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Profiling of the leaves and stems of *Curcuma longa* using LC-ESI-MS and HPLC analysis

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Abstract *Curcuma longa* is a plant belonging to the genus *Curcuma* and is distributed across various Asian regions. This plant is widely known for its rhizomes, which possess a variety of pharmacological properties. However, although the leaves and stems of this plant also contain several health-promoting secondary metabolites, very few studies have characterized these compounds. Therefore, our study sought to quantify the secondary metabolites from the leaves and stems of *Curcuma longa* L. (LSCL) using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) and high-performance liquid chromatography (HPLC). Our LC-ESI-MS analyses detected twenty-one phenolic compounds in the LSCL, among which fifteen compounds were detected via HPLC analysis. Four compounds, namely vanillic acid (0.129 mg/g), *p*-coumaric acid (0.431 mg/g), 4-methylcatechol (0.199 mg/g), and afzelin (0.074 mg/g) were then quantified. These findings suggest that LSCL is rich in secondary metabolites and holds potential as a valuable resource for the development of functional and nutritional supplements in the future.

Keywords *Curcuma longa* L. · High-performance Liquid Chromatography-Electrospray Ionization-mass Spectrometry · Quantitative analysis

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Introduction

Curcuma longa L. (CL), commonly known as turmeric, is a herbaceous perennial plant belonging to the genus *Curcuma* in the Zingiberaceae family, which consists of a variety of plants that grow from rhizomes [1,2]. This plant is extensively cultivated in tropical and subtropical regions, particularly in Asian countries such as India, China, Vietnam, and Thailand [3,4].

The rhizomes of CL have been traditionally used as a medicinal remedy in China for treating inflammation, sepsis, and wounds, as well as for their stimulant, carminative, emmenagogue, astringent, detergent, and diuretic properties [5]. Additionally, powdered CL rhizomes have a wide range of applications for pain management in patients with skin disease, Crohn's disease, and ulcerative colitis [6]. CL is rich in phenolic compounds, which contributes to its numerous therapeutic properties including its energizing, antioxidant, antibacterial, and anticancer effects [7,8]. CL powder is widely utilized as a component in curry dishes and as a food coloring agent due to its deep orange-yellow color when boiled [9]. Additionally, CL essential oils are a potential raw material for the extraction of pharmaceutical compounds, nutraceuticals, and functional food ingredients. The main active compounds in CL essential oil include ar-turmerone, β-turmerone, α-zingiberene, α-turmerone, and germacrone, all of which possess extraordinary antibacterial and antifungal activities [10]. The essential oils of CL also exhibit various biological properties, including antimicrobial and larvicidal effects, as well as repellent, antioxidant, anti-inflammatory, and anticancer properties [6].

Furthermore, CL leaves are also a main ingredient in a variety of dishes in South-East Asia. Some of the bioactive compounds in CL leaves include curcumin, phenolics, and flavonoids [11]. However, although CL leaves possess antioxidant, anti-inflammatory, antitumor, and antibacterial properties, they are often discarded as byproducts or used as animal feed after harvesting [12-14]. Furthermore, the existing literature on CL has mostly focused on rhizomes, whereas the bioactive compounds and functionality of CL leaves and stems remain largely unexplored.

Therefore, our study sought to analyze the secondary metabolites present in the leaves and stems of CL using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) and high-performance liquid chromatography (HPLC) analysis.

Materials and Methods

Plant materials

CL was cultivated by Jindoulguem Corp. (Jindo-gun, Jeollanam-do, Korea) from April to November, 2021. The aerial parts of CL were harvested in November and the collected fresh leaves and stems were dried. The dried leaves and stems were then cut to 2-3 cm pieces using a crusher. The leaves and stems of *C. longa* (LSCL; TLSWE-8510) were obtained from French Korean Aromatics Co., Ltd., Yongin, Korea.

Instruments and reagents

Liquid chromatography was conducted using a Thermo Vanquish UHPLC (Thermo Scientific, Waltham, MA, USA). Mass spectrometry was performed using a high-resolution mass spectrometer (Q Exactive Hybrid Quadrupole-Orbitrap, Thermo Scientific). The remaining analyses were performed with an HPLC system (Waters Alliance e2695 Separations Module, Milford, MA, USA) consisting of an auto-sampler, pump, and photodiode array detector (Waters 2998 PDA detector). HPLC-grade solvents including water and acetonitrile (ACN) were purchased from J. T. Baker (Phillipsburg, PA, USA), and HPLC-grade trifluoroacetic acid (TFA) was purchased from Thermo Scientific. Vanillic acid, *p*-coumaric acid, 4-methylcatechol, and afzelin (Fig. 1) were obtained from the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea.

