A New HPLC-UV Method Using Hydrolyzation with Sodium Hydroxide for Quantitation of Trans-p-hydroxycinnamic Acid and Its Esters in the Leaves of Ligustrum Robustum

Shi-Hui Lu *, Xiao-Na Liang , Xiao-Jin Nong , Ran Chen , Xiu-Xia Li *

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Keywords: trans-p-hydroxycinnamic acid; esters; Ligustrum robustum; HPLC-UV; hydrolyzation; sodium hydroxide; quantification

Article

A New HPLC-UV Method Using Hydrolyzation with Sodium Hydroxide for Quantitation of *Trans-p*-hydroxycinnamic Acid and Its Esters in the Leaves of *Ligustrum robustum*

Shi-Hui Lu [1,2,3,*,†], Xiao-Na Liang [1,†], Xiao-Jin Nong [1], Ran Chen [4] and Xiu-Xia Li [5,*]

[1] College of Pharmacy, Youjiang Medical University for Nationalities, Baise 533000, People's Republic of China; 20221001046@stu.ymun.edu.cn (X.-N.L.); n15777644073@163.com (X.-J.N.)
[2] Guangxi Database Construction and Application Engineering Research Center for in Tracorporal Pharmacochemistry of TCM, Baise 533000, People's Republic of China
[3] Key Laboratory of Youjiang Basin Characteristic Ethnic Medicine Research in Guangxi, Baise 533000, People's Republic of China
[4] Institute of Life Science, Youjiang Medical University for Nationalities, Baise 533000, People's Republic of China; ran625@ymun.edu.cn
[5] Nursing School, Youjiang Medical University for Nationalities, Baise 533000, People's Republic of China
* Correspondence: lushihui0818@126.com (S.-H.L.); axia-883333@163.com (X.-X.L.)
† These authors contributed equally to this article.

Abstract: The leaves of *Ligustrum robustum* have been applied as Ku-Ding-Cha, a functional tea to clear heat and remove toxins; and served as a folk medicine to deal with diabetes, obesity, and hypertension. *Trans-p*-hydroxycinnamic acid and its esters, which were isolated from the leaves of *L. robustum*, might be a new resource to prevent diabetes and its complications because of their strong α-glucosidase and fatty acid synthase (FAS) inhibitory activities and antioxidant effect. However, the quantitative analytical method for *trans-p*-hydroxycinnamic acid and its esters in the leaves of *L. robustum* hasn't been reported so far. In addition, it was difficult and troublesome to analyze no less than 34 *trans-p*-hydroxycinnamic acid esters by usual HPLC. Therefore, a new HPLC-UV method using hydrolyzation with sodium hydroxide for quantitation of *trans-p*-hydroxycinnamic acid and its esters in the leaves of *L. robustum* was developed in the present study. The optimal hydrolyzation conditions were as follows: 1 mL sodium hydroxide aqueous solution (1 M) was added into 1 mL the extract of *L. robustum* and incubated at 80 °C for 2 h , and then 1 mL hydrochloride (1 M) solution was added. HPLC analysis was performed in reverse phase mode using a C-18 column, eluting with methanol-0.1% acetic acid aqueous solution (40:60, *v*/*v*) in isocratic mode at a flow rate of 1.0 mL/min, and detecting at 310 nm. The analytical method was simple and rapid, which simplified greatly the analytical process. The method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. High linearity was demonstrated with correlation coefficient of 1.000 for *trans-p*-hydroxycinnamic acid. The LOD and LOQ values were 1.55 and 4.69 μg/mL, respectively. The relative standard deviations (RSD) of intra-day and inter-day variabilities for *trans-p*-hydroxycinnamic acid were less than 2%. The percentage recovery of *trans-p*-hydroxycinnamic acid was 101.2% ± 0.8%. Finally, the proposed method was applied successfully to the determination and comparison of the contents of *trans-p*-hydroxycinnamic acid and its esters in various extracts of the leaves of *L. robustum*. The 60%-70% ethanol extracts of *L. robustum* showed the highest contents of free *trans-p*-hydroxycinnamic acid (3.96-3.99 mg/g), and the 50%-80% ethanol extracts of *L. robustum* displayed the highest contents of *trans-p*-hydroxycinnamic acid esters (202.6-210.6 mg/g). The method can be applied also to the quality control of the products of *L. robustum*.

