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One single HPLC-PDA/(-)ESI-MS/MS analysis to simultaneously determine 30 components of the aqueous extract of *Rabdosia rubescens*

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ABSTRACT

In China the leaves of *Rabdosia rubescens* have been cooked in water and widely drank to treat inflammatory and pain related diseases. To explore the components that were possibly absorbed by people the aqueous extract of the leaves was prepared, and one single HPLC-PDA/(-)ESI-MS/MS analysis was developed to simultaneously determine the components. Using the HPLC-PDA analysis 39 peaks were found in the aqueous extract, while using the (-)ESI-MS/MS analysis we were able to identify 30 peaks represented components, including 5 nucleic acids, 21 phenolic acids and 4 diterpenoids. On mouse models the *in vivo* anti-inflammation and analgesic actions demonstrate that 0.32 g/kg of the aqueous extract of the leaves of *Rabdosia rubescens* can effectively inhibit the inflammation-induced chronic pain.

1. Introduction

Rabdosia rubescens (RR, Donglingcao in Chinese), a perennial herb, is native to the valley of the Yellow River and the Yangtze River, as well as the Jiyuan Taihang Mount and Wangwu Mount in Henan province of China. In the past 40 years a lot of phytochemical researches of RR were focused on the liposoluble diterpenoids of the nonaqueous extracts with anti-tumor, anti-microbial, anti-inflammatory, anti-catastrophe, and anti-oxidation actions [1–10]. Besides, the aqueous extract of RR is believed to be pharmacologic importance [11], and the leaves of RR have been cooked in water and widely drank to treat inflammatory and pain related diseases, such as stomachache, pharyngitis, sore throat and cough [12,13]. However, to date no phytochemical research of RR was aimed at its aqueous extract. In this context the present paper investigated the chemical profiles of water-extracted components of RR leaves with HPLC-PDA/(-)ESI-MS/MS, evaluated its *in vivo* analgesic activity with tail-flick assay and its *in vivo* anti-inflammatory activity with xylene-induced mouse ear edema assay to provide evidence for the pharmacologic use of the aqueous extract.

2. Materials and methods

2.1. Chemicals, reagents and materials

Oridonin, rosmarinic acid, caffeic acid and chlorogenic acid were purchased from the China Institute for Control of Pharmaceutical and Biological Products. Adenosine diphosphate (ADP) was purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA). HPLC grade acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) was used for HPLC analysis. Redistilled water was prepared with Heal Force-PWVF Reagent Water System (Shanghai CanRex Analyse Instrument Corporation, China). Acetic acid (spectroscopically pure) was purchased from Beijing Chemical Works. The leaves of RR were purchased from Jiyuan.

2.2. Aqueous extract sample

The dry leaves of RR were ground to fine powder with a pulverizer and sifted through a 0.45 mm sieve to provide the powders of the leaves. To a 50 mL-capped conical flask containing 0.5 g of the leaf powder 10 mL of redistilled water was added. To simulate the general tea soaking procedure the suspension was stirred at 80 °C for 30 min, centrifuged (10 min, 4000 rpm) and filtered through a 0.45 μm microporous membrane to provide the sample for HPLC-PDA, HPLC–UV-Q-TOF-MS/MS analysis and bioassays.

2.3. HPLC-PDA analysis

A Waters 2695 HPLC system with a Waters 2996 Photodiode Array Detector was used. The sample was separated on a Kromasil C_{18} (Dikma) reversed-phase column (5 μm, 250 mm × 4.6 mm) protected by a guard column of the same material (5 μm, 10 mm × 4.6 mm). The column thermostat was maintained at 40 °C. Onto the column 50 μL of sample solution was injected for analysis. The mobile phase consisted of solvent A (acetonitrile), solvent B (0.05% acetic acid in water, v/v) and solvent C (0.5% acetic acid in water, v/v). The gradient elution program

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Table 1
HPLC gradient elution conditions.[a]

Time (min)	A (%)	B (%)	C (%)
0.0	5	95	0
10.0	8	92	0
35.0	12	88	0
40.0	12	0	88
150.0	15	0	85
250.0	20	0	80
300.0	25	0	75
380.0	100	0	0

[a] *A* = acetonitrile, *B* = aqueous acetic acid (0.05%, v/v) and *C* = aqueous acetic acid (0.5%, v/v).

was shown in Table 1, and the flow rate was 0.3 mL/min. After each run, the column was washed with acetonitrile and equilibrated to initial conditions for 15 min. UV absorption spectra were recorded online. The PDA detector was set to a scanning range of 200–400 nm.

2.4. HPLC–UV-Q-TOF-MS/MS analysis

A Waters 2795 HPLC system coupled to a Waters 2489 Dual absorbance detector and a Bruker micrO-TOF-Q instrument (Bruker Daltonics, Bremen, Germany) was used. The column, elution gradient, injection volume, time period and flow rate same as that used in HPLC-PDA analysis were used. Mass spectrometry operating conditions of negative ionisation mode were optimized in order to achieve maximum sensitivity values. The source parameters were nebuliser gas 0.8 bar, dry gas 8.0 L/min and dry temperature 200 °C. The scan range was from *m*/*z* 50 to 1500, spectra time in MS mode was 0.5 s. The collision energy for fragmentation ranged from 5 to 16 eV. Each run (TOF-MS-analyser) was calibrated internally with a sodium formate cluster calibrant. The accurate mass and composition for the precursor ions and for the fragment ions were calculated by using the Bruker Data Analysis version 3.4 software.

2.5. Preparative RP-HPLC

The preparative HPLC system used in this experiment was a Waters Delta Prep liquid chromatography equipped with a Waters Prep LC Controller System, a Waters 2487 Dual Absorbance Detector, and an injector (20 mL sample loop). The aqueous extract was separated on a Luster C_{18} (Dikma) reversed-phase column (10 μm, 250 mm × 21.2 mm). The wavelength was fixed at 254 nm. The elution was carried out by a linear A/B gradient (A represented 0.05% aqueous acetic acid, v/v, and B represented acetonitrile). The elution profile was 10–15% B (0–30 min), 15–20% B (30–100 min), 20–30% B (100–150 min), 30–30% B (150–200 min), 30–50% B (200–250 min), and 50–100% B (250–300 min). The flow rate was 2 mL/min. The fractions were collected, detected on MS and then combined.

2.6. Identification of the components

Some peaks in the sample were identified by comparing the retention time, the UV spectra and the MS spectra of the components with those of the peaks in the authentic reference samples (adenosine diphosphate, rosmarinic acid, caffeic acid, chlorogenic acid, and oridonin), some peaks in the sample were identified based on the data reported in the literature, and some peaks in the sample were identified according to the proposed MS fragmentation mechanisms.

