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ORIGINAL ARTICLE

Comparison of Seven Chemical Components in *Callicarpa nudiflora* from Different Regions by Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry and its Analgesic Effect

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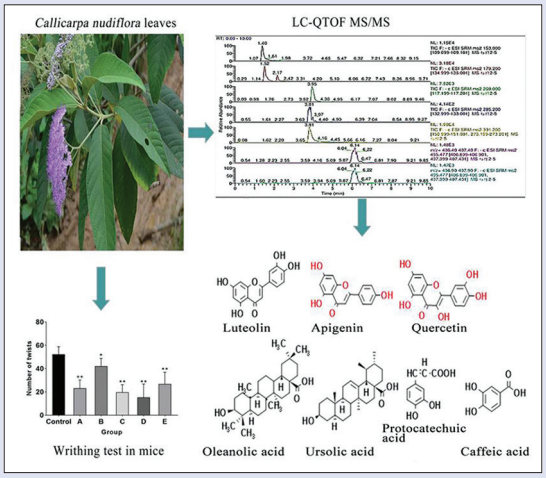
ABSTRACT

Background: *Callicarpa nudiflora* has been usually employed as a traditional Chinese medicine for acesodyne, dispersing edema, and hemostasis; however, its analgesic effects have been poorly considered. **Objectives:** This article focused on the differences in the contents of seven active chemical constituents in *C. nudiflora* grown in different regions. Moreover, we examined the analgesic effect of *C. nudiflora* from five arbitrarily designated regions and studied the relationship between the compound contents and analgesic effect. **Materials and Methods:** A simple and delicate qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry method was first established for the immediate determination of seven active components (luteolin, apigenin, quercetin, oleanolic acid, ursolic acid, protocatechuic acid, and caffeic acid) in *C. nudiflora*. Ethanol extracts were prepared from the mature leaves of *C. nudiflora* from five regions and the writhing test was performed by intragastric administration in mice. SPSS 13.0 and Graph Pad Prism 8.01 software were used to analyze the correlation of all data. **Results:** Calibration curves presented satisfactory linearity, with correlation coefficients >0.99 for all compounds within the concentration range. The compound contents were uppermost in plants from Hainan Province. The contents of the seven active chemical components inclined in the order of caffeic acid > luteolin > apigenin > oleanolic acid > ursolic acid > protocatechuic acid > quercetin. Pharmacological studies showed that *C. nudiflora* from all five regions had obvious analgesic effects. **Conclusion:** These results might be helpful for the screening and cultivation of *C. nudiflora* and its realistic clinical application.

Key words: Analgesic effect, *Callicarpa nudiflora*, determination, liquid chromatography quadrupole time of flight mass spectrometry, writhing test

SUMMARY

- The existing study reports the quantitative fortitude of luteolin, apigenin, quercetin, oleanolic acid, ursolic acid, protocatechuic acid, and caffeic acid present in the ethanol crude extract from *Callicarpa nudiflora* using liquid chromatography quadrupole time of flight mass spectrometry. The ethanol extract of the leaves of *C. nudiflora* was evaluated for their analgesic effect. There were significant differences in the contents of *C. nudiflora* and the analgesic effect may have an optimistic connection with the content of active ingredients.



Abbreviations used: *C. nudiflora: Callicarpa nudiflora*; LC-QTOF MS/MS: Liquid chromatography quadrupole time of flight mass spectrometry; TCM: Traditional Chinese medicine; HPLC: High-performance liquid chromatography; LOD: Limit of detection; LOQ: Limit of quantitative.