Keywords: *trans-p*-hydroxycinnamic acid; esters; *Ligustrum robustum*; HPLC-UV; hydrolyzation; sodium hydroxide; quantification

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1. Introduction

Ligustrum robustum (Roxb.) Blume, a plant of Oleaceae, is distributed widely in Southwest China, Burma, Vietnam, India and Cambodia [1]. The leaves of *L. robustum* have been used as Ku-Ding-Cha, a functional tea to clear heat and remove toxins, in Southwest China for near 2000 years [2,3]. Additionally, *L. robustum* was served as a folk medicine to deal with diabetes, obesity, hypertension, and so on [3,4].

In the previous phytochemical studies [2,5–17], about 90 chemical constituents, including *trans-p*-hydroxycinnamic acid, 34 *trans-p*-hydroxycinnamic acid esters (Figure 1), flavonoid glycosides, lignan glycosides, and other compositions, were isolated and identified from the leaves of *L. robustum*. In our previous biological investigations [14–17], the chemical compositions isolated from the leaves of *L. robustum* were tested and the results showed that: (1) *trans-p*-hydroxycinnamic acid displayed stronger α-glucosidase inhibitory activity than the positive control acarbose; (2) *trans-p*-hydroxycinnamic acid and several *trans-p*-hydroxycinnamic acid esters displayed no weaker fatty acid synthase (FAS) inhibitory activities than the positive control orlistat; (3) *trans-p*-hydroxycinnamic acid and a lot of *trans-p*-hydroxycinnamic acid esters revealed stronger 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ammonium salt (ABTS) radical scavenging effects than the positive control L-(+)-ascorbic acid, and revealed moderate α-amylase inhibitory activities. What is more, *trans-p*-hydroxycinnamic acid esters might be hydrolyzed with catalysis of carboxylesterase or gastric acid in vivo [18,19], and release *trans-p*-hydroxycinnamic acid, meaning that *trans-p*-hydroxycinnamic acid esters are the prodrugs of *trans-p*-hydroxycinnamic acid. In addition, it was reported that natural products with inhibitory activities on α-glucosidase, α-amylase, and FAS as well as an antioxidant effect might be a new resource to prevent diabetes and its complications [17]. Therefore, *trans-p*-hydroxycinnamic acid and its esters isolated from *L. robustum* might be a novel resource for preventing diabetes and its complications.

However, the quantitative analytical method for *trans-p*-hydroxycinnamic acid and its esters in the leaves of *L. robustum* hasn't been reported so far. HPLC is a common and accurate method to analyze simultaneously several compositions [20], but it was difficult and troublesome to determine 34 *trans-p*-hydroxycinnamic acid esters by usual HPLC. In addition, there might be other unknown *trans-p*-hydroxycinnamic acid esters in the leaves of *L. robustum*. In the present study, thus, a new HPLC-UV method using hydrolyzation with sodium hydroxide for quantitation of *trans-p*-hydroxycinnamic acid and its esters in the leaves of *L. robustum* was developed and validated. Moreover, the proposed method was applied successfully to the determination and comparison of the contents of *trans-p*-hydroxycinnamic acid and its esters in various extracts of the leaves of *L. robustum*.

