energy eV
2	6.54	300.9	150.9	-140	-30
4	5.63	447.0	299.5	-120	-36
5	5.33	463.0	299.8	-130	-37
6	5.06	609.0	299.8	-100	-50
9	4.61	447.0	326.5	-200	-33
10	3.47	447.1	356.8	-60	-35
11	3.06	592.9	352.8	-180	-50
12	2.74	609.0	368.7	-160	-50
13	3.13	579.1	368.8	-195	-50
14	3.64	562.9	382.9	-180	-48

4-6, 9-14 had more significant antioxidative effects compared with the others. The successive order of IC₅₀ values of DPPH radical scavenging activity was as follows: 2 > 10 > 4 > 5 > 6 > 9 > 12 > 13 > 11 > 14; The successive order of IC₅₀ values of ABTS radical scavenging activity was as follows: 9 > 2 > 5 > 4 > 10 > 6 > 13 > 12 > 11 > 14; The successive order of FRAP values of total antioxidant capacity was as follows: 9 > 6 > 4 > 2 > 5 > 13 > 12 > 10 > 11 > 14.

The presence of 3', 4'-dihydroxy group (catechol) in the B ring and/or the presence of the 3-OH group in the C ring is often deemed to be essential for radical scavenging activity of flavonoids. In addition, the 5-OH group in combination with 1, 4-pyrone moiety, and C2=C3 double bond may increase the radical scavenging activity (36-38). The fifteen compounds were divided into four categories (Figure 1) by the chemical structure: quercetin derivatives (compound 2, 4, 5, 6), apigenin derivatives (compound 7, 8, 11, 14, 15), kaempferol derivatives (compound 1 3) and

luteolin derivatives (compound 9, 10, 12, 13). The luteolin and quercetin derivatives had significantly higher oxidation resistances than the kaempferol and apigenin derivatives due to the ortho-diphenol structure at 3', 4' position in the B ring. Quercetin derivatives had higher antioxidant activities than luteolin derivatives on the whole, possibly due to the presence of a 3-OH group in the C ring. Compared with the quercetin and luteolin derivatives, apigenin and kaempferol derivatives showed weaker antioxidant activities. So, compounds 2, 4-6, and 9-14 were selected as the antioxidant substance in THL, and an analytical method to determine these antioxidants simultaneously by using ultra-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry was then explored.

Analytical method optimization

The Mass spectra in positive and negative ion modes were compared. It was found that the flavonoids had higher sensitivity in the negative ion mode, which made it easier to confirm molecular ions or protonated molecule ions in the identification of each peak. Therefore, the mass spectrometer was set to collect data in negative ion mode. All MS optimization experiments were carried out in MS scan mode and in product scan mode. Quantitative analysis was carried out in MRM mode. The tune page parameters and conditions for each of the MRM transitions were optimized by infusing the neat standard solution into the mass spectrometer. Each compound was

Figure 2. Total ion chromatograms for standards mixture of THL.

Table 5. Calibration curves, linear ranges, correlation coefficient, LOQ, and LOD of ten compounds.

Compound	Regression equation (n = 6)	Linear range ng/mL	R^2	LOQ ng/mL	LOD ng/mL
2	$Y = 679.15X - 4.99 \times 10^4$	5.0-500.0	0.9986	3.50	1.05
4	$Y = 4454.90X - 1.91 \times 10^5$	5.0-500.0	0.9980	2.35	0.71
5	$Y = 10979.57X + 2.18 \times 10^4$	5.0-500.0	0.9996	1.12	0.34
6	$Y = 17346.63X - 3.08 \times 10^4$	5.0-1000.0	0.9995	1.02	0.31
9	$Y = 14472.60X - 3.64 \times 10^5$	5.0-1000.0	0.9998	1.87	0.56
10	$Y = 7102.75X - 1.20 \times 10^5$	5.0-2000.0	0.9999	2.15	0.65
11	$Y = 1192.95X - 1.29 \times 10^4$	5.0-2000.0	0.9999	2.65	0.80
12	$Y = 1318.13X - 2.46 \times 10^4$	5.0-2000.0	0.9998	2.76	0.83
13	$Y = 811.16X - 7.01 \times 10^3$	5.0-1000.0	0.9999	3.06	0.92
14	$Y = 1212.76X - 2.86 \times 10^4$	5.0-2000.0	0.9998	2.87	0.86

Table 6. The precision, repeatability, stability, and recovery in UPLC-MS/MS analysis for the ten compounds in samples.