Preparation of samples and standard solutions for HPLC

Dried LSCL (10 g) was extracted with EtOH (200 mL) under reflux and evaporated *in vacuo*. The EtOH extract was dissolved in 80% MeOH (50 mg/mL) and filtered through a syringe filter (PVDF, 0.45 μm). Stock solutions of the standard compounds were prepared by dissolving them in 80% MeOH. To prepare the standard calibration curves, working solutions were prepared by diluting the stock solution to the concentrations from 1.5625 to 100 μg/mL.

LC-ESI-MS conditions

Separation was conducted using an LC system consisting of a Thermo Vanquish UHPLC with a Waters Cortex T3 column (150 mm×2.1 mm, particle size 1.6 μm) maintained at 45 ºC. The mobile phase was composed of water (eluent A: 0.1% HCOOH) and acetonitrile (eluent B: 0.1% HCOOH). The gradient started at 3% B and increased to 15% B for 15 min, then increased again to 100% B for 35 min, and was finally held for 5 additional minutes (total run time: 55 min). The column was finally re-equilibrated to 3% eluent B for 5 min. The flow rate was set at 0.25 mL/min. Mass spectrometry was conducted using a high-resolution mass spectrometer equipped with a heated electrospray ion source. The mass spectrometer was operated in both positive and negative ion modes. Survey full-scan MS spectra (*m/z* 100-1500) were acquired using a quadrupole system with a resolution setting of 70000. The spray voltage was set to 3.5 and 3.0 kV for the positive and negative ion modes, respectively. The top ten most intense precursor ions were selected for MS2 fragmentation, and spectra acquisition was performed using a resolution setting of 17500. The remaining MS parameters were as follows: capillary temperature, 320 ºC; sheath gas, 50 AU; sweep gas, 1 AU; auxiliary gas, 10 AU.

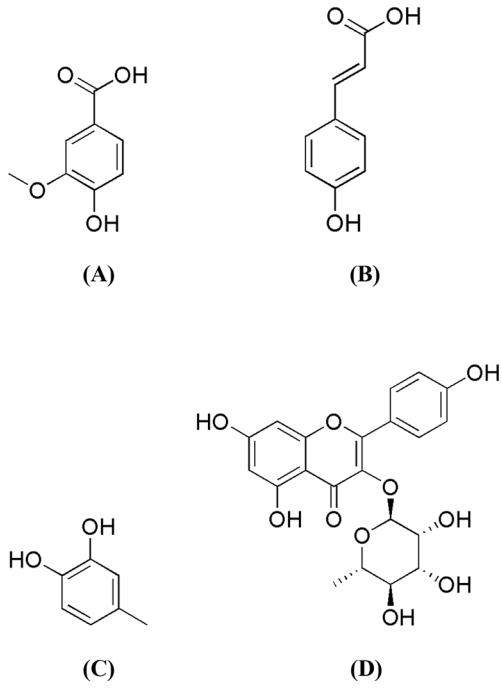


Fig. 1 Chemical structures of vanillic acid (A), *p*-coumaric acid (B), 4-methylcatechol (C), and afzelin (D)

HPLC/PDA conditions

HPLC analysis was performed on a reversed-phase HPLC system using a YMC Pack-Pro C18 column (4.6×250 mm, 5 μm). Detection was carried out at a 254 nm wavelength and an injection volume of 10 μL. Analyses were conducted at 30 ºC with a 0.9 mL/min flow rate using a gradient elution system. The mobile phase was 0.1% TFA in water (A) and ACN (B). The elution program was as follows: 8% B at 0 min and maintained until 5 min, followed by 18% B from 5 to 10 min, 22% B from 10 to 23 min, 50% B from 23 to 38 min, 80% B from 38 to 42 min, and 100% B from 42 to 44 min and maintained until 50 min.

Calibration curve

A calibration curve was prepared by plotting the concentrations of

the standard solution against their respective peak areas. The linearity of the calibration curve was determined based on the correlation coefficient (r^2), and the standard concentrations in the samples were then calculated from the calibration curve. The calibration functions were determined based on the peak area (Y), concentration (X, mg/mL), and mean ± standard deviation (n =3).