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INTRODUCTION

Callicarpa nudiflora, a member of the *Verbenaceae* family, is largely distributed in Hainan, Guangdong, and Guangxi provinces of the P. R. of China.[1] The dried mature leaves of this plant (*C. nudiflorae* Folium) are employed as a medicine, and this plant was designated as a new variety of traditional Chinese medicine (TCM) by the 2015 edition of the Chinese Pharmacopoeia.[2] Similar to Yunnan white medicinal powder, which is a renowned hemostatic medicine in Yunnan Province, P. R. China, *C. nudiflora* is often called Hainan black medicine because its leaves often

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turn black after drying. Modern pharmacological studies display that *C. nudiflora* and its preparation *C. nudiflora* tablets unveil a diversity of pharmacological effects, such as anti-inflammatory,[3] hemostatic,[4] burn- and scald-relieving effects.[5] Phytochemical research specifies that *C. nudiflora* encompasses many constituents, such as flavonoids,[6] terpenoids,[7] and volatile oils and so on.[8] However, as there are diverse growth environments and active constituents in different *C. nudiflora* cultivation regions, the quality of crude drugs is also different. It is difficult to comprehend the standardization of commercial crude drugs. Earlier studies have engrossed on the chemical constituents and quality control of *C. nudiflorae* Folium from different areas: High-performance liquid chromatography (HPLC) analysis,[9] determination of different components,[10] and establishment of HPLC fingerprints.[11] The total flavonoids and verbascoside contents in the same genus of *Callicarpa* L. are detected as the key indicators of quality control,[12] but the existing methods lack specificity and the chemical components of TCM studied are comparatively modest and cannot imitate the difference in chemical composition contents of *C. nudiflorae* Folium from different regions. None of the seven active chemical components stated in this article has been described yet. In addition, the drug has been confirmed to have good analgesic effects in the clinical applications; however, few studies have focused on the analgesic effect of *C. nudiflorae* Folium.

The components of plant metabolites in extracts can be expansively and precisely analyzed, by using the technology of plant metabonomics, particularly for the analysis of multi-component complex systems, which promotes research with a holistic view of TCM.[13,14] In the present study, the leaves of *C. nudiflora* were composed from different regions and a simple and delicate qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF MS/MS) method was first established for the immediate determination of luteolin, apigenin, quercetin, oleanolic acid, ursolic acid, protocatechuic acid, and caffeic acid in a *C. nudiflora* extract. In addition, this article examined the analgesic effect of *C. nudiflora* grown in five arbitrarily selected regions and studied the relationship between the compound contents and the analgesic effect. It was predictable that the results acquired would deliver some references to gauge the screening, cultivation, and the clinical application of *C. nudiflora*.

MATERIALS AND METHODS

Experimental equipment

An ultrasonic unit (B2500S-MT), a diode array detector with a binary high-voltage gradient pump (American Waters Co.), a vacuum pump (DOA-P730-BN), a vacuum dryer (Shanghai BoXun Medical Equipment Factory), a high throughput API 3200 LC/MS/MS system (American Waters Co.), and tandem triple quadrupole linear ion trap mass spectrometer (American Allen-Bradley Co.) were employed in this study.

Chemicals and reagents

Standards of the seven compounds were acquired and were of HPLC grade (≥98% purity, Nanjing Senbeijia Reagent Co.). The information is revealed in Table 1. The extraction solvents were 95% ethanol, methanol and anhydrous acetic acid (analytical purity).

Plant materials

The species employed in this study was recognized as *C. nudiflora* by Professor Tian Jianping, School of Pharmacy, Hainan Medical University, P. R. China specimen was dropped in the Herbarium of Hainan Medical University. In total, twenty samples of *C. nudiflorae* Folium used for content determination were collected in Hainan, Guangdong, and Guangxi provinces from 2018 to 2019. The particulars are shown in Table 2. The samples employed for the study of analgesic effect were Nos. 6, 9, 12, 13, and 16 in Table 2. All samples were desiccated at 50°C, creased into fine powder, sieved through 0.45 mm mesh, placed in a wide-mouth bottle, sealed, and stored in a cool place until succeeding analysis.

Liquid chromatography quadrupole time of flight mass spectrometry analytical conditions

The chromatographic conditions were as trails: A C_{18} column (100 mm × 2.1 mm, 3.5 μm) was used, methanol was used as mobile phase A and aqueous 5×10^{-3} mL/min formic acid was used as mobile phase B. The gradient elution programme was as follows: 0 min, 25% A; 7 min, 80% A and 7.01–9.0 min, 25% A. The flow rate was 0.4 mL/min, the column temperature was 40°C, the exposure wavelength was 280 nm, and the injection volume was 2.0 μL.

