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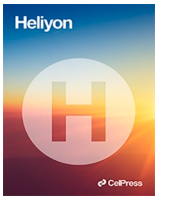

A quality evaluation method of lotus leaf based on its lipid lowering components using QAMS and chemometrics

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A R T I C L E I N F O

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A B S T R A C T

Introduction: Lotus leaf has long been used as food and medicine in China and is well-known for its lipid-lowering effects. However, there is a lack of a comprehensive quality evaluation for lotus leaf due to the absence of consideration of the correlation between various components and their efficacy.

Objectives: This study aims to find out the key bioactive components that can be used for quality evaluation of lotus leaf on lipid-lowering effect.

Methods: Thirteen compounds were characterized in the lotus leaf using ultra-high- performance liquid chromatography-time-of-fight mass spectrometry (UPLC-Q-TOF-MS). Five alkaloids and four flavonoids were identified according to their lipid-lowering activities reported in literatures. Then, the contents of these nine components were analyzed in 39 batches of lotus leaves growing in different locations using high performance liquid chromatography diode-array detector (HPLC-DAD), and further evaluated by quantitative analysis of multi-components by single marker (QAMS) and chemometrics. The anti-adipogenic activity of lotus leaves were evaluated for their inhibitory effect on the PPARγ expression by luciferase assay.

Results: The 39 batches were clustered into two regions, the north and the south, based on the contents of these components. Three alkaloids, nuciferine, *N*-nornuciferine, and asimilobine, and three flavonoids, astragalin, hyperoside, and trifolioside, were found to serve as the key factors behind the region differences. Their contents were higher in Guangchang County of Jiangxi Province than other habitat locations. Moreover, the luciferase assay combined with chemometrics showed that these components were positively correlated with lipid-lowering activity of lotus leaf.

Conclusions: Three alkaloids and three flavonoids were screened out and could be used as key compounds for quality evaluation of lotus leaf on lipid-lowering effect.

1. Introduction

Lotus leaf (LL) refers to the leaves of *Nelumbo nucifera* Gaertn., a plant of the Nelumbonaceae family. The cultivation of LL plants was recorded for the first time in the “The Book of Songs” compiled around 600 BCE in China. For thousands of years, the dried LLs have been widely used as a traditional Chinese medicine, and documented many traditional therapeutic effects, such as heat relief, enhancing the lucid yang, cooling blood, and hemostasis [1]. Previous investigations revealed that LL extracts and their major

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chemical constituents exhibited various bioactivities, including antioxidant, anti-inflammatory, and anti-obesity [2–6]. Especially, its effects of lipid-lowering and inducing weight loss attract more and more attention in recent years. Lotus leaf is not only the principal drug of many Chinese patent drugs with lipid-lowering activity, such as He Dan Tablets, Heye Tiaozhi Tea, Jiangzhining Granules, but also could effectively reduce lipid levels in obese rats in the modern pharmaceutical study [7].

LL plants are widely cultivated in Asia and Africa now. In China, LL plants are growing both in the southern region, including the Jiangxi, Anhui, Jiangsu, and Zhejiang provinces, and the northern region, including the Heilongjiang, Hebei, and Shanxi provinces. Our preliminary investigations revealed that the content of major components of the LLs differ greatly with the habitat location. Hence, it is necessary to determine the key factors that cause the differences, and further evaluate the quality of the herbs.

Alkaloids and flavonoids are reported as bioactive components of LL [8]. The identified alkaloids including nuciferine, *N*-nornuciferine, and roemerine, and flavonoids including hyperoside, isoquercitrin, and astragaline, showed significant inhibitory effect on both adipocyte differentiation and pancreatic lipase activity [9]. The contributions of various alkaloids and flavonoids to the quality of LL are of broad concerns.

A study used HPLC to determine the contents of three alkaloids from LL to evaluate its quality during different growth periods [10]. It is obvious that considering alkaloids only may not reflect the true quality of LL. In the following study, four alkaloids and four flavonoids of 11 batches of LLs were analyzed simultaneously, discussing their correlation with DPPH (2-2 diphenyl hydrazy) radical scavenging activity. So far, simultaneous determination of the contents of alkaloids and flavonoids of LL, as well as their correlation with lipid-lowering activity, has not been explored yet [11].

Quantitative analysis of multi-components by single marker (QAMS) has emerged as a quality control method and commonly used in recent years, which can achieve multi-component-related quality control without reference compounds. In a study, a single alkaloid has been used as an internal reference to determine the contents of two alkaloids and three flavonoids in LL [12]. However, it is difficult to accurately reflect the content of flavonoids by using an alkaloid as reference due to their structural differences. Given the different response and retention of various compounds on the instrument, dual internal references (IRs) instead of single internal could get more reliable results. Therefore, it is pivotal to develop a dual IRs method based on QAMS for a accurate and comprehensive quality evaluation of LLs.

In this study, 13 compounds were firstly characterized from LL using ultra-high performance liquid chromatography-time-of-fight mass spectrometry (UPLC-Q-TOF-MS). Then, LLs of 39 batches were analyzed by establishing an updated QAMS with dual internal references (IRs), resulting in the identification of nine bioactive ingredients, including five alkaloids and four flavonoids. The Partial Least Squares Discriminant Analysis (PLS-DA) model was used to evaluate the LL growing in different locations. The 3T3-L1-Lenti-*PPARγ*-RE-Luc cell strain was used to analyze the correlation between the contents of the nine bioactive ingredients and the inhibition of the expression levels of peroxisome proliferator-activated receptor γ (*PPARγ)*. The volcanic sample map combined with the results obtained from the QAMS was used to analyze the distribution of samples among 39 batches for the first time. Six compounds were