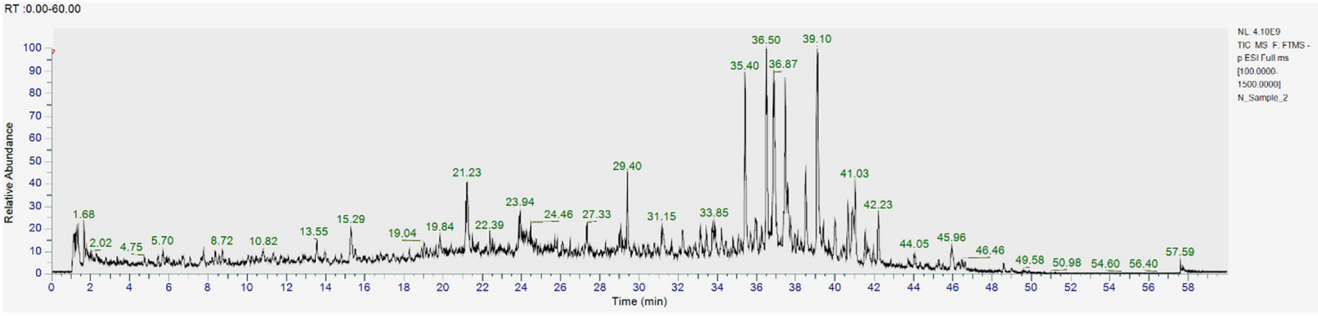
Results and Discussion

CL is a plant that has been widely used since ancient times due to its wide variety of confirmed therapeutic properties. Nowadays, CL rhizomes are still used as a functional food; however, only a few people know the leaves and stems' benefits like antioxidant activity [15]. Previous studies have assessed the total phenolic content, total flavonoid content, and antioxidant properties of CL leaves. One study estimated the antioxidant capacity of fresh and dried CL leaves and the total phenolic content to be 4.12 mg gallic acid equivalent/g dry matter [16]. Another study demonstrated that the total phenolic compound content of a CL leaf water extract was 2.741 mg gallic acid equivalent/g extract, whereas the flavonoid content was 4.776 mg quercetin equivalent/g extract. This study also demonstrated the antioxidant properties of CL leaves using the DPPH and ABTS assays, in addition to assessing the hydrogen peroxide-scavenging activity [17]. Nevertheless, our knowledge of the composition of functional compounds in LSCL remains limited.

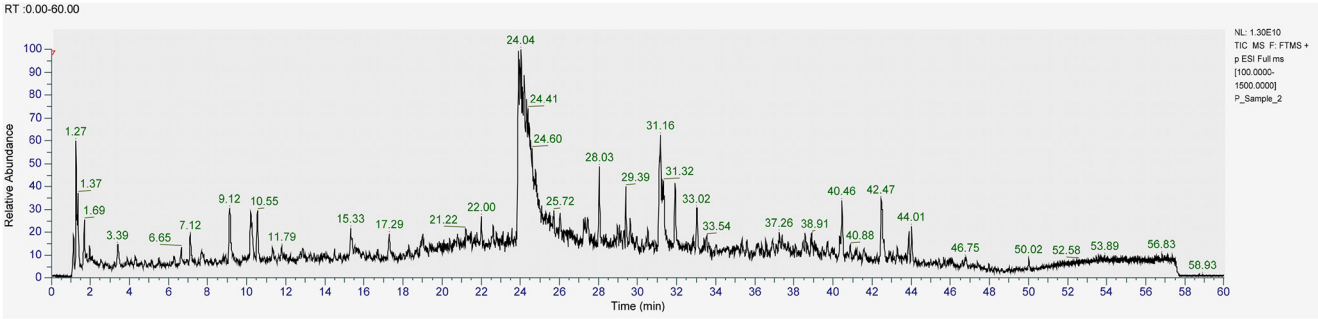
To investigate the phytochemical compounds in LSCL, an EtOH extract of LSCL was analyzed via the LC-ESI-MS method. A total ionization chromatogram in positive and negative modes revealed the presence of 21 compounds (Fig. 2) and an overview profiling of LSCL is presented in Table 1. The first compound isolated was protocatechuic acid (*m/z* 154.0), followed by 4-methylcatechol (*m/z* 124.1) and 4-O-methylgallic acid (*m/z* 184.0) at retention times of 5.20, 6.92, and 7.07 min, respectively. Other compounds were identified later on, with quercetin (*m/z* 302.0) being the last compound detected at 24.18 min.