The mass spectrometric circumstances were as trails: The electrospray ionization source was worked in negative ion mode, the electrospray scanning voltage was set at −4500 V, the ion source temperature (TEM) was 550°C, and the ion source auxiliary pressure was 1369 kPa.

Preparation of standard solutions

The reference compounds caffeic acid, apigenin, protocatechuic acid, oleanolic acid, ursolic acid, quercetin, and luteolin at masses of 1.5, 1.5, 2.0, 2.0, 3.0, 3.5, and 4.0 mg were considered and liquefied in 70% methanol in a 5 mL volumetric flask to yield the stock solutions (0.3, 0.3, 0.4, 0.4, 0.6, 0.7, and 0.8 mg/mL, respectively). The stock solutions were diluted to suitable concentrations by adding 70% methanol for LC-QTOF MS/MS analyses. All solutions were deposited at 4°C and kept away from light.

Preparation of sample solution

C. nudiflora powder (1.0 g) from unlike cultivation regions (sample Nos. 1–20) was weighed put into a 50 mL volumetric flask, mixed with 70% methanol until dissolved and ultrasonically extracted (power: 250 W and frequency: 42 kHz) for 30 min. Due to the change in ultrasonic intensity at diverse positions of the ultrasonic instrument, the position of the conical bottle was staggered during the treatment process. The supernatant was composed and filtered with a 0.22 μm porous membrane filter. Finally, the supernatants (20 μL) were transferred into glass vials before injection into the LC-QTOF MS/MS for examination.

Preparation of *Callicarpa nudiflora* extract

The preparation of *C. nudiflora* extract was acquired according to the methodology planned by Shao *et al.*[4] with slight variations. Dried leaf powder of *C. nudiflora* (1 kg) was extracted three times (1 h/each) with 95% alcohol. The extract was combined and freeze vacuum drying for 24 h to attain the extract of *C. nudiflora*.

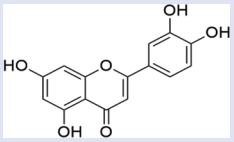
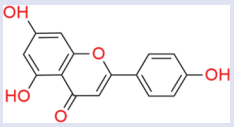
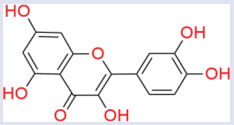
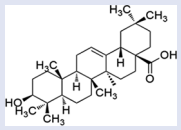
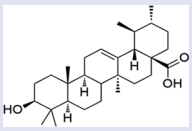
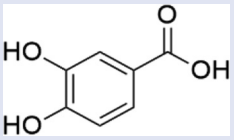
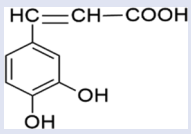
Method validation of determination

The method was authenticated according to the fourth part of the Pharmacopoeia of the People's Republic of China (2015 Edition).[15] The following parameters were dogged: Precision, recovery, linearity, limit of detection (LOD), and limit of quantitation (LOQ). Endorsement was conducted following the procedure of Han, Rui and Ying with slight amendments.[16-18]

Precision

According to the above chromatographic conditions, the mixed reference solution was precisely absorbed, the sample was inoculated incessantly for 6 times, the peak area of each component was documented, performed on different days, with different analysts,

Table 1: The seven compounds in *Callicarpa nudiflora* simultaneously analyzed by tandem liquid chromatography quadrupole time of flight mass spectrometry

Sample	Batch production	Molecular weight	Chemical formula	Chemical structure
Luteolin	160521	286.23	$C_{15}H_{10}O_6$	
Apigenin	161010	270.24	$C_{15}H_{10}O_5$	
Quercetin	CAS: 117-39-5	302	$C_{15}H_{10}O_7$	
Oleanolic acid	161118	456.71	$C_{30}H_{48}O_3$	
Ursolic acid	160813	456.71	$C_{30}H_{48}O_3$	
Protocatechic acid	161210	154.12	$(HO)_2C_6H_3COOH$	
Caffeic acid	160815	180.15	C_9H_8O4	

but with the same equipment. The results were articulated by relative standard deviation (RSD) value.