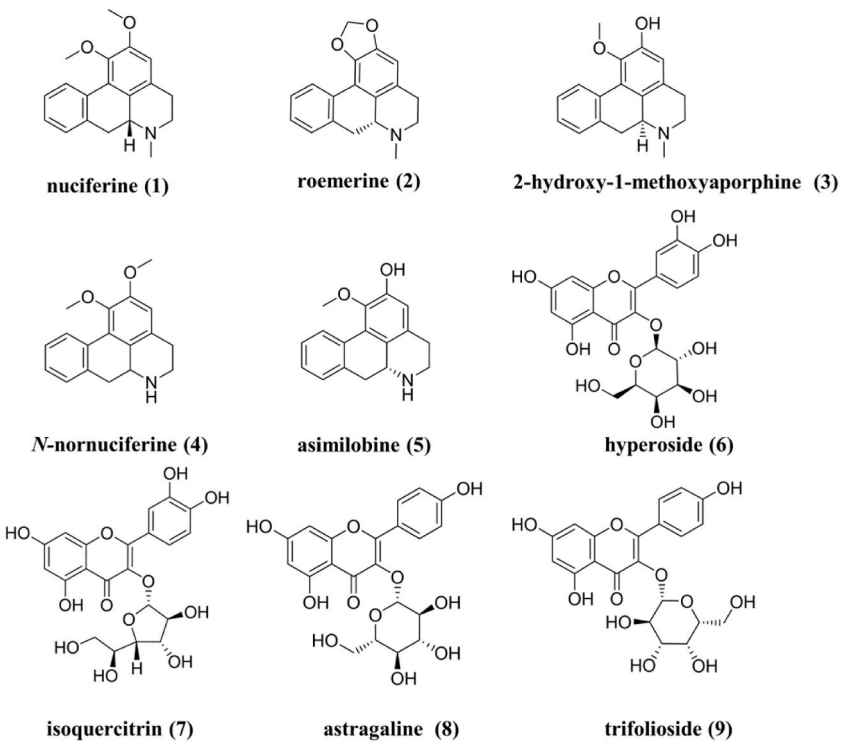

Fig. 1. Chemical structures of nine compounds in lotus leaf.

screened out, which could be served as key factors for quality evaluation of lotus leaf on lipid-lowering effect.

2. Experimental

2.1. Materials and reagents

Chemical standards of nuciferine (Batch no. PRF10070821), *N*-nornuciferine (Batch no. RDD00802201021), roemerine (Batch no. PS010846), hyperoside (Batch no. PRF20080301), and isoquercitrin (Batch no. PRF20050905) were purchased from the Chengdu Puruifa Biotechnology (Chengdu, Sichuan, China). Trifolioside (Batch no. RDDS17102203031) was purchased from Sichuan Vicky Biotechnology (Sichuan, China). Astragaline (Batch no. F18HB175834) and 2-hydroxy-1-methoxyaporphine (Batch no. P29M11S110402) were purchased from Shanghai Yuanye Biotechnology (Shanghai, China). Asimilobine was isolated in the laboratory. All chemicals used as standards for quantitative analysis had a purity > 98.0 %. Their structures are indicated in Fig. 1. HPLC-grade acetonitrile and methanol were purchased from the Fisher Chemical Co., Ltd. (Pittsburgh, PA, USA). HPLC-grade phosphoric acid and triethylamine were purchased from the Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). In total, 39 batches of LLs (Table 1) were collected from 15 provinces of China. All plant materials collected were identified and authenticated by Dr. Wu Lihong of the Shanghai R&D Center for Standardization of Chinese Medicines, based on the 2020 edition of *Chinese Pharmacopoeia*.

Cell culture reagents, including Dulbecco's modified Eagle's medium-high glucose (DMEM-HG), fetal bovine serum (FBS), and penicillin-streptomycin solution were purchased from Gibco Life Technologies (Carlsbad, CA, USA). Cell counting kit-8 (CCK-8) and phosphate-buffered saline (PBS) were purchased from the Dalian Meilun Biotech Co., Ltd. (Dalian, Liaoning, China). Dimethyl sulfoxide (DMSO), tris (hydroxymethyl)methyl aminomethane (Tris), coenzyme A, dithiothreitol (DTT), and adenosine triphosphate (ATP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). D-luciferin-potassium salt was purchased from the Yeasen Biotech Co., Ltd. (Shanghai, China). Ethylene diamine tetraacetic acid (EDTA), magnesium sulfate ($MgSO_4$), hydrochloric acid (HCl), and sodium bicarbonate ($NaHCO_3$) were obtained from the Sino-Pharm Chemical Reagent Co., Ltd. (Shanghai, China). T0070907, a *PPAR*γ inhibitor, was obtained from Selleck Chemicals (Houston, TX, USA).

2.2. Instruments

A pH 400 pH meter purchased from the Shanghai Anlailisi Instrument Technology Co., Ltd. (Shanghai, China) was used. Purified water of a reference quality of Milli-Q was used as a mobile phase for chromatography and was purchased from the Millipore Co. (Boston, MA, USA). High-resolution mass spectrometry (HRMS) was performed using a 1290 UPLC system and a 6545 ultrahigh definition quadrupole time-of-flight (UPLC-Q-TOF) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Method validation and robustness of relative correction factor (RCF) studies were performed using the 1260 Infinity I and II systems (Agilent Technologies, Santa Clara, CA, USA). The Infinity I system consisted of a quaternary pump (G1311B), an autosampler (G1330B), a column temperature controller (G7116A), a variable wavelength UV detector (G1315C), and an analytical workstation. The Infinity II system consisted of a quaternary pump (G7111B), an autosampler (G7129A), a column temperature controller (G7116A), a variable wavelength UV detector (G1315D), and an analytical workstation. All analytes were separated on a reversed-phase, C_{18} column (4.6 mm × 150 mm, 3 μm; Fortis Technologies Ltd, Chester, UK). For method validation and determination of the RCF robustness, T3 (4.6 mm × 150 mm, 3 μm; Waters, Milford, MA, USA) and Kromasil C_{18} (4.6 mm × 150 mm, 3 μm; Akzo Nobel N.V, Bohus, Sweden) columns were used. A BB150 CO_2 incubator and a Synergy H4 Microplate Reader were purchased from BioTek Instruments, Inc.

Table 1
Detailed information of 39 batches of lotus leaf.

Sample code	Origin (Province)	Sample code	Origin (Province)
S1	Anhui	S21	Sichuan
S2	Anhui	S22	Shandong
S3	Anhui	S23	Shandong
S4	Hubei	S24	Shandong
S5	Hubei	S25	Shandong
S6	Hunan	S26	Shandong
S7	Hunan	S27	Shandong
S8	Hunan	S28	Shandong
S9	Hunan	S29	Shandong
S10	Hunan	S30	Shanxi
S11	Henan	S31	Shanxi
S12	Heilongjiang	S32	Shanghai
S13	Jiangxi	S33	Yunnan
S14	Jiangxi	S34	Zhejiang
S15	Jiangxi	S35	Zhejiang
S16	Jiangxi	S36	Zhejiang
S17	Jiangsu	S37	Zhejiang
S18	Jiangsu	S38	Zhejiang
S19	Liaoning	S39	Zhejiang
S20	Liaoning		

(Winooski, VT, USA).