2.7. Xylene-induced ear edema assay [14]

Male ICR mice weighing 20 ± 2 g, were housed in a 12/12 light/dark cycle at 21 ± 2 °C for 2 days before being used. Food and tap water were supplied *ad libitum*. The assessments described here were performed based on a protocol reviewed and approved by the ethics committee of Capital Medical University. The committee assures the welfare of the animals was maintained in accordance to the requirements of the animal welfare act and according to the guide for care and use of laboratory animals.

Mice (weighing 20 ± 2 g) were randomly divided into three groups (each 12 mice). The mice in vehicle control group were administrated orally normal saline (NS), the mice in positive control group were administrated orally a suspension of aspirin in NS at a dosage of 30 mg/kg and a concentration of 0.3 mg/mL, while the mice in the test group were administrated orally a suspension of the aqueous extract in NS at a dosage of 0.32 g/kg (corresponding to the dose per person daily) and a concentration of 35.5 mg/mL. Thirty minutes after administration 30 μL of xylene was applied to both the anterior and posterior surfaces of the right ear of the mouse. The left ear was considered as control. Two hours after xylene application, the mice were sacrificed and both ears were removed. Using a cork borer with a diameter of 7 mm, several circular sections were taken and weighed. The increase in weight caused by the irritant was measured through subtracting the weight of the untreated left ear section from that of the treated right ear section. The statistical analysis of the data was carried out by use of Student's *t* test, $p < 0.05$ is considered significant.

2.8. Pain threshold assay [14]

Male ICR mice weighing 20 ± 2 g, were housed in a 12/12 light/dark cycle at 21 ± 2 °C for 2 days before being used. Food and tap water were supplied *ad libitum*. The assessments described here were performed based on a protocol reviewed and approved by the ethics committee of Capital Medical University. The committee assures the welfare of the animals was maintained in accordance to the requirements of the animal welfare act and according to the guide for care and use of laboratory animals.

The mice were randomly divided into three groups of 12 mice, named the test group, vehicle control group, and positive control group. The mice in vehicle control group were administrated orally NS, the mice in positive control group were administrated orally a suspension of aspirin in NS at a dosage of 30 mg/kg and a concentration of 0.3 mg/mL, while the mice in the test group were administrated orally a suspension of the aqueous extract in NS at a dosage of 0.32 g/kg (corresponding to the dose per person daily) and a concentration of 35.5 mg/mL.

Thirty minutes after administration the pain thresholds were measured at 30 min intervals. This measurement was totally carried out for 180 min. The potency of analgesia was indicated by pain threshold variation. The values were calculated according to equation PTV = AAPT/BPT wherein PTV = pain threshold variation, BPT = basic pain threshold and AAPT = pain threshold after administration − basic pain threshold. All values of the pain threshold variation for each animal were averaged and constituted one sample.

3. Results and discussion

3.1. Peaks of HPLC-PDA and total ion current of (-)ESI-Q-TOF-MS

To optimize the conditions of HPLC-PDA analysis a series of mobile phases including acetonitrile/water, methanol/water and

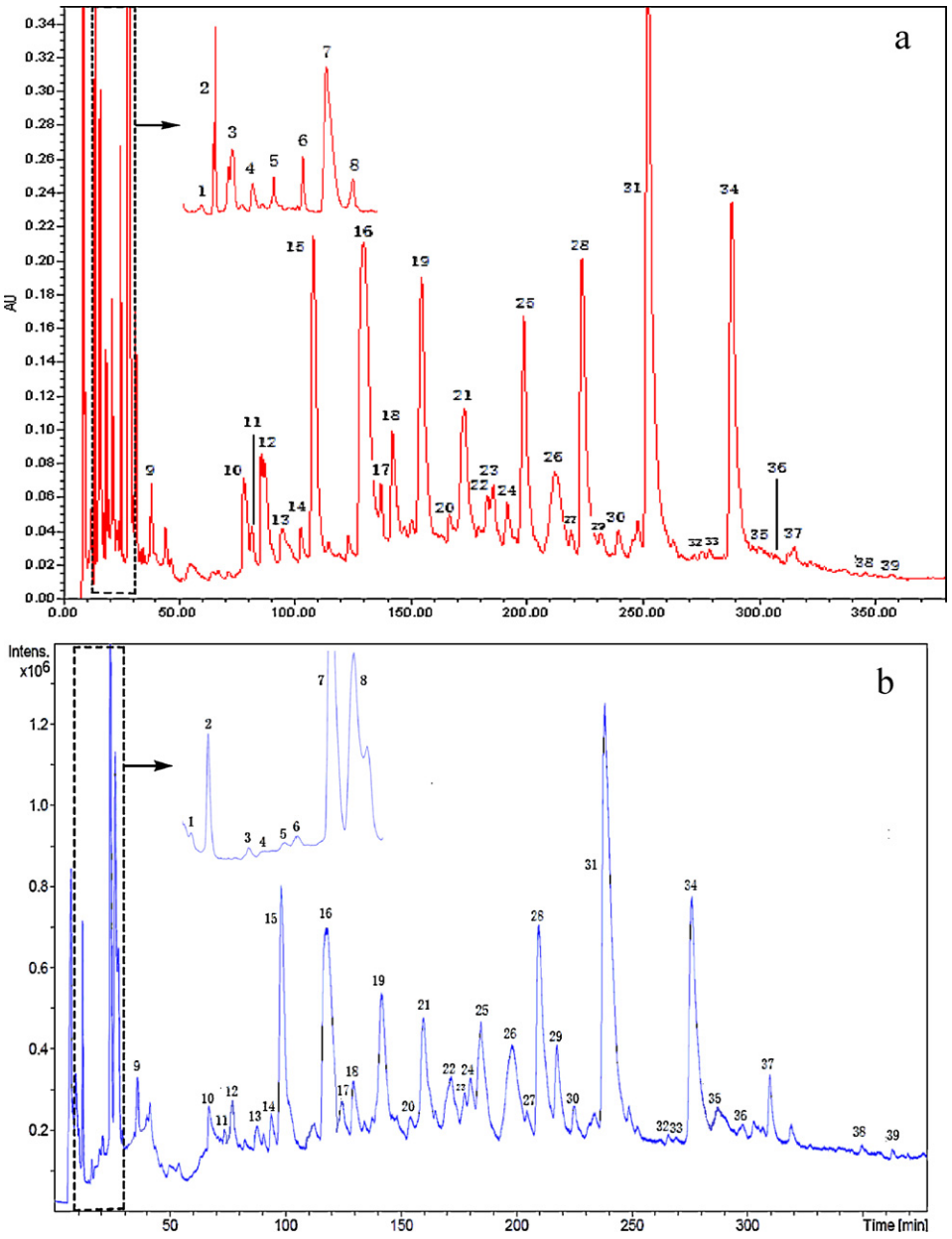


Fig. 1. Chromatogram of HPLC-PDA (a) and total ion current chromatogram of (-)ESI-Q-TOF-MS (b) for the aqueous extract of the leaves of RR.

methanol/water (0.05% acetic acid), restraining the peak tailing of phenols with acetic acid modified mobile phase were tested, a gradient elution performed at a flow rate of 0.3 mL/min, the components were monitored at 254 nm of wavelength and an elution time of 380 min was used. In this case the HPLC-PDA analysis gave completely separated 39 peaks (Fig. 1a).