Recovery

C. nudiflora powder (1.0 g) from sample number 1 was evaluated put into a 10 mL volumetric flask, and the mixed reference solution with diverse mass concentration was added 1 mL, respectively. The recovery experiment of standard addition was performed with the same operation stages and experimental conditions as preparation of sample solution. The peak area was assessed and recorded, and the recovery rate was considered.

Linearity, limit of detection, and limit of quantitation

An appropriate amount of prepared standard solution was located into a conical flask, and methanol solution was added and mixed well. The mass concentrations of the standard solutions were 5 μg/L, 10 μg/L, 25 μg/L, 50 μg/L, 1100 μg/L, and 250 μg/L. After the peak areas of each reference substance were dogged, the regression equations were considered. The LOD was the compound concentration yielding a signal-to-noise (S/N) ratio of 3:1 and the LOQ was the concentration yielding an S/N ratio of 10:1. Lastly, the contents of chemical components in separate samples were calculated, and the relevant data were administered by SPSS 13.0 (Bontz Inc., Beijing, China).

Experimental animals

Kunming mice were delivered by Hunan Slyk Jingda Experimental Animal Limited Company, and the production licence number of experimental animals is SCXK (Xiang) 2019-0004. This study was directed in accordance with the Experimental Animal Administration regulations issue by the State Committee of Science and Technology of the People's Republic of China. All procedures defined here had prior approval from the Institutional Animal Care and Use Committee at the Hainan Medical University (Haikou, China).

Acetic acid-induced writhing in mice

Dry extracts of five samples were diluted with normal saline and ultrasonic suspensions were organized for the animal study. Sixty healthy male and female mice house at a room temperature of 18°C and weighing 18–22 g were arbitrarily alienated into one control group (normal saline group) and five experimental groups (ten mice in each group). In the experimental groups, the mice were intragastrically administered extracts of *C. nudiflora* (0.2 mL/10 g). After 30 min, 0.6% acetic acid (0.2 mL/10 g) was intraperitoneally injected to induce pain. The number of writhing mice and writhing times in 20 min were calculated. The inhibition rate of the writhing reaction was gained. The results were statistically examined by the SPSS software version 13.0.

Table 2: Comprehensive information for the twenty samples of *Callicarpa nudiflora* folium studied

No	Growing location
1	Wuzhishan, Hainan province
2	Shishan, Haikou, Hainan province
3	Changjiang, Hainan province
4	Baisha, Hainan province
5	Chengmai, Hainan province
6	Ding'an, Hainan province
7	Tunchang, Hainan province
8	Wupo, Tunchang, Hainan province
9	Tunchang, Hainan province
10	Danzhou, Hainan province
11	Yulin, Guangxi province
12	Yunfu, Guangdong province
13	Haikou, Hainan province
14	Xishui, baisha, Hainan province
15	Luoshuai, Baisha, Hainan province
16	Hongkan, Baisha, Hainan province
17	Yunmen, Baisha, Hainan province
18	Hongmao, Hainan province
19	Shiyun, Fanggeng, Hainan province
20	Shiyun, Shengtong, Hainan province