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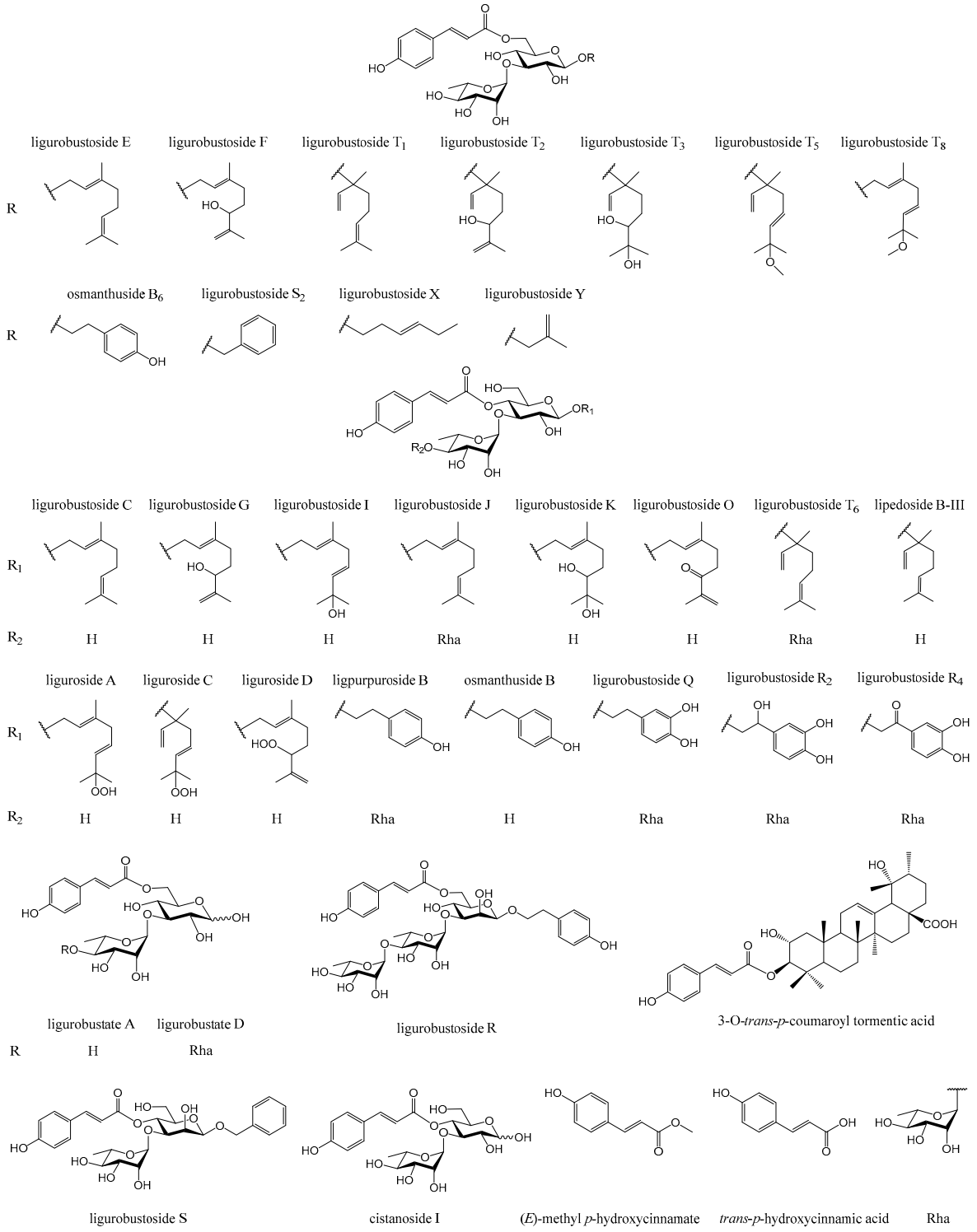

Figure 1. Structures of *trans-p*-hydroxycinnamic acid and its esters isolated from the leaves of *L. robustum*.

2. Results and Discussion

2.1. Method Development

The raw powder of the dried leaves of *L. robustum* was extracted with 70% (*v*/*v*) ethanol (25 mL/g) under reflux for 1 h. The conditions of hydrolyzation of *trans-p*-hydroxycinnamic acid esters in the extracting solution of *L. robustum* were optimized by changing various parameters, i.e., catalyst of hydrolyzation (hydrochloride or sodium hydroxide), incubation temperature (30-90 °C), and period