To optimize the conditions of ESI-Q-TOF-MS analysis a negative ion modes was used to obtain the full-scan mass spectra. On negative ion mode the all peaks in the full-scan mass spectrum not only had higher sensitivity, but also could be well labeled in total ion current profile (Fig. 1b).

It was noticed that the retention time of the peaks in Fig. 1a from HPLC-PDA and Fig. 1b from HPLC–ESI-MS/MS did not completely match each other. This could be attributed to the fact that the HPLC-PDA and the HPLC–ESI-MS/MS were analyzed on Waters 2695 HPLC system and Waters 2795 HPLC system, respectively.

3.2. Assignment of 30 peaks

From the full-scan mass spectrum of (-)ESI-Q-TOF-MS $[M-H]^-$ and $[2\ M-H]^-$ values of 30 peaks were obtained. Based on the $[M-H]^-$, $[2\ M-H]^-$, daughter ions and λ_{max} of 30 peaks were obtained and are listed in Table 2.

It is well documented that for small molecules Q-TOF is able to give accurate mass (error < 5 mDa) [15]. With sodium formate cluster as reference ion and analyzing the data with Bruker Data Analysis version 3.4 software the molecular formulas of 30 peaks were defined. In respect of the calculated mass the errors of the found mass were less than 5 mDa. Based on the MS, MS/MS and UV spectra 30 peaks were assigned to 5 nucleic acids (peaks 1–5), 21 phenolic acids (peaks 7–10, 12, 14–28 and 31) and 4 diterpenoids (peaks 34, 36, 37 and 39). This means that in contrast to nonaqueous extract, in which the diterpenoids are the major components, the

Table 2
λ_{max}, t_R and (−)ESI-Q-TOF-MS of 30 peaks.

Peak no	t_R (min)	CE[a] (eV)	MI(f)/MI(c) (Da)	Daughter ion	λ_{max} (nm)
1	10.1	12	346.0524/346.0558	211	258
2	10.6	8	362.0459/362.0507	211	258
3	12.2	7	344.0376/344.0402	150	255
4	16.1	8	328.0428/328.0452	134	258
5	17.5	8	282.0831/282.0844	150	256
7	24.1	5	311.0367/311.0409	179, 149, 135	327, 218
8	26.2	10	197.0439/197.0455	179, 135	280
9	38.9	8	491.0799/491.0830	329, 293, 197, 179, 135	315
10	67.4	15	649.1392/649.1410	469, 353, 295, 163, 149	273
12	75.2	5	325.0538/325.0565	193, 131, 113	270
14	94.6	10	353.0845/353.0878	191, 179, 173, 161, 155, 135	327, 291
15	98.5	5	295.0426/295.0459	179, 133, 115	327
16	118.8	6	473.0707/473.0725	311, 293, 179, 149, 113	327
17	124.8	10	671.1232/671.1254	539, 359, 179, 149	274, 328
18	130.0	12	571.1111/571.1093	527, 483, 439,329, 285, 241, 197, 179, 135	276, 325
19	141.7	12	537.1030/537.1038	493, 357, 313, 295, 197, 179, 161, 135	276, 330
20	154.2	9	179.0344/179.0350	135	328
21	159.8	7	457.0787/457.0776	311, 295, 293, 277, 179, 163, 149	315, 291
22	172.3	15	539.1026/539.1195	521, 495, 477, 359, 341, 315, 297, 197	277
23	177.1	15	527.1003/527.1042	483, 439, 329, 303, 285, 241, 197	283, 330
24	180.5	6	487.0911/487.0882	325, 307, 293, 219, 193, 179, 135	277, 327
25	184.9	12	751.1341/751.1363	707, 663, 533, 509,465, 329, 311, 285, 267, 197	277, 320
26	198.2	10	735.1573/735.1567	717, 519, 539, 339, 197, 179	268, 336
27	204.9	16	719.1615/719.1618	539, 519, 475, 359, 197	277, 330
28	209.5	16	717.1493/717.1461	519, 475, 359, 339, 197	256, 283, 346
31	238.0	10	359.0778/359.0772	197, 179, 161, 135	289, 331
34	275.1	15	363.1773/363.1813	345, 327, 309, 297, 291, 281, 271	240
36	296.3	15	361.1630/361.1657	343, 325, 299, 271	280
37	309.2	10	407.2069/407.2075	347, 329, 311	236
39	362.8	8	347.1855/347.1864	329, 311, 299, 285	238

[a] CE = collision energy, MI(c) = calculated molecular ion and MI(f) = found molecular ion.

aqueous extract with phenolic acids and nucleic acids as its major components.

3.2.1. Assignment of 4 nucleic acids (peaks 1–5)

By comparing its mass and UV spectra with that of adenosine monophosphate (AMP) peak 1 (t_R = 10.1 min) was assigned to AMP. By utilizing their MS^1 and MS^2 spectra and the literature data peak 2 (t_R = 10.6 min), peak 3 (t_R = 12.2 min), peak 4 (t_R = 16.1 min) and peak 5 (t_R = 17.5 min) were assigned to guanosine monophosphate (GMP) [16], cyclic guanosine monophosphate (cGMP) [17], cyclic adenosine monophosphate (cAMP) [18] and guanosine [18], respectively. To explain the assignment peak 5 was used as an example here. Based on the MS^1 spectra, $[M-H]^-$/282 and $[2\ M-H]^-$/565, the molecular weight of peak 5 was calculated to 283. The N rule suggested the number of nitrogen atom of peak 5 was odd. In respect of the error (<5 mDa) of the experimental molecular weight four formulas, $C_5H_{12}N_7O_7$, $C_6H_8N_{11}O_3$, $C_9H_{16}N_1O_9$ and $C_{10}H_{12}N_5O_5$, could be the candidates of peak 5. Further more the fragment negative ions of 150 Da and 133 Da match $[M-H-ribose]^-$ and $[M-H-ribose-NH_3]^-$, respectively, and the reported λ_{max} of guanosine ($C_{10}H_{12}N_5O_5$) closes 256 nm. Thus peak 5 was assigned to guanosine (Fig. 2).