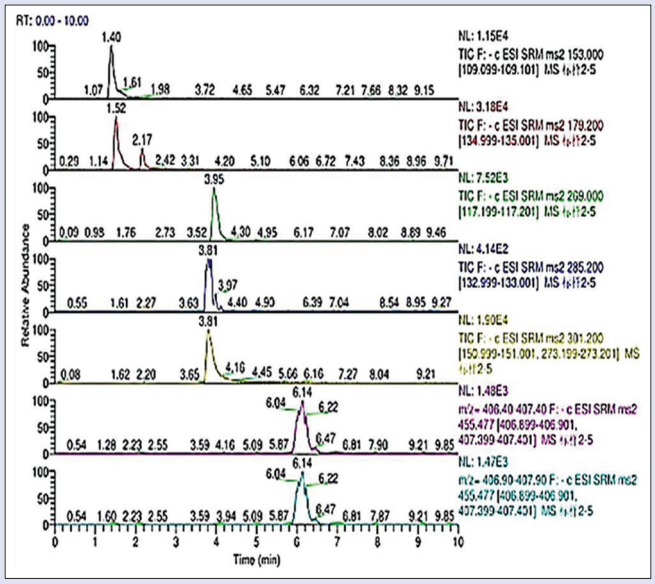


Figure 1: Selective ion chromatography of mixed reference substance of *Callicarpae nudiflorae* Folium. The mass spectrum information of reference substance is on the rightof the graph. From top to bottom are protocatechic acid, caffeic acid, apigenin, luteolin, quercetin, ursolic acid and oleanolic acid

RESULTS AND DISCUSSION

Optimization of liquid chromatography quadrupole time of flight mass spectrometry parameters

The conformation of the mobile phase is very vital in the selection of chromatographic conditions and controls the ionization efficiency of the samples to be assessed. The results display that the concentration of formic acid can affect the ionization efficiency, but it had no palpable effect on the separation of the components to be dogged. The impact on data of the specific concentration was as trails: When the concentration of formic acid was 2.0 mmol/L, the ionic strength of each index in the total ion flow diagram of the mixed standard sample was 1.0×10^5 and the signal was comparatively low. When the concentration of formic acid was 5.0 mmol/L, the ionic strength was 1.0×10^7, which revealed that the ionization efficiency of the mixed standards was knowingly developed. However, when the concentration of formic acid in the mobile phase was 10.0 mmol/L, the ionic strength was 3.4×10^7 and the signal augmented. Compared with the methanol system, the acetonitrile/water system had no obvious effect on the resolution and ionization efficiency of chromatographic peaks. Considering that the toxicity of methanol is less than that of acetonitrile, methanol was nominated for use as mobile phase A. Considering the economy and ionization efficiency, an aqueous solution containing 5.0 mmol/L formic acid was lastly designated as mobile phase B. According to the references and experimental testing, the ideal gradient conditions were designated as follows: 0 min, 25% A; 7 min, 80% A and 7.01–9.0 min, 25% A.

Qualitative analysis of *Callicarpa nudiflora*

The LC-QTOF MS/MS chromatograms of the seven reference standards and twenty samples are revealed in Figures 1 and 2. Under the detection conditions employed in this experiment, the retention times of the seven compounds were all in the range of 1.4–6.14 min, with good peak shape, no intrusions from impurities were detected and the baseline was steady. The method had high specificity, could precisely assess the content of seven chemical constituents in the mixed sample of *C. nudiflora* and had high sensitivity.

Ion pair chromatography can effectually separate difficult-to-separate mixtures, as shown in Figure 3, which is the extractive ion pair chromatogram attained in multiple reaction monitoring mode for numerous indicators to be assessed in the extract of *C. nudiflora*. According to the analysis of Figure 3, it could be realised that all seven targeted chemical components were well detached and the run time was shorter than those of other methods. Separation and detection could be finished in a short time of 3.5 min.

Method validation of *Callicarpa nudiflora*

Method validation studies disclosed that the linear regression equations, linear ranges, LODs and LOQs of oleanolic acid, ursolic acid, caffeic acid, luteolin, protocatechuic acid, quercetin and apigenin are presented in Table 3. The repeatability RSDs of all seven compounds in the sample solution did not surpass 2%. The low RSD values advise that the test procedure had good repeatability. The recovery rates (R%) of the seven compounds in the validation study demonstrated that the method was consistent when analyzing the seven compounds in *C. nudiflora*. The accuracy RSDs did not outstrip 5%. The quantitative limit was 0.2–3.0 μg/L for an S/N ratio of 10:1 and the detection limit was 0.05–1.0 μg/L for an S/N ration of 3:1. The grades of the validation studies verified that the method was steadfast when analyzing the seven target compounds in *C. nudiflora*.