2.3. UPLC-HRMS analysis

UPLC-Q-TOF MS was used to characterize the components of the 70 % methanol-derived extracts obtained from the LL. The mobile phase comprised acetonitrile (A) and 0.1 % formic acid in water (B). The steps used for gradient elution were: 0–2 min of 15 %–20 % A; 2–12 min of 20 %–20 % A; 12–20 min of 20 %–22 % A; 20–30 min of 22 %−2 6 % A. The column temperature was maintained at 30 °C, the flow rate at 1.0 mL/min, and 3 μL of the sample was injected. The sample solution was analyzed through Q-TOF MS, with the electrospray ionization (ESI) source set at both positive and negative ion modes. The operational parameters of ESI-MS were as follows: the capillary voltage was ± 4 kV; the fragmentor at 140 V; and a nozzle voltage of 500 V. The scan range was *m/z* 50–1000. N_2 was used as the sheath gas at 12 L/min and 350 °C; the nebulizer gas at 30 psi; and the drying gas at 8 L/min and 325 °C.

2.4. Preparation of standard stock and sample solutions

A standard stock solution containing a mixture of asimilobine (1), 2-hydroxy-1-methoxyaporphine (2), hyperoside (3), isoquercitrin (4), trifolioside (5), astragaline (6), *N*-nornuciferine (7), nuciferine (8), and roemerine (9) was prepared in 70 % methanol. The solution was then diluted with 70 % methanol to the required concentrations to prepare the working solutions (Table 2). All standard solutions were stored at 4 °C, and filtered through a 0.22 μm membrane before injecting into the HPLC system.

For preparing the samples to be used for HPLC, the LLs were ground to a fine powder and passed through a 50-mesh sieve. The powder was accurately weighed to a sample of 0.5 g which was transferred to a 50 mL conical flask with a stopper. After adding 20 mL of 70 % methanol, the sample was extracted by ultrasonication (power of 300 W; frequency of 40 kHz) for 30 min. Next, the conical flask was cooled to room temperature, and 70 % methanol was added to its original weight. Finally, the supernatant was filtered using a 0.22 μm nylon membrane and syringe filters before injection into the HPLC system.

As samples for assaying the *PPARγ* inhibition activity, 1 mL of the sample prepared for HPLC was obtained and blow-dried with N_2. DMSO was added to prepare a sample containing 70 μg/mL of LL extracts.

2.5. Chromatography conditions and method validation

Elution was performed using acetonitrile (A) and an aqueous solution of 0.2 % phosphoric acid and 0.2 % triethylamine (B) as the mobile phases using the following gradient program: 0–2 min of 15 %–20 % A; 2–12 min of 20 %–20 % A; 12–20 min of 20 %–22 % A; and 20–30 min of 22 %–26 % A. The column temperature was maintained at 30 °C, the flow rate was 1.0 mL/min, the sample injection volume was 3 μL, and the detection λ was 270 nm. Each sample was injected thrice in parallel.

The method validation, including linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, repeatability, stability, and accuracy, was conducted following the 2020 edition of the *Chinese Pharmacopoeia*. The linearity was established using the peak areas of six different concentrations for each compound. The LOD and LOQ values were calculated at a signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively. The intraday and interday precision of the instrument was evaluated based on the relative standard deviation (RSD) values for six repetitive injections of each of the three concentrations. The repeatability was determined by analyzing six replications of each sample. Recovery tests were measured by spiking the nine samples with a known content determined from sample S15 using known amounts of each analyte (Table 3).

2.6. Chemometric analysis

The external standard method (ESM) and the QAMS method with two IRs were used to calculate the contents of the nine analytes in the 39 batches of LLs. Relative errors (REs) were calculated to verify the feasibility of the QAMS method. The content histogram and volcano plot of the nine analytes in the 39 batches were analyzed using the GraphPad Prism software (version 9.4.1; La Jolla, California, USA). For the chemometric analysis, PLS-DA was applied to classify the specific criteria for the quality of LLs using the SIMCA software (version 14.1; MKS Instruments, Umetrics, Sweden). In the PLS-DA model-based analysis, the variable importance in

Table 2
Linear relationship of nine target components.

Components	Regression equation	R [2]	Range of linearity (mg/mL)	LOD (ng/mL)	LOQ (ng/mL)
Asimilobine	y = 6861.3x + 1.3349	1.0000	0.001–0.100	22.89	76.28
2-hydroxy-1-methoxyaporphine	y = 9225.1x − 6.8622	1.0000	0.002–0.200	15.58	51.95
Hyperoside	y = 5668.6x + 11.699	0.9998	0.010–1.000	68.11	227.02
Isoquercitrin	y = 5402.3x + 22.006	0.9997	0.010–1.000	67.54	225.14
Trifolioside	y = 5803.0x − 0.1388	0.9998	0.001–0.020	40.54	134.41
Astragaline	y = 5832.7x + 2.077	0.9997	0.001–0.200	44.22	147.41
N-nornuciferine	y = 8272.2x + 3.7226	1.0000	0.002–0.200	40.54	135.14
Nuciferine	y = 8682.0x + 2.8348	0.9998	0.010–1.000	18.80	62.68
Roemerine	y = 9445.8x + 6.8573	0.9997	0.001–0.100	23.43	78.13

Note: LOD, limit of detection; LOQ, limit of quantification.

Table 3
Results of precision, repeatability, stability, and recovery tests using the proposed HPLC method.

Components	Precision RSD (%)		Repeatability	Stability	Recovery	
	interday	intraday	RSD (%)	RSD (%)	Mean (%)	RSD (%)
Asimilobine	1.50	1.44	0.28	1.51	98.33	1.63
2-hydroxy-1-methoxyaporphine	1.20	1.21	0.32	1.18	100.35	3.35
Hyperoside	0.36	1.20	0.25	1.69	100.19	2.96
Isoquercitrin	1.29	1.07	0.27	1.10	100.45	1.62
Trifolioside	1.97	1.89	0.99	2.38	100.84	1.74
Astragaline	1.86	1.05	0.64	1.49	101.72	1.19
N-nornuciferine	0.71	0.97	0.26	1.46	100.20	2.95
Nuciferine	0.54	0.56	0.30	1.22	100.49	1.01
Roemerine	0.77	1.01	1.54	1.04	102.68	0.98

Note: RSD, relative standard deviation.

projection value (VIP) was used as an indicator for screening the variables; components with VIP values > 1 were considered to contribute to the grouping significantly. Volcano plot analysis was performed using the Origin 2021 software (Origin Lab Company, Northampton, MA, USA). Canonical correlation analysis module was performed using the SPSS 26.0 (IBM, New York, USA). The inhibition rate of LL on *PPARγ* expression and the content of nine components were imported into SPSS software. Then canonical correlation analysis was selected, inhibition rate of LL on *PPARγ* was defined as set 1, and the content of nine components was defined as set 2, other parameters set to default. Finally, the correlation coefficients between the nine components and the inhibition rate were calculated, respectively.