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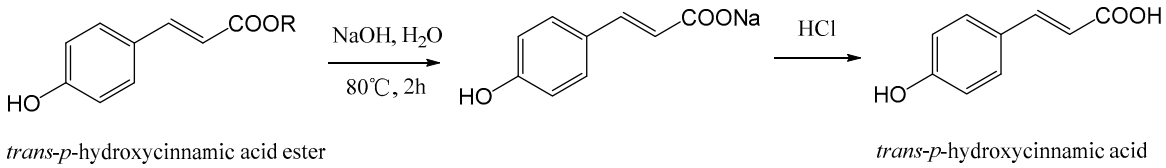
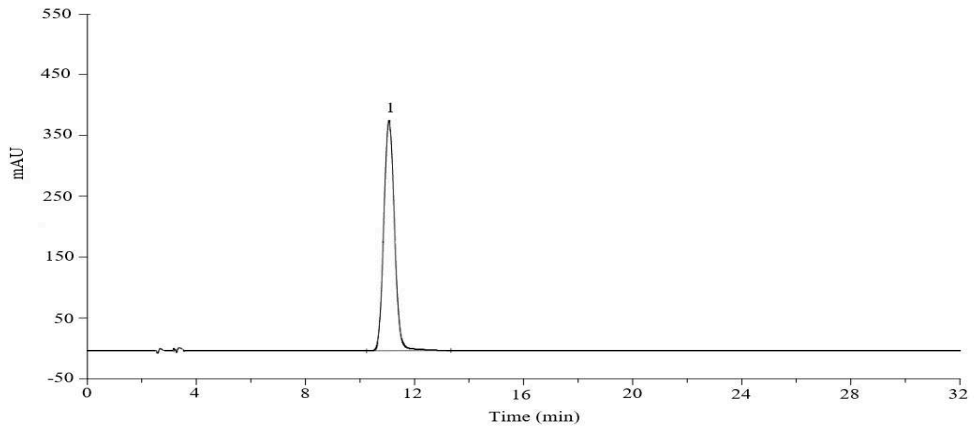
of incubation (1-6 h). As a result (Supplementary Table S1), the optimal hydrolyzation conditions were as follows: 1 mL sodium hydroxide aqueous solution (1 M) was added into 1 mL the extracting solution of *L. robustum* and incubated at 80 °C for 2 h , and then 1 mL hydrochloride (1 M) solution was added (Figure 2).



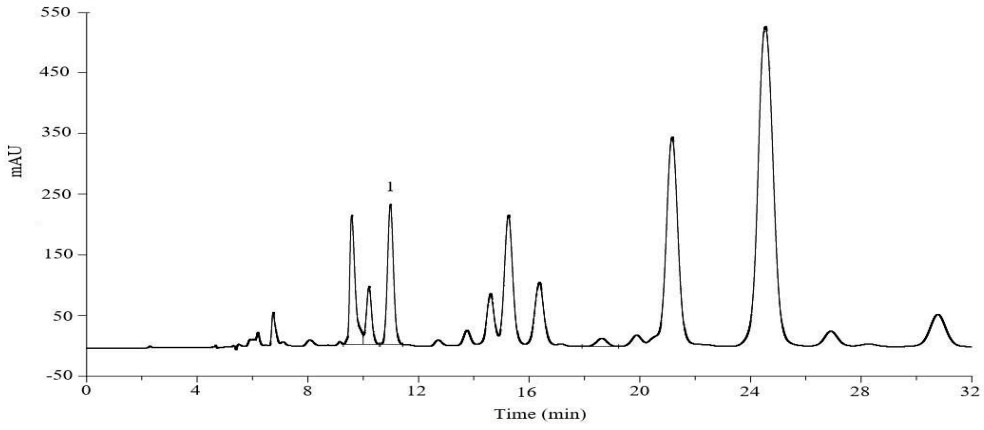
trans-p-hydroxycinnamic acid ester *trans-p*-hydroxycinnamic acid

Figure 2. Hydrolyzation of *trans-p*-hydroxycinnamic esters.

In order to quantify the free *trans-p*-hydroxycinnamic acid in the original extracting solution and the potential *trans-p*-hydroxycinnamic acid in the hydrolyzed extracting solution, several chromatographic items were considered. The first factor was the stationary phase, where C-18 column gave ideal effect if compared to C-8 column. The second factor was the mobile phase. Methanol, acetonitrile and ultrapure water in different volumes were tried, and methanol-water (40:60, *v*/*v*), without glacial acetic acid, showed acceptable resolution value but with tailed peak. Consequently, glacial acetic acid was added to improve the resolution and peak shape. Furthermore, the UV detector was successfully applied for the detection of the compositions, and the wavelength of 310 nm gave the maximum sensitivity at 30 °C (Supplementary Figure S1). Generally, the optimal HPLC performance (Figure 3) was observed when using C-18 column (4.6 mm × 250 mm, 5 μm), eluting with methanol-0.1% acetic acid aqueous solution (40:60, *v*/*v*) at a flow rate of 1.0 mL/min, and detecting at 310 nm.