3.2.2. Assignment of 21 phenolic acids

The (-)ESI-Q-TOF-MS demonstrated that there were 21 phenolic acids in the water extract of RR including phenolic acid, phenolic acid consisted of two molecule residues, phenolic acid consisted of three molecule residues, phenolic acid consisted of four molecule residues and phenolic acid consisted of five molecule residues. The $[M-H]^-$ ions of phenolic acid usually lost CO_2 or CO or H_2O. The $[M-H]^-$ ions of the others predominantly lost danshensu or caffeic acid and formed $[M-H-198]^-$ and $[M-H-180]^-$, respectively.

3.2.2.1. Phenolic acids (peaks 8 and 20). The (-)ESI-Q-TOF-MS demonstrated that peak 8 (t_R = 26.2 min) gave molecular ions of $[M-H]^-$/197 and $[2\ M-H]^-$/395. Since the fragmentation of $[M-H]^-$/197 formed daughter ions $[M-H-H_2O]^-$/179 and $[M-H-H_2O-CO_2]^-$/135, peak 8 contained a hydroxyl and a carboxylic group. Thus peak 8 was assigned to 2-hydroxy-3-(3,4-dihydroxyphenyl)propanoic acid (danshensu, the hydrated form of caffeic acid) [19].

The (-)ESI-Q-TOF-MS demonstrated that peak 20 (t_R = 154.2 min) gave molecular ions of $[M-H]^-$/179 and $[2\ M-H]^-$/359. Since the fragmentation of $[M-H]^-$/179 formed daughter ions $[M-H-CO_2]^-$/135, peak 20 contained a carboxylic group. Besides, the t_R and mass of the authentic standard caffeic acid were the same as that of peak 20. Thus peak 20 was assigned to caffeic acid.

3.2.2.2. Phenolic acids (peaks 7, 12, 14, 15 and 31) consisted of two molecule residues. The (-)ESI-Q-TOF-MS demonstrated that peak 7 (t_R = 24.1 min) gave molecular ions of $[M-H]^-$/311 and $[2\ M-H]^-$/623. Since the fragmentation of $[M-H]^-$/311 formed daughter ions of *m*/*z*/149 and *m*/*z*/179, peak 7 contained a tartaric acid and a caffeic acid, which was further supported by the signals of decarboxylation of caffeic acid (*m*/*z*/135). Thus peak 7 was assigned to caffeoyltartaric acid. Furthermore, the λ_{max} (327, 241 and 290 nm) allowed us to assign peak 7 been consisted of one tartaric acid residue with one *trans*-caffeic acid residue (Fig. 3) rather than one tartaric acid residue with one *cis*-caffeic acid residue (λ_{max}: 321, 277 and 232 nm) [20].

The (-)ESI-Q-TOF-MS demonstrated that peak 12 (t_R = 75.2 min) gave molecular ions of $[M-H]^-$/325 and $[2\ M-H]^-$/651. Since the fragmentation of $[M-H]^-$/325 formed daughter ions of *m*/*z*/193 (base peak), *m*/*z*/131(tartaric acid losing water) and $[M-H-194-2H_2O]^-$/113, peak 12 consisted of one tartaric acid residue and one ferulic acid residue. Thus peak 12 was assigned to fertaric acid [21].

The (-)ESI-Q-TOF-MS demonstrated that peak 14 (t_R = 94.6 min) gave molecular ions of $[M-H]^-$/353 and $[2\ M-H]^-$/707. Since the

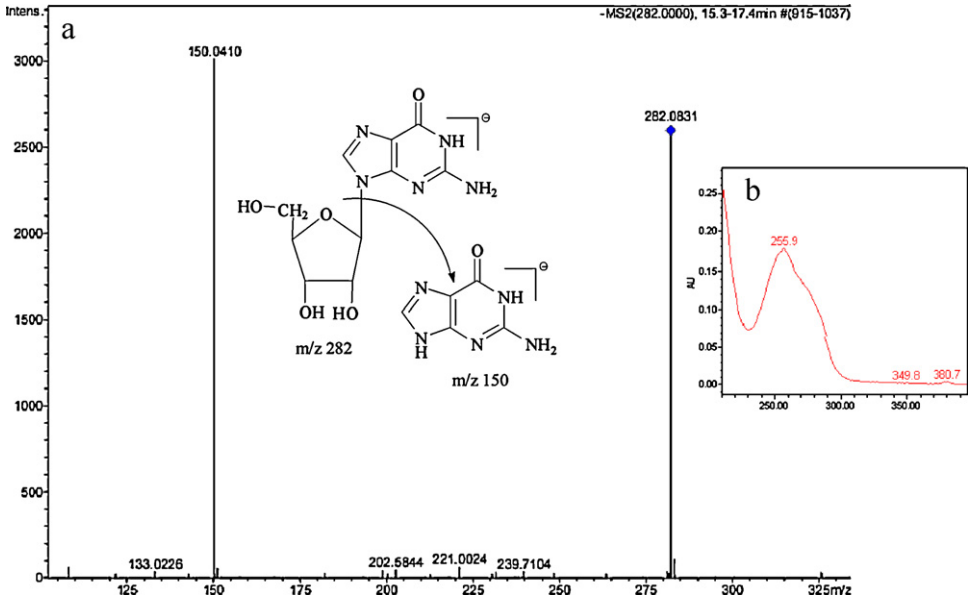


Fig. 2. MS^2 spectrum and proposed MS fragmentation pathway (a), and UV absorption spectrum (b) for peak 5.

fragmentation of $[M-H]^-$/353 formed daughter ions of *m*/*z*/191 and *m*/*z*/179, peak 14 consisted of one quinic acid residue and one caffeic acid residue. Furthermore, the t_R and mass of the authentic standard chlorogenic acid were the same as that of peak 14. Thus peak 14 was assigned to chlorogenic acid.

The (-)ESI-Q-TOF-MS demonstrated that peak 15 (t_R = 98.5 min) gave molecular ions of $[M-H]^-$/295 and [2 M−H]$^-$/591. Since the fragmentation of $[M-H]^-$/353 formed daughter ions of *m*/*z*/179 and *m*/*z*/133, peak 15 consisted of one caffeic acid residue and one malic acid residue. Thus peak 15 was assigned to caffeoylmalic acid [22].

The (-)ESI-Q-TOF-MS demonstrated that peak 31 (t_R = 238.0 min) gave molecular ions of $[M-H]^-$/359 and [2 M−H]$^-$/719. Since the fragmentation of $[M-H]^-$/359 formed daughter ions of *m*/*z*/197 and *m*/*z*/179, peak 31 consisted of one danshensu residue and one caffeic acid residue. The dehydration fragments were also observed at *m*/*z*/161, $[197-2H_2O]^-$ and $[179-H_2O]^-$, which evidenced the existence of danshensu residue and caffeic acid residue. Besides, the t_R and mass of the authentic standard rosmarinic acid were the same as that of peak 31. Thus peak 31 was assigned to rosmarinic acid.