2.7. Luciferase assay

Peroxisome proliferator-activated receptor gamma (*PPARγ*) plays a crucial role in preadipocyte differentiation, adipocyte differentiation, glucose metabolism, and the complete generation of fat [13]. The 3T3-L1-Lenti-*PPARγ*-RE-Luc cells reported in a previous study by our group [14] were used to evaluate the lipid-lowering activity of the LL-extracts through inhibiting the expression of *PPARγ* [15]. The cells were seeded at a density of 4×10^3 cells/well in two 96-well plates. After 12 h of culture, the cells were incubated with T0070907 (positive control) and LL-extracts for 48 h. The cytotoxic effects of all samples were evaluated using the CCK-8 assay. Briefly, 10 μL of the CCK-8 working solution was added to the cells and incubated for 30 min. The absorbance at 450 nm was measured using a microplate reader. The calculation was performed using the formula:

$$\text{Cell viability} = [(A_s - A_b)/(A_c - A_b)] \times 100\%$$

where, A_s: the absorbance value of the sample well; A_b: the absorbance value of the blank sample; and A_c: the absorbance value of the control well.

The medium was removed, and 50 μL of cell lysate was added. Then 20 μL of the supernatant from each lysate was transferred to another 96-well plate, and the luciferase reaction was started by adding the luciferase assay buffer consisting of a mixture of 200 μL of Tris-HCl (pH 7.8), 10 μL of 1 M $NaHCO_3$, 50 μL of 0.5 M $MgSO_4$, 2 μL of 0.5 M EDTA, 15.4 mg of DTT, 0.46 mg of coenzyme A, 0.7 mg of potassium luciferin, and 1.4 mg of ATP, and then diluted to 10 mL with deionized water. The luminescence values of *PPARγ* were measured at 230 nm using a microplate reader.

Rate of inhibition of *PPARγ* was calculated using the formula:

$$\text{Expression of target gene} = [1\text{-}(L_s - L_b)/(L_c - L_b)] \times 100\%$$

where, L_s: the luminescence value of the sample well; L_c: the luminescence value of the control well; and L_b: the luminescence value of the blank sample. This experiment was repeated three times in four replications for each treatment.

3. Results and discussion

3.1. Determination of quantitative compounds

A total of 13 components: *N*-Norarmepavine, Quercetin 3-*O*-arabinopyranosyl-galactopyranoside, armepavine, asimilobine, 2-hydroxy-1-methoxyaporphine, hyperoside, isoquercitrin, *N*-demethylcoclaurine, trifolioside, astragalin, *N*-Nornuciferine, nuciferine, and roemerine from the LL were identified and characterized by UPLC-Q-TOF MS by comparing their MS [2] fragment ions with standard compounds and references (Fig. S1, Table S1). Of these, nine components, including five alkaloids: asimilobine, 2-hydroxy-1--methoxyaporphine, *N*-nornuciferine, nuciferine, and roemerine; and four flavonoids: hyperoside, isoquercitrin, trifolioside, and astragaline, have been reported to possess lipid-lowering activities. Hyperoside, isoquercitrin, and astragaline stimulated lipolysis in the white adipose tissues of mice [16]. Asimilobine effectively inhibited the proliferation and differentiation of 3T3-L1 cells and reduced the accumulation of fat droplets in them [17]. Nuciferine, 2-hydroxy-1-methoxyaporphine, *N*-Nornuciferine, and roemerine have received considerable attention owing to their bioactivities, including anti-hyperlipidemic and anti-obesity effects [18].

Trifolioside reduced the sugar and lipid levels in the blood [19]. Therefore, these five alkaloids and four flavonoids, closely associated with lipid-lowering effects, were selected as critical components for the quality control of LL.

3.2. Optimization of the method for sample solution preparation

To accurately determine the contents of these nine components, the composition of the solvents: methanol, ethanol, ethylacetate, dichloromethane, 70 % methanol, and 50 % methanol; methods of extraction: ultrasonic, cold immersion, and heating reflux; extraction time: 15, 30, and 45 min; and solid:liquid ratio: 1:20, 1:40, and 1:60 for use with the LL sample, S15 were optimized. Ethyl acetate and dichloromethane, when used as extraction solvents, could not detect any flavones; while the meager contents of nuciferine and 2-hydroxy-1-methoxy-aporphine could be ascertained and those of the other three alkaloids were undetectable. The contents of the nine components were determined to be higher when 70 % methanol was used rather than 50 % or 100 % methanol. There was no significant difference in the extraction efficiencies of either ultrasound- or heated reflux-based methods used for 1 h each; hence, ultrasound was selected. Ultrasonic treatment was carried out for 15, 30, and 45 min. The contents of the nine compounds were detected to be lower than those of the others at 15 min, but there were no significant differences at 30 and 45 min, indicating that the compounds had been completely extracted at 30 min. The extraction efficiency was higher when the material: liquid ratio was 1:40 than at either 1:20 or 1:60. The highest final extraction efficiency was obtained by adding 20 mL of 70 % methanol to 0.5 g of LL samples for ultrasonic-based extraction for 30 min (Tables S2–S6).

3.3. Optimization of the liquid chromatography conditions

For the purpose of accurately determining the contents of the five alkaloids and four flavonoids under the same optimal chromatographic conditions, the composition and pH value (1.5–11.0) of the mobile phase, the chromatographic columns used (Cortecs T3, Kromasil C18, and Fortis C18), and the detection λ (254, 270, and 360 nm) were standardized. Acetonitrile was used as mobile phase A, while mobile phase B consisted of an aqueous solution of formic acid and triethylamine (B1), or formic acid and ammonium formate (B2), or phosphoric acid and triethylamine (B3) (Figs. S2–S5). The parameters of resolution, tailing factor, and symmetry of the nine components followed the analysis requirements (Tables S7–S9).