Overall, all of the detected compounds were classified as phenolic compounds, which are widely distributed in nature and can be found in various plants, fruits, vegetables, and other sources. Phenolic compounds have gained significant attention due to their potential health benefits and antioxidant properties. Particularly, they exhibit a range of bioactivities, including anti-inflammatory, antimicrobial, anticancer, and cardiovascular protective effects. Moreover, these compounds are known for their ability to scavenge free radicals and prevent oxidative damage in the body [18-21].

HPLC analysis was conducted based on the LC-ESI-MS results. A total of 15 compounds were identified, with 11 compounds showing trace content. Therefore, only the remaining four compounds, namely vanillic acid, *p*-coumaric acid, 4-methylcatechol, and afzelin, were quantified. The ESI-MS data of these four compounds are described in Fig. 3 and the HPLC chromatogram of the



(A)



(B)

Fig. 2 Total ion chromatograms of EtOH extracts from LSCL in negative (A) and positive (B) ionization modes using LC-ESI-MS

Table 1 LC-ESI-MS profiling of LSCL in positive and negative ionization modes

Retention time (min)	Molecular weight	Proposed structure
5.20	154.0	protocatechuic acid[1]
6.92	124.1	4-methylcatechol[1]
7.07	184.0	4-*O*-methylgallic acid[1]
10.01	168.0	5-methoxysalicylic acid[1]
10.16	179.1	phenacetin[2]
10.24	150.1	cuminyl alcohol[2]
10.83	168.0	vanillic acid[1]
11.40	180.0	caffeic acid[1]
12.96	234.2	lidocaine[2]
13.95	152.0	2-hydroxy-5-methoxybenzaldehyde[1]
15.27	164.0	*p*-coumaric acid[1]
16.22	196.1	hydroferulic acid[1]
17.70	194.1	ferulic acid[1]
18.27	610.2	quercetin 3-*O*-neohesperidoside[1]
19.34	610.2	rutin[1]
19.51	594.2	kaempferol 3-*O*-neohesperidoside[1]
19.63	464.1	hyperoside[1]
22.37	578.2	lespenefril[1]
22.38	286.0	luteolin[2]
22.49	432.1	afzelin[1]
24.18	302.0	quercetin[1]

[1] = positive ion mode
[2] = negative ion mode

ethanol extracts of LSCL is presented in Fig. 4. In the chromatogram, the four compounds were well separated, with retention times of 15.02 min for vanillic acid, 19.79 min for *p*-coumaric acid, 21.14 min for 4-methylcatechol, and 9.82 min for afzelin. The linear calibration curve equations of vanillic acid, *p*-coumaric acid, 4-methylcatechol, and afzelin were $Y = 25394X + 5740.7$, $Y = 6289.1X + 4565.3$, $Y = 9112.6X + 1984.1$, and $Y = 16116X + 5399.1$, respectively, where Y represents a given peak area and X represents the compound concentration. The correlation coefficients (r^2) were all above 0.9991, indicating that the quantification method had excellent linearity (Table 2). Next, the content of each compound in LSCL was determined using the calibration curve equation. The chromatogram of the sample is shown in Fig. 4, and the quantitative analysis results are summarized in Table 3.

In general, *p*-coumaric acid exhibited the highest content in LSCL (0.431 mg/g), followed by 4-methylcatechol (0.199 mg/g), vanillic acid (0.129 mg/g), and finally afzelin (0.074 mg/g). Vanillic acid and *p*-coumaric acid are phenolic acids that are abundant in many plant species and are widely consumed through the diet [22]. Vanillic acid is present in vanilla beans, strawberries, raspberries, olives, cereals, whole grains, fruits, green tea, and wines [23]. This compound is known to possess anticancer, antiobesity, antidiabetic, antibacterial, anti-inflammatory, and antioxidant properties [24-25]. Similarly, *p*-coumaric acid has also been linked to multiple health benefits such as anti-melanogenic effects in different experimental settings including human studies [26-28]. 4-Methylcatechol, a polyphenolic compound, is produced during the metabolism of quercetin and has shown promising potential as a useful anti-melanoma agent due to its ability to inhibit cell survival and induce apoptosis in melanoma cells [29-30]. On the other hand, afzelin is a flavonoid compound belonging to the flavonol subgroup. Previous studies have demonstrated that afzelin exhibits strong antioxidant activity, anti-inflammatory properties, and potential anticancer effects, particularly inhibiting the growth of breast and colorectal cancer cells and inducing apoptosis in certain cancer types [31-32]. Collectively, these compounds exhibit diverse biological activities and contribute to the therapeutic properties of LSCL.