(**A**)



(**B**)

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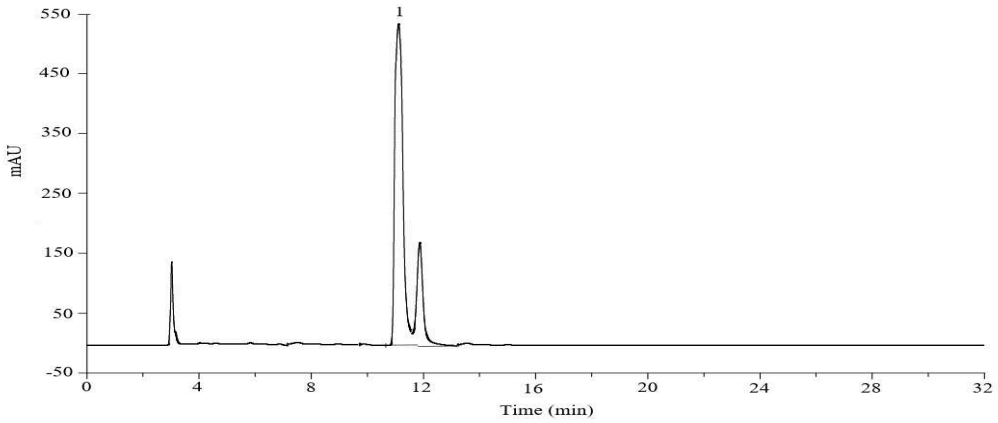

(C)

Figure 3. HPLC chromatograms of *trans-p*-hydroxycinnamic acid (**A**), the original extract of *L. robustum* (**B**), and the hydrolyzed extract of *L. robustum* (**C**).

The total concentration of *trans-p*-hydroxycinnamic esters (C_t, μmol/mL) in the extracting solution of *L. robustum* was calculated by follow equation:

$$C_t = C_p - C_f \quad (1)$$

C_p (μmol/mL): The concentration of potential *trans-p*-hydroxycinnamic acid (molecular weight: 164.16) in the hydrolyzed extracting solution of *L. robustum*. It was calculated as C_p (μg/mL) / 164.16.

C_f (μmol/mL): The concentration of free *trans-p*-hydroxycinnamic acid in the original extracting solution of *L. robustum*. It was calculated as C_f (μg/mL) / 164.16.

Additionally, osmanthuside B (molecular weight: 592.59) was considered as a representation of *trans-p*-hydroxycinnamic acid esters because of its moderate molecular weight and high content in the leaves of *L. robustum*. Consequently, C_t (μg/mL) might be calculated approximatively as C_t (μmol/mL) × 592.59.

2.2. Method Validation

2.2.1. Linearity and Calibration Curve

The calibration curve for *trans-p*-hydroxycinnamic acid was obtained by external standard method, using six concentrations of the standard, with three injections per concentration (Supplementary Table S2). The chromatogram peak areas were plotted against the corresponding concentrations of the standard solutions to establish the calibration curve (Supplementary Figure S2), and linear regression equation was calculated by the least squares method. This HPLC method showed linear regression at concentrations from 11.0 to 352.0 μg/mL, and the correlation coefficient (r^2) was 1.000 (Table 1), indicating excellent linearity.

Table 1. Results of regression equation, correlation coefficient, linear range, LOD and LOQ for *trans-p*-hydroxycinnamic acid by HPLC.

No.	Regression Equation	r^2	Linear Range (μg/mL)	Residual STD (σ)	Calibration Curve Slope (S)	LOD (μg/mL)	LOQ (μg/mL)
1	Y = 69.34X + 349.8	1.000	11.0-352.0	51.25	69.34	2.44	7.39
2	Y = 69.46X + 358.0	1.000	11.0-352.0	24.50	69.46	1.16	3.53
3	Y = 69.46X + 347.1	1.000	11.0-352.0	21.83	69.46	1.04	3.14
Integration (n=3)	Y = 69.42X + 351.6	1.000	11.0-352.0	32.53	69.42	1.55	4.69

Limit of detection (LOD) = 3.3 × σ/S; Limit of quantification (LOQ) = 10 × σ/S; σ, residual standard deviation; S, calibration curve slope.