When B1 was used as the mobile phase, only five peaks related to asimolobine, hyperoside, isoquercitrin, trifolioside, and astragaline were detected. With B2, the resolution of asimilobine, nuciferine, and roemerine did not meet the minimal separation criterion. Only when B3 (an aqueous solution of 0.2 % phosphoric acid and 0.2 % triethylamine at a pH ± 2) was used as the mobile phase the resolution and symmetry parameters of all nine peaks met the analytical requirements. With the Cortec T3 column, the peaks of 2-hydroxy-1-methoxyaporphine, superoside, and isoquercitrin did not meet the separation requirements, with the symmetry of *N*-nornuciferine being poor. Using the Kromasil C18 column, the two peaks indicating nuciferine and roemeinine were indiscernible. With the Fortis C18 column, the symmetry of separation of the nine compounds met the analytical requirements. The UV absorption curves

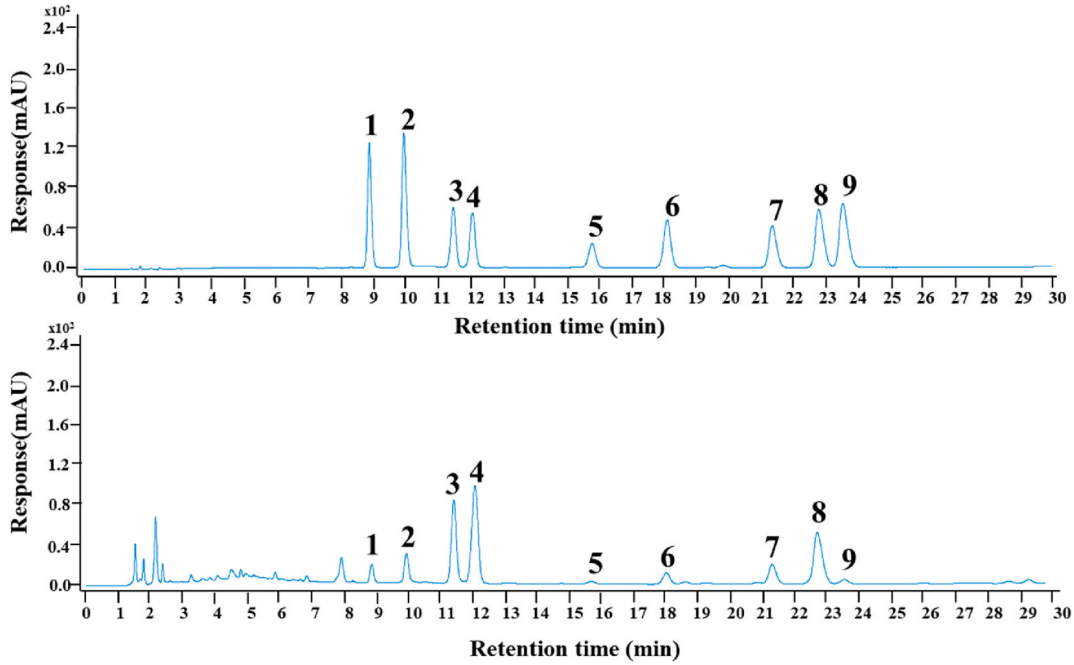

Fig. 2. Chromatograms of (A) lotus leaf reference substance and (B) S15 sample: 1, asimilobine; 2, 2-hydroxy-1-methoxyaporphine; 3, hyperoside; 4, isoquercitrin; 5, trifolioside; 6, astragaline; 7, *N*-nornuciferine; 8, nuciferine; 9, roemerine.

of the five alkaloids and four flavonoids were compared, with all nine peaks showing a high response and a moderate peak area at 270 nm. Finally, an aqueous solution of 0.2 % phosphoric acid and 0.2 % triethylamine (pH 2) as the mobile phase B, Fortis C18 (4.6 mm × 150 mm, 3 μm) as the chromatographic column, and a detection λ of 270 nm were selected as the optimal conditions for the simultaneous determination of the alkaloids and flavonoids (Fig. 2A and B).

3.4. Validation of HPLC

3.4.1. Linearity and sensitivity

The calibration curves of asimilobine, 2-hydroxy-1-methoxyaporphine, hyperoside, isoquercitrin, trifolioside, astragaline, *N*-nornuciferine, nuciferine, and roemerine were constructed by plotting the peak area (y-axis) versus the concentration (x-axis). The LOD and LOQ values for the nine analytes were estimated at the S/N ratios of 3 and 10, respectively. The results are shown in Table 2. A good linearity ($r > 0.999$) was obtained for the indicated concentration ranges.

3.4.2. Precision, repeatability, stability, and recovery

The intraday and interday precisions of each analyte were studied by measuring their concentrations in the three samples on a single or three consecutive days, respectively. The repeatability was determined by analyzing six different S15 solutions extracted from the same batch of LL. The stability test was conducted by analyzing each S15 solution at 0, 2, 4, 6, 12, 24, 48, and 72 h after preparation. Three different quantities (50 %, 100 %, and 150 %) of the authentic standards were added to the S15 sample. The samples were then extracted and analyzed using the proposed method by performing the experiments in triplicates at each concentration, and the % recovery rate was calculated using HPLC. The results are shown in Table 3 and Figs. S10–S14.

3.4.3. Calculation of the RCF values

Compounds with a high content and similar properties were selected as the IR. In particular, nuciferine and hyperoside were chosen as the IR for the alkaloids (asimilobine, 2-hydroxy-1-methoxy-aporphine, *N*-nornuciferine, and roemerine) and the flavonoids (isoquercitrin, trifolioside, and astragalin), respectively. The RCFs were determined using the QAMS method with two IRs to determine the contents of all nine components simultaneously in the LLs. The RCFs of each compound were calculated according to equation (1), and the contents according to equation (2). The results are summarized in Table 4.

$$f_x = \frac{As/Ws}{Ax/Wx} \quad \text{Equation 1}$$

$$W_x = \frac{Ax \times Ws}{As} \times f_x \quad \text{Equation 2}$$

where, f_x is the relative correction factor, As is the peak area of the IR, Ax is the peak area of the other compounds, Ws is the concentration of the IR, and Wx is the concentration of the other compounds.

3.4.4. Robustness of the RCFs

To investigate the robustness of the QAMS method, the mixed standard solution was analyzed under different conditions. The RCFs and relative retention time (RRT) of the other target peaks to the IR were calculated using different flow rates, column temperatures, and column types according to the average method and assessed in terms of the RSD (<5 %) [20]. The flow rate was set at 0.8/1.0/1.2 mL/min and the temperature at 28 °C/30 °C/32 °C. The mixed standard solution was injected into two instruments using three different columns to evaluate the robustness of the RCF. The results summarized in Tables S15 and S16 indicate that the RSD of the RCFs and the RRT was 0.05 %–2.47 %, demonstrating the suitability of the method for the quantitative analysis of alkaloids and flavonoids in the LL with reliability.