3.2.2.3. Phenolic acid (peaks 9, 16, 18, 19, 21, 22 and 24) consisted of three molecule residues. The (-)ESI-Q-TOF-MS demonstrated that peak 9 (t_R = 38.9 min) gave molecular ions of $[M-H]^-$/491. Since the fragmentation of $[M-H]^-$/491 formed daughter ions of *m*/*z*/197, *m*/*z*/179 and *m*/*z*/149, peak 9 consisted of one danshensu residue, one caffeic acid residue and one tartaric acid residue. In addition, the fragments $[M-H-162]^-$/329, $[M-H-198]^-$/293, *m*/*z*/311 (caffeoyl-tartaric acid) and *m*/*z*/135 (decarboxylated caffeic acid) were also observed, which evidenced the existence of caffeic acid residue and danshensu residue. Thus peak 9 was assigned to caffeoyldihydroxyphenyllactoyltartaric acid [20].

The (-)ESI-Q-TOF-MS demonstrated that peak 16 (t_R = 118.8 min) gave molecular ions of $[M-H]^-$/473 and [2 M−H]$^-$/947. Since the fragmentation of $[M-H]^-$/473 formed daughter ions of *m*/*z*/179 and *m*/*z*/149, peak 16 consisted of two caffeic acid residues and one tartaric acid residue. In addition, the fragments $[M-H-162]^-$/311(caffeoyltartaric) and $[M-H-162-18]^-$/293 were also observed, which evidenced the existence of caffeic acid residue and tartaric acid residue (Fig. 4). Thus peak 16 was assigned to dicaffeoyltartaric acid (chicoric acid) [20].

The (-)ESI-Q-TOF-MS demonstrated that peak 18 (t_R = 130.0 min) gave molecular ions of $[M-H]^-$/571. Since the fragmentation of $[M-H]^-$/571 formed daughter ion of *m*/*z*/197, peak 18 contained one danshensu residue. In addition, the prominent ions $[M-H-44]^-$/527, $[M-H-(44\times2)]^-$/483, $[M-H-(44\times3)]^-$/439

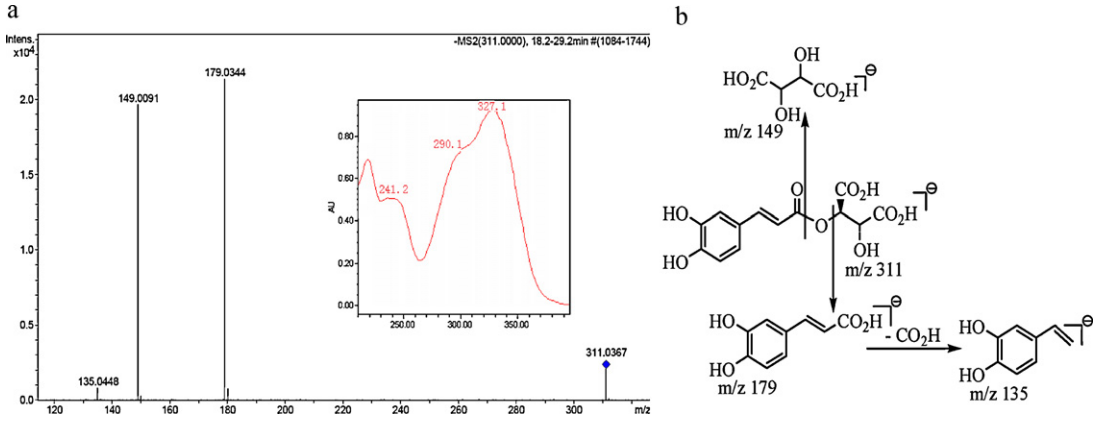


Fig. 3. MS^2 spectrum and UV absorption spectrum (a), and proposed MS fragmentation pathway (b) for peak 7.

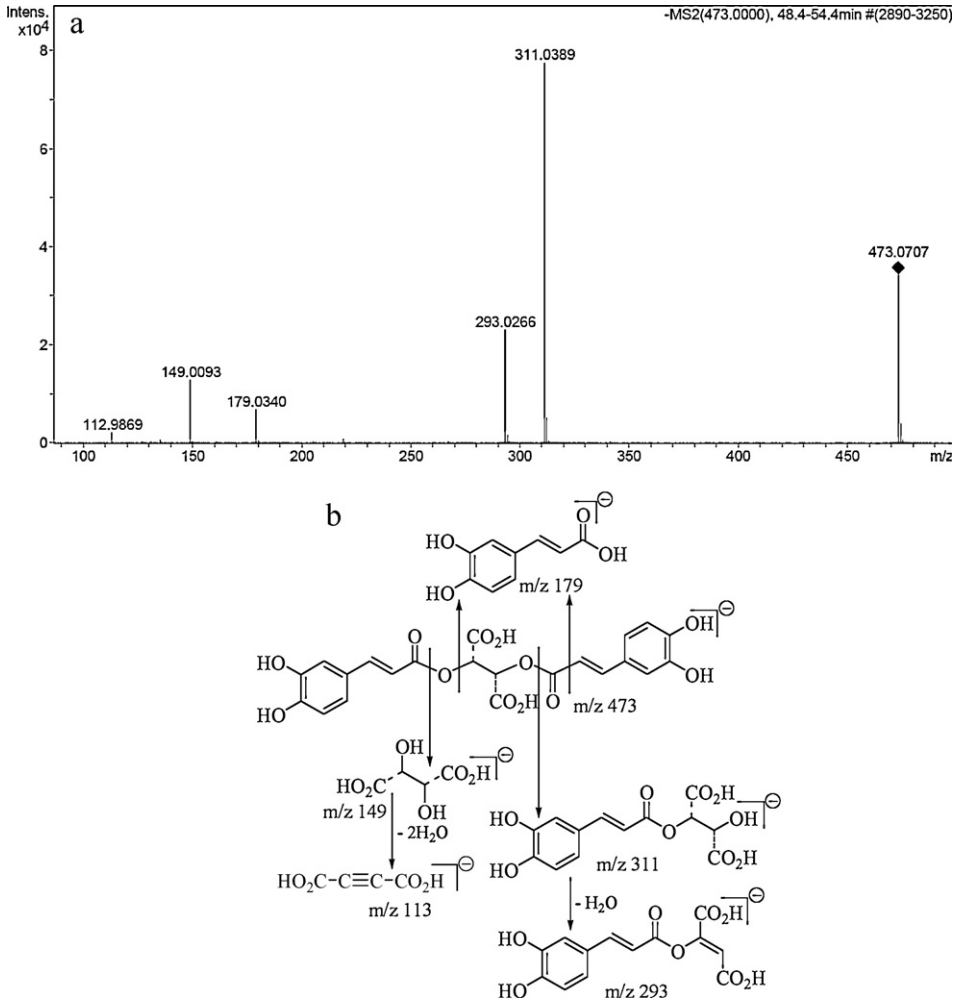


Fig. 4. MS^2 spectrum (a) and proposed MS fragmentation pathway (b) for peak 16.

showed that peak 18 at least contained three carboxylic groups. Besides, the ions [M−H-(44 × 3)–198]$^-$/241 were also observed, which evidenced the existence of danshensu acid residue and three carboxylic groups. Thus peak 18 was assigned to yunnaneic acid E [19].