Comparison of seven chemical constituents in *Callicarpa nudiflora* from different regions

The contents of the compounds in the samples are accessible in Table 4 and Figure 4. Comparing of the contents of the seven chemical elements in *C. nudiflora* grown in different regions, the contents inclined in the order of caffeic acid > luteolin > apigenin > oleanolic acid > ursolic acid > protocatechuic acid > quercetin. The consequences exposed that the luteolin and quercetin contents of *C. nudiflora* were the uppermost in plants from Hainan Province, followed by those from Guangdong and

Table 3: The calibration and validation data obtained for the quantification of the seven compounds

Compounds	Equation	Linear range (μg/L)	R^2	Limits		Accuracy	
				LOD (μg/L)	LOQ (μg/L)	RSD %	R %
Protocatechic acid	Y=423X+9300	5-5000	0.9965	0.05	0.2	1.13	99.3
Caffeic acid	Y=327X+12435	10-2500	0.9984	0.10	0.3	0.82	97.8
Ursolic acid	Y=334X+11270	5-10000	0.9901	0.20	0.5	1.47	99.7
Oleanolic acid	Y=489X+7658	5-2500	0.9932	0.50	1.5	1.21	98.5
Luteolin	Y=216X+8647	10-10000	0.9958	0.05	0.2	0.74	98.4
Apigenin	Y=552X+10326	5-5000	0.9976	0.40	1.2	1.04	98.2
Quercetin	Y=211X+7159	5-5000	0.9979	1.00	3.0	0.94	99.6

LOD: Limit of detection; LOQ: Limit of quantitation; RSD: Relative standard deviation

Table 4: Comparison of seven chemical constituents in *Callicarpae nudiflorae* Folium from different areas (mg/g)

Luteolin	Apigenin	Quercetin	Oleanolic acid	Ursolic acid	Protocatechc acid	Caffeic acid
0.1569	0.0848	0.0289	0.0279	0.0279	0.0123	0.4515
0.0846	0.0199	0.0153	0.0158	0.0158	0.0304	1.3842
0.1073	0.0124	0.0106	0.0166	0.0166	0.0288	0.7847
0.0983	0.0183	0.0079	0.0194	0.0194	0.0163	1.1173
0.1462	0.0159	0.0075	0.0215	0.0215	0.0332	0.9457
0.1595	0.0180	0.0123	0.0531	0.0531	0.0157	0.0250
0.1202	0.0109	0.0071	0.0233	0.0233	0.0209	0.5449
0.1380	0.0169	0.0058	0.0237	0.0237	0.0196	0.4166
0.1678	0.0142	0.0074	0.0155	0.0155	0.0215	0.4000
0.1333	0.0204	0.0076	0.0403	0.0403	0.0134	2.5077
0.1698	0.0146	0.0154	0.0074	0.0074	0.0240	0.3000
0.5475	0.0752	0.0082	0.0525	0.0525	0.0179	0.6520
0.0454	0.0207	0.0069	0.0161	0.0161	0.0277	1.7928
0.1867	0.0057	0.0043	0.0014	0.0014	0.0157	0.2419
0.1545	0.0900	0.0036	0.0500	0.0050	0.0460	0.2110
0.1762	0.0400	0.0021	0.0177	0.0177	-	-
0.1623	0.0450	0.0185	0.0175	0.0169	-	0.2600
0.1471	0.0860	0.0025	0.0123	0.0123	-	0.1400
0.2130	0.0760	0.0054	0.0110	0.0110	0.0310	0.0900
0.2870	1.0010	0.0068	-	-	0.0100	0.0600