3.4.5. Assessment of the QAMS method and ESM

Based on the results obtained from the determination of RCF, the ESM and QAMS methods were used to quantify the 39 batches of

Table 4
Relative correction factors of alkaloids and flavonoids in lotus leaf.

Concentration	*f* asimilobine/ nuciferine	*f* 2-hydroxy-1-methoxy-aporphine / nuciferine	*f N*-nornuciferine / nuciferine	*f* roemerine / nuciferine	*f* isoquercitrin. / hyperoside	*f* trifolioside / hyperoside	*f* astragalin / hyperoside
1	1.2270	0.9600	1.0572	0.8749	1.0187	0.9774	0.9570
2	1.2220	0.9779	1.0145	0.8833	1.0588	0.9994	0.9431
3	1.2536	0.9637	1.0370	0.8709	1.0595	0.9590	0.9899
4	1.2531	0.9483	1.0376	0.8869	1.0244	0.9899	0.9630
5	1.2415	0.9359	1.0363	0.9047	1.0309	0.9808	0.9771
6	1.2730	0.9404	1.0458	0.9130	1.0621	0.9872	0.9728
Mean	1.2451	0.9544	1.0342	0.8889	1.0424	0.9823	0.9672
RSD/%	1.52	1.65	1.13	1.87	1.90	1.40	1.70

LLs to evaluate and verify the feasibility of QAMS. The results summarized in Tables S17 and S18 reveal that the RE values of these two methods were < 5.0 %. No significant differences were observed between the two methods, illustrating that the proposed QAMS method was accurate and reliable for the simultaneous quantification of the nine analytes in LL.

3.5. Quantitative analysis of the nine components of LL

3.5.1. Contents of the nine analytes in LL

In this study, the LL samples S1 – S39 were collected from different habitats in the northern and southern provinces of China. A box plot analysis of the nine analytes in the 39 batches of LLs was conducted using the GraphPad Prism software. Fig. 3 and Table S19 indicate that the average content of each decreased in the order: isoquercitrin > hyperoside > nuciferine > *N*-nornuciferine > 2-hydroxy-1-methoxy-aporphine > astragalin > roemerine > asimilobine > trifolioside. Isoquercitrin showed the highest median and mean values of 17.2470 and 19.0558 mg/g, respectively; while trifolioside showed the lowest at 0.0522 and 0.1480 mg/g, respectively. The large difference between the lowest and highest contents of hyperoside and nuciferine, indicated that their contents varied greatly in the 39 batches, which may be the main components responsible for the differences observed in the LLs from the various regions. The quality of LLs was further evaluated by chemometrics.

3.5.2. PLS-DA

To further explore the specific compounds responsible for the variations in the quality of LLs from different habitats, the contents of the nine analytes in the 39 batches were imported into the SIMCA-P software to perform a PLS-DA multivariate statistical analysis. The values of $R^2X(cum) = 1.000$, $R^2Y(cum) = 0.775$, and $Q^2(cum) = 0.458$ obtained indicated that the model fitted the data precisely and demonstrated good stability and excellent predictive ability. The model was tested by performing a permutation test and 200 validations, which indicated that the slopes of the regression lines of R^2 and Q^2 were > 1, indicating that the model did not overfit, and the prediction results were reliable (Fig. 4C).

The PLS-DA model divided the 39 batches into Classes A and B (Fig. 4A). The LLs in Class A were collected from the southern provinces, such as Jiangxi, Sichuan, Zhejiang, Hunan, and Hubei, while those in Class B were from the northern provinces, such as Shandong, Heilongjiang, and Liaoning. The occurrence of variations in Classes A and B may be attributed to the differences in precipitation and temperature between the two regions. The LL mainly grows in aqueous environments such as lakes, reservoirs, swamps, and water pools in the mountains. Sufficient precipitation makes them grow vigorously. Consequently, the content of each component was significantly higher in the LLs collected from the southern regions, which are rainier. The quality of water also has a great influence on the growth of LLs, which is better near the rivers than inland [21]. Similarly, the temperature differences between the northern and southern regions are also significant. Although LL is grown up from July to October, since the temperature in the south is higher than in the north, the LL in the south matured earlier. Based on a VIP value > 1 as the screening index, the three flavonoids: hyperoside, trifolioside, and astragaline, and the three alkaloids: *N*-nornuciferine, nuciferine, and asimilobine, may be assumed to be crucial for the differences between the northern and southern regions (Fig. 4B). Therefore, as alkaloids and flavonoids might play essential roles in the quality of LL, both types of components should be considered as the basis for controlling the quality of LL.

3.5.3. Volcano plot

The sample S15 originated from Guangchang County, Fuzhou City, Jiangxi Province, referred to as "the hometown of the lotus" since ancient times, and is one of the areas producing LL. Therefore, the differences in the levels of the nine components between S15 and LLs from other areas were further explored. The *P* values and fold change (FC) values of the nine analytes in S15 and samples from the other areas were calculated, and the volcano diagram was obtained (Fig. 5). Values of $P < 0.05$ and FC > 2 or < 0.5 indicated a significant increase or decrease in the contents, respectively. The distribution of the nine components in the remaining 38 batches is

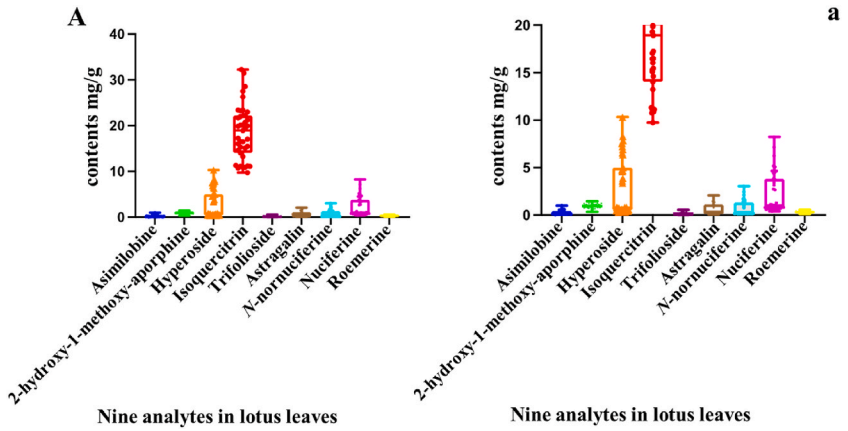

Fig. 3. Box diagram of nine chemical markers in lotus leaf samples (S1–S39) from different habitats (Note: a is the enlarged graph of A).