The (-)ESI-Q-TOF-MS demonstrated that peak 19 (t_R = 141.7 min) gave molecular ions of [M−H]$^-$/537 and [2 M−H]$^-$/1075. Since the fragmentation of [M−H]$^-$/537 formed daughter ion of *m*/*z*/197, peak 19 contained one danshensu residue. Besides, the prominent ions [M−H-44–198]$^-$/295, [M−H-180]$^-$/357, [M−H-44]$^-$/493, [M−H-180–44]$^-$/313, [M−H-180–44 × 2]$^-$/269 and [M−H-180–44 × 2–18]$^-$/251 were also observed, which evidenced the existence of danshensu acid residue, carboxylic and hydroxy groups. Thus peak 19 was assigned to lithospermic acid [19].

The (-)ESI-Q-TOF-MS demonstrated that peak 21 (t_R = 159.8 min) gave molecular ions of [M−H]$^-$/457 and [2 M−H]$^-$/915. Since the fragmentation of [M−H]$^-$/457 formed daughter ions of *m*/*z*/149, *m*/*z*/163 and *m*/*z*/179, peak 21 consisted of one tartaric acid residue, one *p*-hydroxycinnamic acid residue, and one caffeic acid residue. Besides, the prominent ions [M−H-164]$^-$/293, [M−H-162]$^-$/295 and [M−H-180]$^-$/277 were also observed, which evidenced the existence of *p*-hydroxycinnamic acid residue and caffeic acid residue. Thus peak 21 was assigned to *p*-hydroxycinnamoyltartaric acid monocaffeoyl-ester [20].

The (-)ESI-Q-TOF-MS demonstrated that peak 22 (t_R = 172.3 min) gave molecular ions of [M−H]$^-$/539 and [2 M−H]$^-$/1079. It was found that the profile of MS^2 spectra of peak 22 was similar to that of peak 19, the difference between the molecular weights of peak 22 and peak 19 was 2 Da, and the difference between peak 22 yielded ions of *m*/*z* 495, 359, 315, 297, 271 as well as 253 and peak 19 yielded ions was also 2 Da. Thus peak 22 was tentatively assigned to 3-(3,4-dihydroxyphenyl)-2-{3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-2,3-dihydrobenzofuran-4-yl]-propionyloxy}-propionic acid (new compound 1, NC1). Due to SciFinder Scholar database gives no structure of this molecular weight, peak 22 may be a new compound. The (-)ESI-Q-TOF-MS spectra and the proposed MS fragmentation pathway are shown in Fig. 5a and b.

The (-)ESI-Q-TOF-MS demonstrated that peak 24 (t_R = 180.5 min) gave molecular ion of [M−H]$^-$/487. Since the fragmentation of [M−H]$^-$/487 formed daughter ions of *m*/*z*/193, *m*/*z*/179 and *m*/*z*/113 (149–2H_2O), peak 24 consisted of one ferulic acid residue, one caffeic acid residue and one tartaric acid residue. Besides, ions [M−H-162]$^-$/325, [M−H-180]$^-$/307, [M−H-176]$^-$/311 and [M−H-194]$^-$/293 were also observed, which evidenced the existence of caffeic acid residue and ferulic acid residue. Thus peak 24 was assigned to caffeoylferuloyl-tartaric acid [23].

3.2.2.4. Phenolic acids (peaks 10, 17, 23, 26, 27 and 28) consisted of four molecule residues. The (-)ESI-Q-TOF-MS demonstrated that peak 10 (t_R = 67.4 min) gave molecular ion of [M−H]$^-$/649. Since the fragmentation of [M−H]$^-$/649 formed daughter ions of *m*/*z*/353,