-: The content of chemical components <0.0005 mg/g

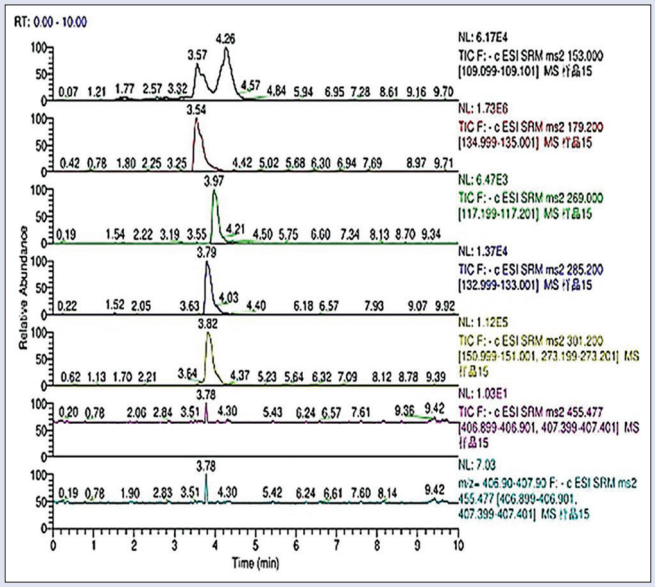


Figure 2: Selective ion chromatography of mixed samples of *Callicarpae nudiflorae* Folium. The mass spectrum information of reference substance is on the right of the graph. From top to bottom are protocatechic acid, caffeic acid, apigenin, luteolin, quercetin, ursolic acid and oleanolic acid

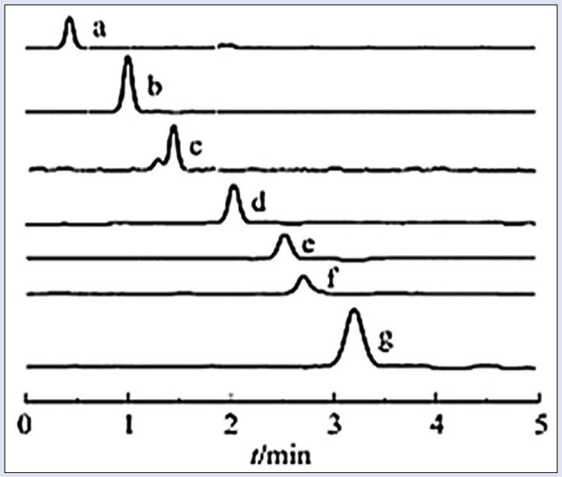


Figure 3: Extraction ion flow in multistage reaction mode of *Callicarpae nudiflorae* Folium

Guangxi. There were substantial differences in the contents of chemical components in plants grown in different regions. The quality of the crude drug also mottled with region. In clinical applications, the choice of the origin of crude drugs is very imperative. Compared with *C. nudiflora* plants grown in Hainan Province, those grown in Guangdong and Guangxi

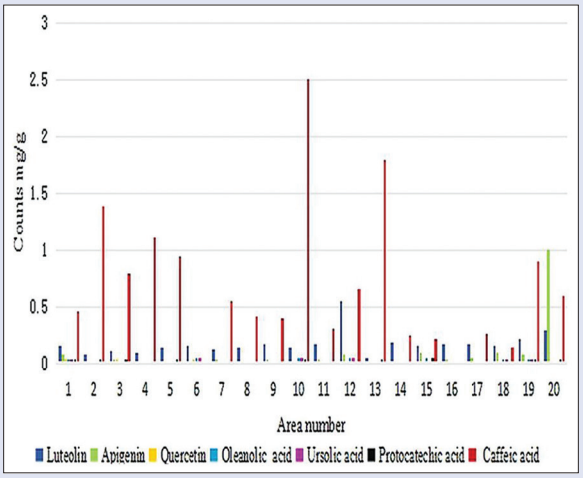


Figure 4: Comparison of content of seven chemical constituents in *Callicarpae nudiflorae* Folium from different areas