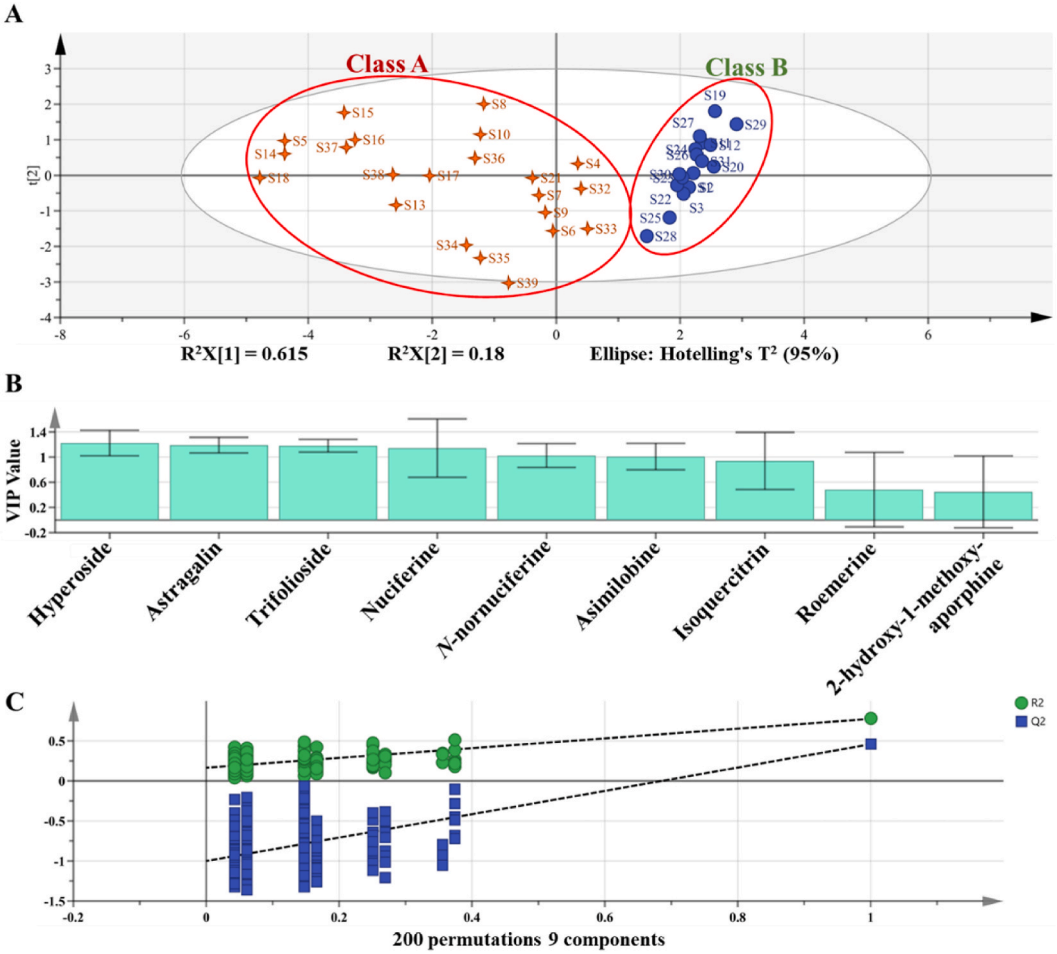

Fig. 4. PLS-DA model for 39 batches of lotus leaf. A: scatter plot of PLS-DA distribution for S1–S39 samples; B: VIP values of nine analytes; C: 200 substitution tests for nine analytes.

shown in Fig. 5A and the distribution of individual components in Fig. 5B–J. The details of specific samples are indicated in Table S20. The contents of isoquercitrin were higher in the ten batches of S7, 11, 12,19, 20, 27, 29, 30, 31, and 39 than in S15, while there were no significant differences with the remaining 28 batches. The contents of roemerine in the 38 batches were similar to that of S15 without any marked differences. The contents of the three flavonoids: astragalin, hyperoside, and trifolioside; and the three alkaloids: nuciferine, *N*-nornuciferine, and asimilobine were significantly lower in most batches compared to S15. In general, the levels of isoquercitrin in S15 were lower than those in the LLs from other places, while the contents of the three flavonoids: astragalin, hyperoside, and trifolioside; and the three alkaloids: nuciferine, *N*-nornuciferine, and asimilobine were higher in S15 than the LLs from other areas.

3.5.4. Analysis of the correlation between the inhibition of PPARγ expression and the contents of the nine selected compounds from the 39 batches of LLs

The 3T3-L1-Lenti-*PPARγ*-RE-Luc cells were used to evaluate the potential lipid-lowering activities of the 39 batches of LLs. Firstly, the cell viability assay indicated that all 39 batches had no notable cytotoxic activity (all > 90 %) (Table S21). Further, the luciferase assay indicated that the LLs had significantly inhibit the expression of *PPARγ* ($P < 0.001$) (Fig. S6 and Table S22). The correlations between the contents of the nine analytes in the 39 batches of samples and the rate of inhibition of *PPARγ* expression by the LL extracts were analyzed using SPSS 26.0 (Table S23). The contents of trifolioside, astragalin, hyperoside, *N*-nornuciferine, and nuciferine had a marked positive correlation with the inhibitory effect on *PPARγ* expression ($r > 0.8$). Asimilobine showed a significant positive correlation, while isoquercitrin exhibited a negligible negative correlation ($0.3 < r < 0.8$) [22]. Roemerine and 2-hydroxy-1-methoxy-aporphine demonstrated no correlation ($r < 0.3$). Thus, the six compounds: trifolioside, astragalin, hyperoside, *N*-nornuciferine, nuciferine, and asimilobine that were positively correlated with the inhibition of *PPARγ* expression were considered to be critical components of LL with a lipid-lowering effect. T0070907 is an inhibitor of *PPARγ*, with an inhibition rate of 20.24 % at 5 μM. The LL extracts with *PPARγ*-expression-inhibition rate > 20 %, i.e., S5, 8, 10, 13, 14, 15, 16, 17, 18, 21, 34, 36, 37, and 38 were considered to possess a strong activity. The contents of six of these compounds in the 39 samples were summed and sequenced. The first 14 samples with high contents identified, i.e., S5, 8, 10, 13, 14, 15, 16, 17, 18, 21, 34, 36, 37, and 38, were the same as the samples with a marked