Compound	Precision RSD (%)		Repeatability (RSD, %, n = 6)	Stability (RSD, %, n = 6)	Recovery (% , n = 3)	
	Intraday (n = 6)	Interday (n = 6)			Mean	RSD
2	1.66	3.47	3.51	2.12	96.39	3.44
4	1.89	2.53	3.47	3.44	99.27	2.97
5	2.07	2.53	2.68	1.73	95.77	4.26
6	1.53	1.77	3.55	2.76	104.84	3.67
9	2.34	2.49	2.94	3.58	102.73	2.54
10	1.55	2.05	1.97	2.91	101.89	4.01
11	1.93	2.36	2.86	3.55	98.45	4.37
12	2.84	3.09	3.84	3.86	103.26	2.88
13	2.45	2.56	2.65	1.94	100.78	3.89
14	2.64	2.48	3.83	2.68	97.26	2.47

studied individually to achieve the best scan time, Declustering Potential (DP), and Collision Energy (CE). The MRM transitions for the tested compounds were shown in Table 4, and the total ion chromatogram of ten markers was shown in Figure 2.

Analytical method validation

A fast and sensitive UPLC-MS/MS method was developed for the simultaneous quantification of ten compounds in THL. The method for quantitative analysis was validated by determining the linearity, intra-day and inter-day precisions, LOD and LOQ, stability, and accuracy. The calibration curves were prepared by injecting standard solutions at seven concentration levels and showed good linearity in the test ranges, ranging from 0.9980 to 0.9999 (Table 5). The overall LODs and LOQs were in the range of 0.31-1.05 ng/mL and 1.02-3.50 ng/mL, respectively. The overall recoveries ranged from 95.77% to 104.84% with RSD less than 5%. The RSD values of stability and repeatability, intra-day and inter-day variations of the ten analytes were all

less than 4% (Table 6). The overall validation results showed that the UPLC-MS/MS method has good linearity, accuracy, precision, and sensitivity, and could be used for the quantification analysis of antioxidants in THL.

Quantitative analysis of a sample

The proposed UPLC-MS/MS method was subsequently applied to determine the ten compounds' content in THL from the different habitats. The results were shown in Table 7. It can be seen from Table 7 that the contents of the ten major antioxidant components in THL from different habitats were very different. Besides, some compounds were undetectable in some batches due to the content below the LOD, which might be related to the different germplasm resources of *T. hemsleyanum* and its environmental diversity of different planting bases. The contents of quercetin, quercitrin, and isoquercitrin in samples from different habitats were all lower than 100 µg/g. The contents of rutin in THL from

Table 7. Compounds contents of THL from different habitats ($\bar{x} \pm s$, n = 3, $\mu\text{g/g DW}$).