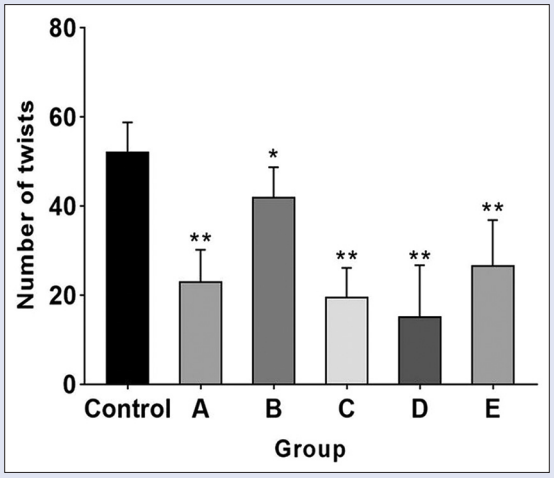


Figure 5: Difference analysis of writhing reaction in mice. Control was the blank control group. A-E are derived from the No 6, 9, 12, 13, 16 samples in Table 2, respectively. Data are expressed as mean ± standard deviation (at column width). *P < 0.05, **P < 0.01 versus the control group

provinces presented inferior quality, which also follows to the fact that the producing area of authentic crude drugs of *C. nudiflora was* Hainan Province.[19] In addition, the content of compounds in *C. nudiflora* grown in Shiyun village of Hainan Province was the finest, and the cultivation of high-quality varieties of *C. nudiflora* can be carried out in this area.

Writhing reaction in mice

Inhibition rate = (mean number of writhing times in control group-average number of writhing times in medication group)/average number of writhing times in control group × 100%. The effect of the extract of *C. nudiflorae* Folium on the writhing reaction of mice tempted by acetic acid is presented in Table 5 and Figure 5. The outcomes exhibited that there were noteworthy differences between the control and treated mice, with P values between $P < 0.05$ and $P < 0.01$ in one-way analysis of variance. The results also disclosed that the ethanolic extract of mature dried leaves of *C. nudiflora* had an obvious inhibitory effect on writhing encouraged by acetic acid in mice.

Earlier studies have exposed that flavonoids, such as rhododendron and Rutin, extracted from TCMs can ominously diminish the number of writhing reactions in mice.[20,21] Myrrha is mostly composed of volatile oil and terpenoids, which can suggestively inhibit pain induced by acetic acid.[22] It can be inferred that the analgesic effect of TCMs is primarily linked to their contents of flavonoids and terpenoids. To discover the analgesic effect of the active components of *C. nudiflora* from different areas, five samples in Table 2 were aimlessly selected to prepare *C. nudiflorae* Folium extracts. In the writhing test of mice, it was found that all *C. nudiflorae* Folium extracts had inhibitory effects on the writhing reaction of mice induced by acetic acid, but the degree of inhibition was diverse. The inhibition rates for sample No. 9 were the lowest at 19.35%. According to the results, it was found that the contents of seven components in sample No. 9 were also the lowest compared with those in the other four samples. The grades established that the active chemical composition of *C. nudiflorae* Folium from twenty areas was altered and that the content directly affected its pharmacological action. This study further established the analgesic effect of *C. nudiflorae* Folium in clinical application and its pharmacodynamic material basis may be correlated to the contents of flavonoids and terpenoids.

Table 5: Effect of extract of *Callicarpae nudiflorae* folium on writhing reaction in mice induced by acetic acid

Group	Dose (mL/10 g)	Number of animals	Twisting times	Inhibition rate (100%)
Control	-	10	52.20±6.58	-
A	0.2	10	23.10±7.09**	55.75
B	0.2	10	42.10±6.57*	19.35
C	0.2	10	19.60±6.48**	62.45
D	0.2	10	15.20±11.52**	70.88
E	0.2	10	26.70±10.12**	48.85

*P < 0.05 Correlation coeficient >0.9500; **P < 0.01 Correlation coeficient >0.9900

CONCLUSION

In conclusion, the outcomes of the methodology validation were decent and the quantitative method employed in this experiment can be productively applied to the routine quality control of the leaves of *C. nudiflora*. There were substantial differences in the content of *C. nudiflora* in different regions. Among the five regions, Haikou City of Hainan province had the uppermost content of chemical constituents and the greatest analgesic effect. This study laid a groundwork for quality screening and cultivation of *C. nudiflora*.

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Conflicts of interest

There are no conflicts of interests.

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