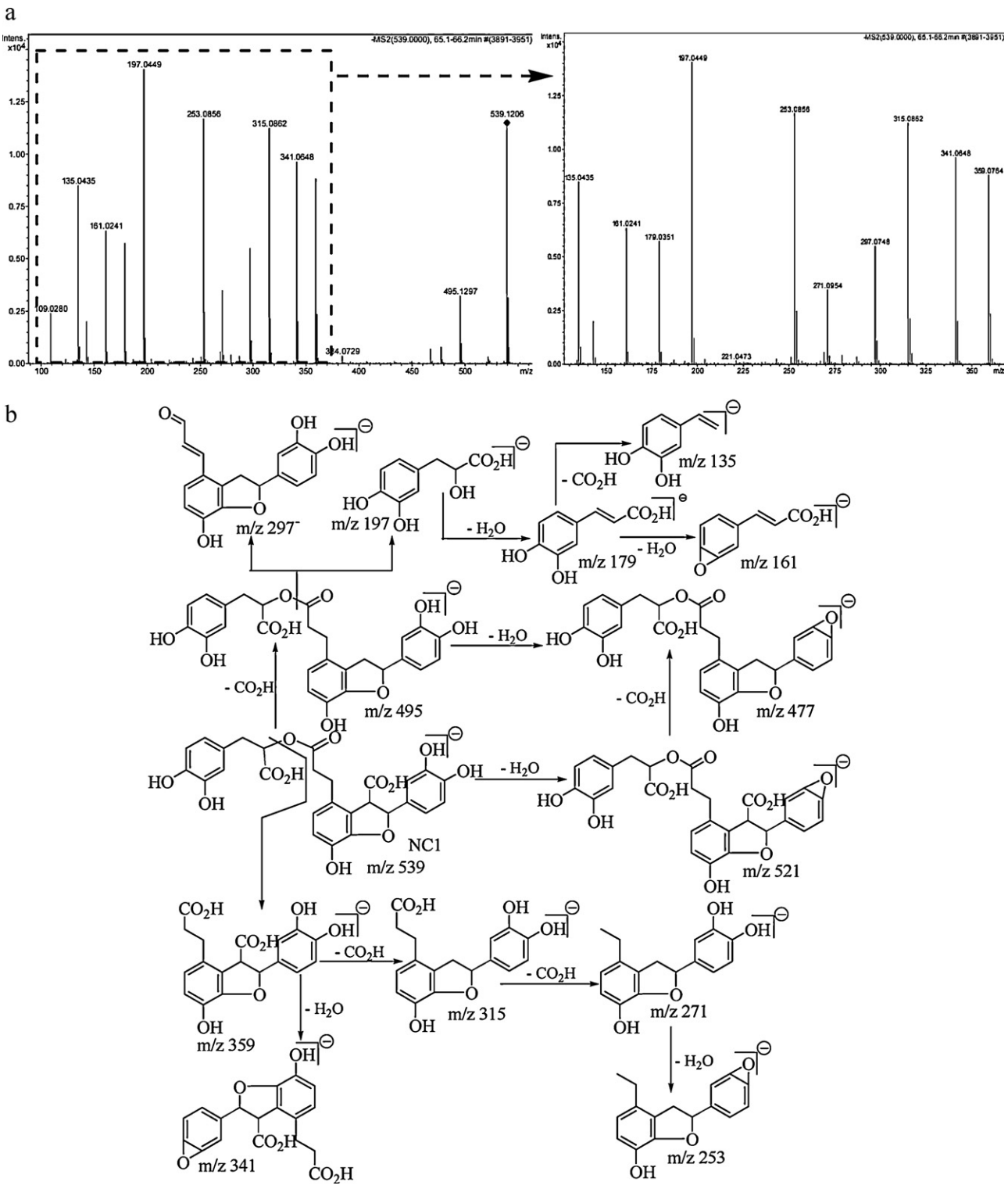


Fig. 5. MS^2 spectrum (a) and proposed MS fragmentation pathway (b) for peak 22 (NC1).

m/*z*/163 and *m*/*z*/149, peak 10 consisted of one chlorogenic acid residue, one *p*-hydroxycinnamic acid residue and one tartaric acid residue. The prominent ions *m*/*z*/295 and *m*/*z*/469 may represent the combination of one tartaric acid and one *p*-hydroxycinnamic acid, and the combination of one quininic acid, one tartaric acid and one *p*-hydroxycinnamic acid, respectively. Thus, the compound corresponding to peak 10 was tentatively assigned to 2-(3-caffeoylquinic acid)-3-(P-hydroxy cinnamic acid) tartaric acid (NC2 in Fig. 6). Due to SciFinder Scholar database gives no structure of this molecular weight, peak 10 may be a new compound.

The (-)ESI-Q-TOF-MS demonstrated that peak 17 (t_R = 124.8 min) gave molecular ion of $[M-H]^-$/671. Since the fragmentation of $[M-H]^-$/671 formed daughter ions of *m*/*z*/197, *m*/*z*/149 and *m*/*z*/359, this molecule contained one danshensu residue and one tartaric acid residue. The prominent ions *m*/*z*/539 and *m*/*z*/311 may represent the combination

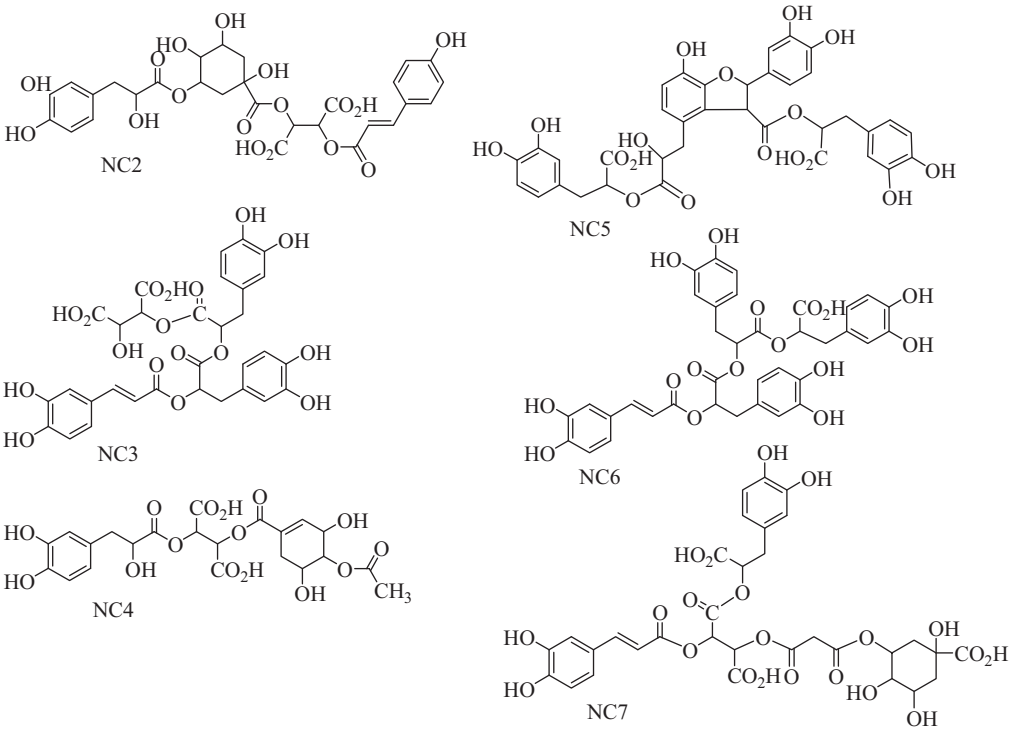


Fig. 6. Structures of new phenolic acids (NC2–NC7).

of one rosmarinic acid and one danshensu, and the combination of one tartaric acid and one danshensu, respectively. Thus, the compound corresponding to peak 17 was tentatively assigned to 2-(2-(2-(3-(3,4-dihy droxyphenyl)acryloyloxy)-3-(3,4-dihydroxyphenyl)propanoyloxy)-3-(3,4-dihydroxyphenyl)propanoyloxy)-3-hydroxysuccinic acid (NC3, Fig. 6). Due to SciFinder Scholar database gives no structure of this molecular weight, peak 17 may be a new compound.

The (-)ESI-Q-TOF-MS demonstrated that peak 23 (t_R = 177.1 min) gave molecular ions of $[M-H]^-$/527. Since the fragmentation of $[M-H]^-$/527 formed daughter ions of *m*/*z* 197, this molecule contained one danshensu residue. The prominent *m*/*z*/329, *m*/*z*/303 and *m*/*z*/215 may represent the combination of one tartaric acid and one danshensu, the combination of one tartaric acid and one shikimic acid, and the combination of one shikimic acid and one acetic acid, respectively. Thus, the compound corresponding to peak 23 was tentatively assigned to 2-[3-(3,4-dihydroxyphenyl)-2-hydroxypropionyloxy]-3-(4-acetoxy-3,5-dihydroxy cyclohex-1-enecarbonyloxy)-succinic acid (NC4 in Fig. 6). Due to SciFinder Scholar database gives no structure of this molecular weight, peak 23 may be a new compound.