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2.2.2. Limit of Detection and Limit of Quantification

The standard deviation of the Y-intercept in the regression equation was used as the residual standard deviation [21]. Consequently, the limit of detection (LOD) and limit of quantification (LOQ) were 1.55 and 4.69 μg/mL, respectively (Table 1).

2.2.3. Precision

In order to validate the precision of this HPLC-UV method, the *trans-p*-hydroxycinnamic acid standard was determined at three different concentrations, and the relative standard deviations (RSD) were calculated from the results of repeated measurements for each concentration. The measurement was performed three times in the same day to obtain the intra-day variability, and carried out three times in three different days to give the inter-day variability. The RSDs of intra-day and inter-day variabilities for *trans-p*-hydroxycinnamic acid were less than 2% (Table 2), indicating the precision of this method was accord with the criterion recommended by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines [21].

Table 2. Intra-day and inter-day variabilities for *trans-p*-hydroxycinnamic acid.

Concentration (μg/mL)	Intra-Day Variability (n = 3)		Inter-Day Variability (n = 3)	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
44.0	44.5 ± 0.4	0.9	44.3 ± 0.6	1.4
88.0	88.9 ± 0.5	0.6	88.4 ± 0.7	0.8
176.0	177.2 ± 0.5	0.3	176.8 ± 0.7	0.4

RSD, relative standard deviation.

2.2.4. Accuracy

To evaluate the accuracy of this analytical method, a recovery experiment was performed. The raw powder of the dried leaves of *L. robustum*, which was spiked with 100% or 50% of its native content of free *trans-p*-hydroxycinnamic acid, was extracted with 70% (*v*/*v*) ethanol under reflux. The concentration of free *trans-p*-hydroxycinnamic acid in the original extract was analyzed and calculated by the regression equation showed in Table 1, and the percentage recovery was calculated by repeated measurements of the analyte. As showed in Table 3, the percentage recovery of *trans-p*-hydroxycinnamic acid was 101.2% ± 0.8%, demonstrating an acceptable accuracy of this method since the acceptance criteria of the percentage recovery were 90%-107% [22].

Table 3. Results of accuracy validation for *trans-p*-hydroxycinnamic acid.

Added Weight (mg)	Observed Weight (mg) [a]	Recovery (%) [a]
4.0	4.04 ± 0.02	101.0 ± 0.5
2.0	2.03 ± 0.02	101.5 ± 1.0
Average (n = 6)		101.2 ± 0.8

[a] Data are expressed as mean ± standard deviation (n = 3).

2.2.5. System Suitability Parameters

The resolution of *trans-p*-hydroxycinnamic acid from other compositions was more than 1.5. The column efficiency for *trans-p*-hydroxycinnamic acid was more than 15000 N/m.

2.3. Quantification of Trans-p-hydroxycinnamic Acid and Its Esters in Various Extracts of L. robustum.

The dried leaves (10.00 g) of *L. robustum* were extracted with 90 mL ethanol aqueous solution (30%, 40%, 50%, 60% ,70%, 80%, *v*/*v*) under reflux for 60 min, and the contents of *trans-p*-hydroxycinnamic acid and its esters in the extracts were determined by the above developed and validated method. The results are presented in Table 4. The 60%-70% ethanol extracts of *L. robustum* showed the highest contents of free *trans-p*-hydroxycinnamic acid (3.96-3.99 mg/g), while the 30% ethanol extract of *L. robustum* showed the lowest content of free *trans-p*-hydroxycinnamic acid (2.26

mg/g) . In addition, the 50%-80% ethanol extracts of *L. robustum* displayed the highest contents of *trans-p*-hydroxycinnamic acid esters (202.6-210.6 mg/g), while the 30% ethanol extract of *L. robustum* displayed the lowest content of *trans-p*-hydroxycinnamic acid esters (125.8 mg/g). Taken together, 60%-70% ethanol was the optimal extraction solvent for *trans-p*-hydroxycinnamic acid and its esters.

Table 4. Quantification of *trans-p*-hydroxycinnamic acid and its esters in the extracts of *L. robustum* depending on extraction solvent [a].