Sample	2	4	5	6	9	10	11	12	13	14
S-1	12.5 ± 0.5	10.0 ± 0.4	10.8 ± 0.4	3.7 ± 0.1	382.4 ± 7.1	546.4 ± 7.9	2139.8 ± 34.2	1112.1 ± 13.4	465.2 ± 8.4	2058.2 ± 27.4
S-2	15.7 ± 0.2	7.6 ± 0.2	11.9 ± 0.1	13.7 ± 0.2	483.7 ± 8.6	714.3 ± 9.3	2190.4 ± 38.3	2330.1 ± 19.5	1731.2 ± 21.3	4269.9 ± 60.6
S-3	12.3 ± 0.3	7.2 ± 0.4	12.5 ± 0.5	4.9 ± 0.3	176.7 ± 5.3	246.1 ± 4.8	2621.1 ± 47.5	1792.6 ± 27.9	1330.1 ± 20.8	5001.6 ± 79.3
S-4	21.0 ± 0.7	11.1 ± 0.3	52.1 ± 0.4	5.8 ± 0.1	1930.3 ± 24.3	2757.6 ± 41.4	4238.0 ± 63.3	2648.8 ± 34.8	525.9 ± 7.6	2218.4 ± 28.8
S-5	12.4 ± 0.2	7.8 ± 0.2	29.4 ± 0.8	4.6 ± 0.2	793.7 ± 10.4	1169.0 ± 17.8	2618.1 ± 39.6	1545.7 ± 16.3	1119.8 ± 12.6	4669.0 ± 47.5
S-6	12.2 ± 0.3	7.5 ± 0.3	62.5 ± 2.1	22.1 ± 0.3	816.0 ± 7.3	1063.4 ± 21.8	2722.6 ± 47.2	2326.7 ± 28.7	1502.6 ± 17.1	4533.4 ± 54.3
S-7	12.7 ± 0.4	12.3 ± 0.3	18.9 ± 0.6	18.3 ± 0.2	136.7 ± 5.6	205.3 ± 6.9	1541.6 ± 25.9	1150.9 ± 14.3	1063.1 ± 9.3	3461.2 ± 31.7
S-8	12.7 ± 0.1	45.1 ± 0.9	42.1 ± 1.8	63.4 ± 0.7	202.9 ± 4.7	261.0 ± 5.3	19.6 ± 0.6	25.7 ± 0.7	14.1 ± 0.2	21.7 ± 0.5
S-9	12.5 ± 0.2	10.2 ± 0.2	31.5 ± 0.5	944.2 ± 18.7	5.4 ± 0.1	4.3 ± 0.1	6.0 ± 0.2	6.2 ± 0.1	/	9.3 ± 0.2
S-10	14.1 ± 0.8	10.2 ± 0.3	71.2 ± 1.4	1916.3 ± 31.7	6.2 ± 0.2	6.2 ± 0.2	10.0 ± 0.2	9.5 ± 0.4	5.6 ± 0.1	16.1 ± 0.3
S-11	12.7 ± 1.2	28.7 ± 0.7	19.8 ± 0.6	22.5 ± 0.8	297.4 ± 7.6	428.1 ± 12.9	186.8 ± 4.9	54.0 ± 1.2	42.9 ± 1.8	157.1 ± 3.3
S-12	12.7 ± 0.5	10.6 ± 0.1	43.7 ± 1.6	784.6 ± 12.4	4.2 ± 0.1	/	/	/	/	/
S-13	12.4 ± 0.2	26.7 ± 0.9	35.9 ± 0.8	20.7 ± 0.3	328.5 ± 9.4	417.0 ± 9.2	222.1 ± 8.7	30.6 ± 1.1	14.3 ± 0.2	98.8 ± 3.2
S-14	12.4 ± 0.5	11.4 ± 0.2	33.4 ± 0.3	33.3 ± 0.6	809.7 ± 12.3	1100.4 ± 17.1	360.3 ± 11.3	86.7 ± 1.8	109.2 ± 2.3	414.1 ± 9.8
S-15	15.7 ± 0.2	63.5 ± 1.4	59.4 ± 2.1	1006.1 ± 13.1	332.5 ± 6.8	438.8 ± 8.3	186.7 ± 6.3	38.8 ± 0.6	/	9.2 ± 0.3
S-16	13.0 ± 0.6	10.7 ± 0.4	77.6 ± 2.8	16.0 ± 0.5	1562.3 ± 23.9	2243.4 ± 31.9	/	14.2 ± 0.8	/	/
S-17	17.2 ± 0.6	7.6 ± 0.2	53.3 ± 1.7	684.0 ± 8.6	12.1 ± 0.3	14.5 ± 0.4	42.4 ± 1.3	48.9 ± 0.4	26.6 ± 0.4	65.7 ± 1.4

Note: The samples numbers were the same as those in Table 1.

Yunnan, Guangxi, Guangdong, and Guizhou were greater than other habitats. The contents of vicenin-2, lucenin-2, lucenin-1, and vicenin-1 in THL from Zhejiang province were much higher than in other regions. The contents of orientin and isoorientin in THL from Guizhou, Guangdong, and Guangxi were lower than those from other regions.

CONCLUSION

In this study, fifteen flavonoids were isolated and identified from THL, four of which were obtained from *T. hemsleyanum* for the first time. Compounds 2, 4-6, and 9-14 showed significant antioxidant activities. In addition, a new simple and sensitive UPLC-MS/MS method was developed for the separation and quantification of these ten antioxidant compounds, which would provide an evaluable reference for the quality control of *T. hemsleyanum*. The contents of ten compounds in the THL from different habitats were significantly different. The results suggested that THL could be a new source for functional foods and a potential candidate for antioxidant agents.

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Conflict of interest

All the authors declare that they have no conflict of interest.

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