A similar study was conducted to identify flavonoids in CL leaves, revealing the presence of 10 key compounds, including diosmetin, quercitrin, rutin, miquelianin, taxifolin, myrictrin, puerarin, narirutin, naringin, and quercetin, among 30 flavonoid samples [5]. In contrast, our study examined the composition of all phytochemical compounds, not only flavonoids. It is also worth noting that the chemical composition of plants may vary depending on the geographical location, harvest time, light, pH, temperature, and weather conditions [33]. These environmental

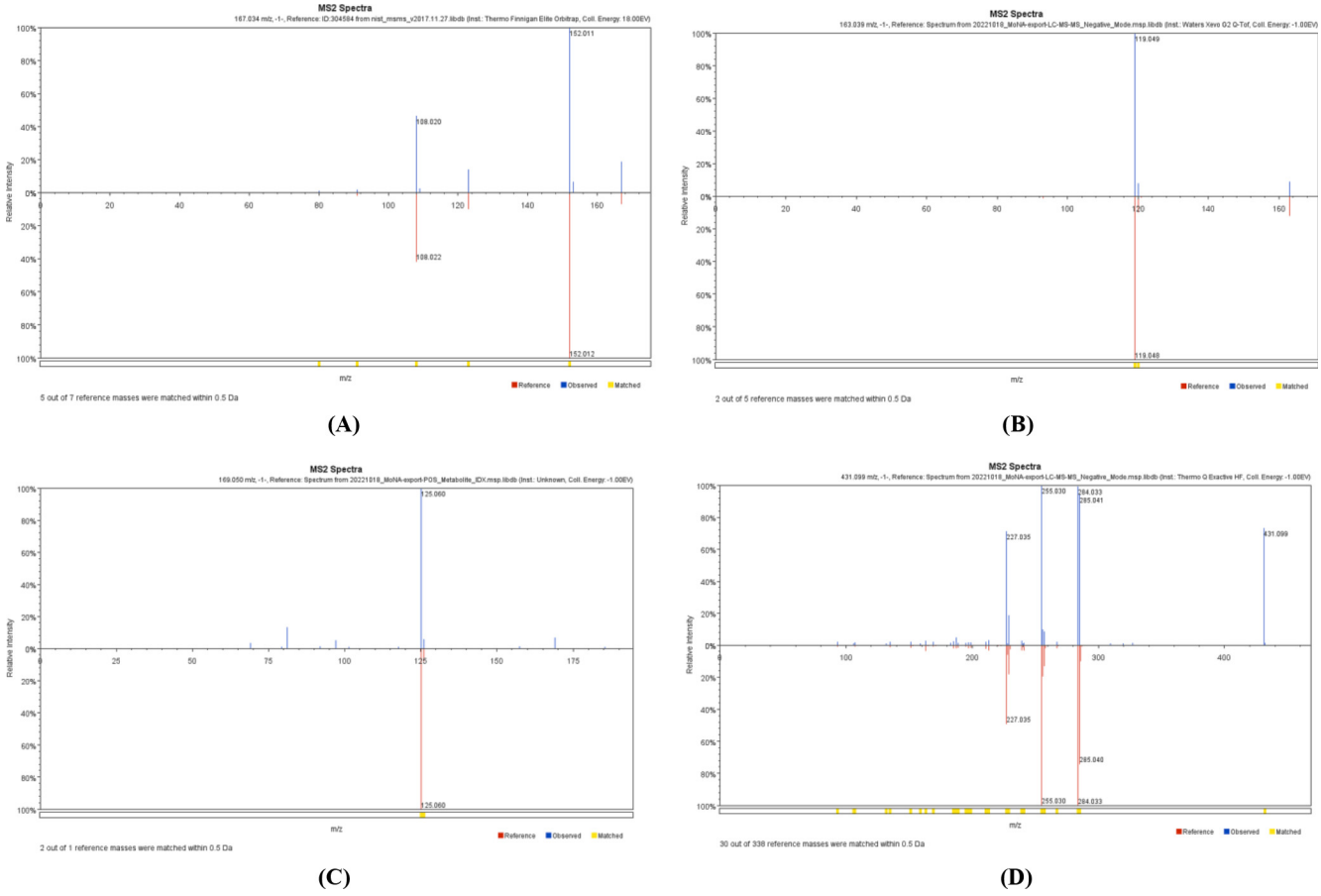


Fig. 3 ESI-MS data of vanillic acid (A), *p*-coumaric acid (B), 4-methylcatechol (C), and afzelin (D)