Sample	Free *Trans-p*-hydroxycinnamic Acid (mg/g) [b]	Potential *Trans-p*-hydroxycinnamic Acid (mg/g) [b]	*Trans-p*-hydroxycinnamic Acid Esters (mg/g) [c]
30%EtOH	2.26 ± 0.02 a	37.1 ± 0.7 a	125.8 ± 2.6 a
40%EtOH	3.34 ± 0.03 b	54.8 ± 0.9 b	185.8 ± 3.3 b
50%EtOH	3.77 ± 0.02 c	59.9 ± 1.0 c	202.6 ± 3.6 c
60%EtOH	3.99 ± 0.03 e	62.2 ± 0.8 d	210.1 ± 2.9 c
70%EtOH	3.96 ± 0.03 e	62.3 ± 1.0 d	210.6 ± 3.6 c
80%EtOH	3.91 ± 0.02 d	61.5 ± 0.8 d	207.9 ± 2.9 c

[a] Data are expressed as mean ± standard deviation (n = 3). Means with the same letter are not significantly different (one-way analysis of variance, α = 0.05). [b] Content of *trans-p*-hydroxycinnamic acid = *trans-p*-hydroxycinnamic acid weight (mg) / *L. robustum* weight (g). [c] Content of *trans-p*-hydroxycinnamic acid esters = *trans-p*-hydroxycinnamic acid esters weight (mg) / *L. robustum* weight (g). .

3. Materials and Methods

3.1. Chemicals and Reagents

Methanol (HPLC) was afforded by Saimo Fisher Scientific Co., Ltd. (Shanghai, China). Ethanol (AR) was purchased from Chengdu Kelong Chemical Co., Ltd. (Chengdu, Sichuan, China). Glacial acetic acid, sodium hydroxide, and hydrochloride (AR) were acquired from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The standard of *trans-p*-hydroxycinnamic acid (>98%purity) was isolated and identified in our laboratory from the leaves of *L. robustum*, as previously described [14]. Ultrapure water was obtained from an ultra-pure water purifier system (Chengdu Yuechun Scientific Co., Ltd., Chengdu, Sichuan, China).

3.2. Plant Material

The dried leaves of *L. robustum* (Ku-Ding-Cha) were purchased from Junlian Qing-Shan-Lu-Shui Tea Co., Ltd. (Yibin, Sichuan, China). The material was crushed by hand before extraction.

3.3. Hydrolyzation of Extracting Solution and Preparation of Solutions

Standard solution of *trans-p*-hydroxycinnamic acid: the stock solution of *trans-p*-hydroxycinnamic acid was diluted with 40% (*v*/*v*) methanol to obtain 6 standard solutions at 11.0, 22.0, 44.0, 88.0, 176.0, 352.0 μg/mL, and filtered through a 0.45 μm PTFE syringe filter (Millipore, Billerica, MA, USA) before HPLC analysis.

Test solution of original extract: the original extracting solution of *L. robustum* was diluted with 40% (*v*/*v*) methanol and percolated using a 0.45 μm PTFE syringe filter prior to HPLC measurement.

Test solution of hydrolyzed extract: 1 mL sodium hydroxide aqueous solution (1 M) and 1 mL the extracting solution of *L. robustum* were mixed and incubated at 80 °C for 2 h, then 1 mL hydrochloride (1 M) solution was added. After cooled down to room temperature, the above mixture solution was transferred to 10 mL volumetric flask, and the volume was filled with 40% (*v*/*v*) methanol. The diluted solution was filtered with a 0.45 μm PTFE syringe filter prior to HPLC determination.

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3.4. HPLC Determination of Trans-p-hydroxycinnamic Acid and Its Esters

HPLC analysis was performed on a LC-20AT HPLC system (Shimadzu Corporation, Kyoto, Japan) with a SPD-20A UV-VIS detector and a binary pump. The standard solution or test solution (20 μL) was injected onto a Phenomenex Luna C18(2) 100A column (4.6 mm × 250 mm, 5 μm) thermostated at 30 °C. The components were eluted with methanol-0.1% acetic acid aqueous solution (40:60, *v*/*v*) at a flow rate of 1.0 mL/min in isocratic mode. The signals at 310 nm were monitored. The results were processed in LabSolutions Analysis Station (Shimadzu Corporation, Kyoto, Japan).