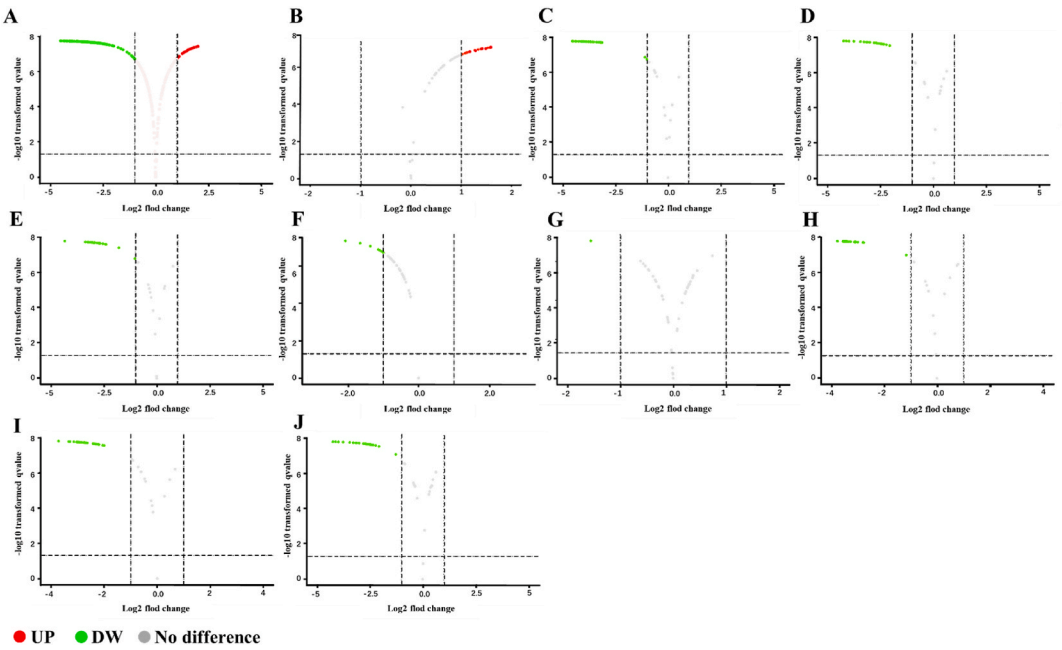

Fig. 5. Volcano plot of nine analytes in lotus leaf samples S1–S39 A: Nine analytes; B: isoquercitrin; C: hyperoside; D: astragalin; E: trifolioside; F: 2-hydroxy-1-methoxy-aporphine; G: roemerine; H: *N*-nornuciferine; I: nuciferine; J: asimilobine.. (Note: The red dots indicate a significant increase, the green dots indicate a significant decrease, and the gray dots indicate no significant change.)

bioactivity, suggesting a significant correlation between the content and the bioactivity. These results indicated that the three alkaloids and three flavonoids contributed to the lipid-lowering activity, and six components had significance lipid-lowering activity ($P < 0.001$) compared with the control group by luciferase reporter assay, as shown in Fig. 6A and B. Finally, the three alkaloids: *N*-nornuciferine, nuciferine, and asimilobine; and the three flavonoids: trifolioside, hyperoside, and astragaline may be considered as crucial components affecting the quality of LL.

3.6. Discussion and conclusions

To detect the five alkaloids and four flavonoids of LL simultaneously and meet the conditions of quantitative analysis, the mobile phase used was acetonitrile combined with an aqueous solution of 0.2 % phosphoric acid and 0.2 % triethylamine (pH 2) for HPLC. The disadvantage of phosphoric acid and triethylamine being non-volatile does not allow them to be used for mass spectrometry. Using UPLC-HRMS, the 13 components of LLs were characterized using acetonitrile and an aqueous solution of 0.1 % formic acid as the mobile phase. Of the flavonoids, isoquercitrin and hyperoside (peaks 3 and 4 in Fig. 2) were a pair of isomer compounds, and the chemometrics analysis showed that only the content of hyperoside has correlation with *PPARγ* expression. So, a good chromatographic separate for these two compounds is essential. Our chromatographic method gave an excellent separation of these two isomers.

In conclusion, the established QAMS method enabled the simultaneous determination of five alkaloids: nuciferine, *N*-nornuciferine, asimilobine, 2-hydroxy-1-methoxyaaporphine, and roemerine; and four flavonoids: isoquercitrin, hyperoside, astragalin, and trifolioside under one same chromatographic condition. Using dual internal standards instead of single internal standard gave more reliable results when involving two different kinds of structures. Secondly, QAMS combined with chemometric methods such as PLS-DA and a volcano plot was firstly applied in quality control of the herbal medicine, which finally figured out six bioactive compounds related to the lipid-lowering effects of LL. Eventually, three alkaloids and three flavonoids were found to be the crucial components for the quality evaluation of LL on lipid-lowering effect. Our findings give a better understanding of the chemical basis of LL relevant to lipid-lowering effect and provide scientific evidence for a comprehensive quality evaluation of this famous herbal medicine.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Limin Ouyang: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

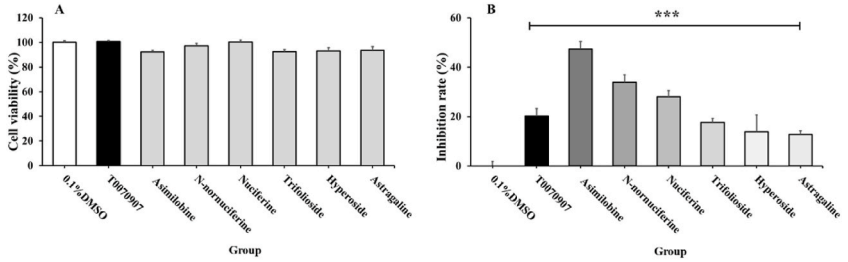

Fig. 6. The inhibitory effect of six compounds on PPARγ expression at 5 μM (A) Cell viability of 3T3-L1-Lenti-PPARγ-RE-Luc cell by CCK-8 assay; (B) Inhibition rate of PPAR-γ expression by luciferase reporter assay; 0.1 % DMSO and T0070907 standed for vehicle control and the positive control at 5 μM * Represents comparison with control group ***$P < 0.001$.

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 A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23009.

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