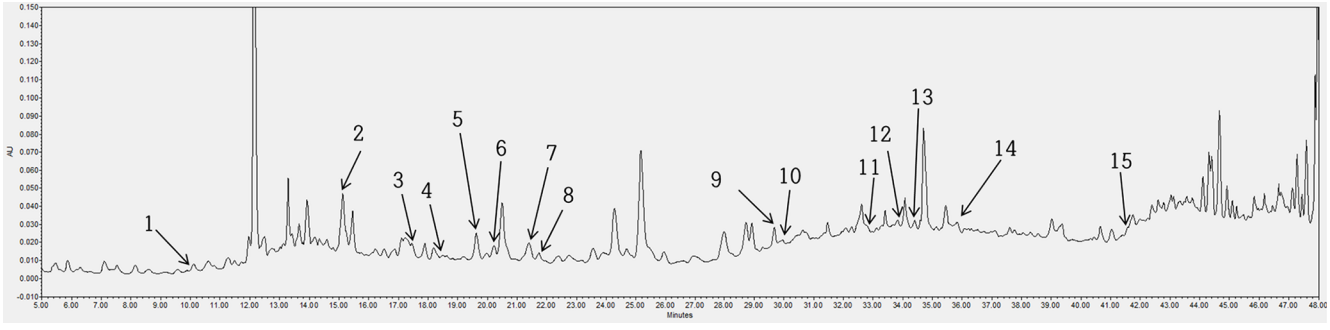


Fig. 4 HPLC chromatogram of the EtOH extracts of LSCL. **1**: protocatechuic acid, **2**: vanillic acid, **3**: quercetin 3-*O*-neohesperidoside, **4**: lidocaine, **5**: *p*-coumaric acid, **6**: hyperoside, **7**: 4-methylcatechol, **8**: ferulic acid, **9**: afzelin, **10**: phenacetin, **11**: 5-methoxysalicylic acid, **12**: luteolin, **13**: quercetin, **14**: 2-hydroxy-5-methoxybenzaldehyde, **15**: cuminyl alcohol

variations could explain why some of the compounds reported in previous research were not detected in our study.

In conclusion, this study used LC-ESI-MS to characterize the phytochemical compounds present in LSCL, after which the contents of these components were determined via HPLC analysis. A total of 15 phenolic compounds were identified in LSCL and four of them were quantified. Among them, *p*-coumaric acid exhibited the highest content (0.431 mg/g), whereas afzelin exhibited the lowest levels (0.074 mg/g). The present study can hardly be compared with existing literature because studies employing CL leaves are scant. The authors believe, to the best of their knowledge, that the present study is the first of its kind. These findings suggest that despite its relatively low phenolic compound levels, LSCL possesses several potential biological activities and could be utilized as a functional food ingredient in the future.

Table 2 Calibration curves for quantitative standards of LSCL

Compound	t_R	Calibration equation	Correlation factor, r^2
vanillic acid (**2**)	15.02	Y = 25394X + 5740.7	0.9991
p-coumaric acid (**5**)	19.79	Y = 6289.1X + 4565.3	0.9991
4-methylcatechol (**7**)	21.14	Y = 9112.6X + 1984.1	0.9999
afzelin (**9**)	29.82	Y = 16116X + 5399.1	0.9993

t_R = retention time
Y = peak area, X = concentration of the standard (μg/mL)
r^2 = correlation coefficient for five data points in the calibration curve

Table 3 Quantification of phytochemical contents in LSCL via HPLC analysis

Compound	Contents (mg/g)
protocatechuic acid (**1**)	tr
4-*O*-methylgallic acid	ND
vanillic acid (**2**)	0.129±0.000
caffeic acid	ND
quercetin 3-*O*-neohesperidoside (**3**)	tr
lidocaine (**4**)	tr
rutin	ND
kaempferol 3-*O*-neohesperidoside	ND
p-coumaric acid (**5**)	0.431±0.002
hydroferulic acid	ND
hyperoside (**6**)	tr
lespenefril	ND
4-methylcatechol (**7**)	0.199±0.001
ferulic acid (**8**)	tr
afzelin (**9**)	0.074±0.000
phenacetin (**10**)	tr
5-methoxysalicylic acid (**11**)	tr
luteolin (**12**)	tr
quercetin (**13**)	tr
2-hydroxy-5-methoxybenzaldehyde (**14**)	tr
cuminyl alcohol (**15**)	tr

tr: trace, ND: not detected

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