3.5. HPLC Validation

The analytical method was validated for linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision and accuracy according to the relevant ICH guidelines [21].

3.5.1. Linearity

The standard solutions of *trans-p*-hydroxycinnamic acid at different concentrations (11.0-352.0 μg/mL) were introduced to the HPLC system in triplicate. The calibration curve of *trans-p*-hydroxycinnamic acid was drawn by plotting the peak areas against the corresponding concentrations. The correlation coefficient (r^2) of the regression equation was obtained to validate the linearity.

3.5.2. Limit of Detection and Limit of Quantification

The LOD value was calculated as $3.3\sigma/S$, while the LOQ value was calculated as $10\sigma/S$, in which σ was the residual standard deviation of the regression equation, and S was the calibration curve slope.

3.5.3. Precision

In order to validate the precision of the analytical method, the standard solutions of *trans-p*-hydroxycinnamic acid at three different concentrations (44.0, 88.0, 176.0 μg/mL) were used to evaluate the intra-day and inter-day variabilities. The standard solutions were analyzed in triplicate in the same day to obtain the intra-day variability, while measured three times in three different days to give the inter-day variability.

3.5.4. Accuracy

In order to validate the accuracy of the analytical method, the recovery of *trans-p*-hydroxycinnamic acid was determined by the standard addition method. *Trans-p*-hydroxycinnamic acid standard (4.0 or 2.0 mg) was added into the dried leaves of *L. robustum* (1.000 g) which contained natively 4.1 mg/g free *trans-p*-hydroxycinnamic acid, and then extracted with 70% (*v*/*v*) ethanol (25 mL/g) under reflux for 1 h. The free *trans-p*-hydroxycinnamic acid in the original extract was analyzed by the HPLC method. And the percentage recovery of *trans-p*-hydroxycinnamic acid was obtained from the results.

3.6. Statistical analyses

Statistical analyses were carried out on GraphPad Prism 5.01. Every sample was determined in triplicate. The results are recorded as mean ± standard deviation (SD). Difference of means between groups was analysed by one-way analysis of variance (ANOVA) on statistical package SPSS 25.0. The difference between groups was believed to be significant when $P < 0.05$.

4. Conclusions

In the present study, a new optimized HPLC-UV method for the quantification of *trans-p*-hydroxycinnamic acid and its esters in the leaves of *L. robustum* was developed and validated in accordance with ICH guidelines. Because it was difficult and troublesome to analyze no less than 34

trans-p-hydroxycinnamic acid esters by usual HPLC, these esters were hydrolyzed with sodium hydroxide, and then the potential *trans-p*-hydroxycinnamic acid was determined by HPLC-UV. The above analytical method was simple and rapid, which simplified greatly the analytical process. Additionally, the methodology validation, including linearity, LOD, LOQ, precision, and accuracy, showed that the new HPLC-UV method was acceptable. To the best of our knowledge, it is the first HPLC method using hydrolyzation for quantification of many carboxylic esters. Finally, the novel method was used successfully to measure the contents of *trans-p*-hydroxycinnamic acid and its esters in various extracts of the leaves of *L. robustum*. The method can be applied also to the quality control of the products of *L. robustum*. Nevertheless, the shortcoming of the method is that the total content of the *trans-p*-hydroxycinnamic acid esters is clear but the specific content of every ester is not clear.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: The results of hydrolyzation of *trans-p*-hydroxycinnamic acid esters in the extract of *L. robustum*; Table S2: The concentrations and peak areas of *trans-p*-hydroxycinnamic acid standard; Figure S1: UV spectrum of *trans-p*-hydroxycinnamic acid; Figure S2: Calibration curve for *trans-p*-hydroxycinnamic acid.

Author Contributions: Conceptualization, S.-H.L.; methodology, S.-H.L. and X.-N.L.; formal analysis, S.-H.L. and X.-N.L.; investigation, X.-N.L., X.-J.N., R.C., X.-X.L., and S.-H.L.; data curation, S.-H.L.; writing—original draft preparation, S.-H.L. and X.-N.L.; writing review and editing, X.-X.L.; supervision, S.-H.L.; funding acquisition, S.-H.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in Supplementary materials.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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