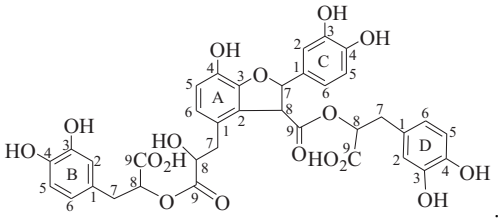
The (-)ESI-Q-TOF-MS demonstrated that peak 26 (t_R = 198.2 min) gave molecular ions of $[M-H]^-$/735 and [2 M−H]$^-$/1471. The difference between molecular weight of peak 26 and peak 28 was 18 Da, while their MS^2 spectra were similar and yielded a series of same fragments such as *m*/*z*/717, 537, 519, 493, 359, 339 and 197. These allowed us deduced peak 26 been the addition product of H_2O toward the double bond of salvianolic acid B (peak 28). Thus peak 26 was assigned to 4-{2-[1-carboxy-2-(3,4-dihydroxyphenyl)-ethoxycarbonyl]-2-hydroxyl-ethyl}-2-(3,4-dihydroxyphenyl)-7-hydroxy-2,3-dihydrobenzofuran-3-carboxylic acid-1-carboxy-2-(3,4-dihydroxyphenyl)-ethyl ester (NC5 in Fig. 6). The ^{1}H and ^{13}C NMR data of Table 3 also support the deduction. Due to SciFinder Scholar database gives no structure of this molecular weight, peak 26 may be a new compound.

The (-)ESI-Q-TOF-MS demonstrated that peak 27 (t_R = 204.9 min) gave molecular ions of $[M-H]^-$/719. Since the fragmentation of $[M-H]^-$/719 formed daughter ions of *m*/*z*/197 and *m*/*z*/359, peak 27 contained one danshensu residue and one rosmarinic acid residue. The [M−H-180]$^-$/539 may represent the combination of one rosmarinic acid residue and one danshensu residue. Thus peak 27 was assigned to 2-(2-(2-(3-(3,4-dihydroxyphenyl)acryloyloxy)-3-(3,4-dihydroxyphenyl)propanoyloxy)-3-(3,4-dihydroxyphenyl)propanoyloxy)-3-(3,4-dihydroxyphenyl)propanoic acid (NC6 in Fig. 6). The ^{1}H and ^{13}C NMR of Table 4 also support the deduction. Due to SciFinder Scholar database gives no structure of this molecular weight, peak 27 may be a new compound.

The (-)ESI-Q-TOF-MS demonstrated that peak 28 (t_R = 209.5 min) gave molecular ions of $[M-H]^-$/717 and [2 M−H]$^-$/1435. Since the fragmentation of $[M-H]^-$/717 formed daughter ion of *m*/*z* 197, peak 28 contained one danshensu residue. The prominent ions [M−H-198]$^-$/519, [M−H-180]$^-$/537 and [M−H-180–18–44]$^-$/475, which evidenced the existence of danshensu residue and carboxylic group. Furthermore ions [M−H-198–180]$^-$/339 and [M−H-198–198]$^-$/321 matched least two danshensu residues of peak 28. On the other hand, the spectrum of peak 28 was the same as that of salvianolic acid B [19]. Thus peak 28 was assigned to salvianolic acid B.

3.2.2.5. Phenolic acid (peak 25) consisted of five molecule residues. The (-)ESI-Q-TOF-MS demonstrated that peak 25 (t_R = 184.9 min) gave molecular ions of $[M-H]^-$/751. Since the fragmentation of $[M-H]^-$/751 formed daughter ions of *m*/*z*/197 and *m*/*z*/179, peak 25 contained one danshensu residue and one caffeic acid residue. The prominent ions [M−H-44]$^-$/707, [M−H-198]$^-$/553 and [M−H-198–44]$^-$/509 were also observed, which evidenced the existence of danshensu residue and carboxylic group. The ions *m*/*z*/311, *m*/*z*/241 and *m*/*z*/553 matched the combination of caffeic acid with tartaric acid, the combination of malonic acid with quininic acid and the combination of caffeic acid, tartaric acid, malonic acid and

Table 3
^{1}H NMR and ^{13}C NMR of NC5 (peak 26) in D_2O.



	Position	Fragment A	Fragment B	Fragment C	Fragment D
^{1}H NMR	2		6.45 (s, 1 H)	6.59 (s, 1 H)	6.51 (s, 1 H)
	5	6.41 (d, *J* = 8.0 Hz, 1 H)	6.56 (d, *J* = 8.0 Hz, 1 H)	6.76 (d, *J* = 8.0 Hz, 1 H)	6.60 (d, *J* = 8.0 Hz, 1 H)
	6	6.52 (d, *J* = 8.0 Hz, 1 H)	6.62 (d, *J* = 8.0 Hz, 1 H)	6.82 (d, *J* = 8.0 Hz, 1 H)	6.65 (d, *J* = 8.0 Hz, 1 H)
	7	3.03 (m, 2 H)	3.03 (m, 2 H)	6.02 (d, *J* = 4.1 Hz, 1 H)	3.03 (m, 2 H)
	8	4.51 (dd, *J* = 4.5 Hz, *J* = 8.9 Hz, 1 H,)	5.26 (dd, *J* = 4.5 Hz, *J* = 8.9 Hz, 1 H)	4.52 (d, *J* = 4.5 Hz, 1 H)	5.31 (dd, *J* = 4.5 Hz, *J* = 8.9 Hz, 1 H)
^{13}C NMR	1	130.21	133.22	134.28	133.36
	2	124.92	115.31	115.61	114.25
	3	148.27	148.29	147.97	146.98
	4	142.61	146.58	146.58	144.97
	5	116.23	118.23	117.32	116.69
	6	123.13	122.64	121.19	122.39
	7	35.26	37.49	86.28	37.85
	8	72.54	74.89	58.67	74.47
	9	171.37	173.26	174.37	173.10

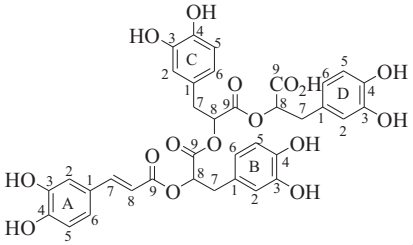
quininic acid, respectively. Thus peak 25 was tentatively assigned to 2-[3-(3,4-dihydroxyphenyl)-acryloyloxy]-3-[2-(5-carboxy-2,3,5-trihydroxycyclohexyloxycarbonyl)-acetoxy]-succinic acid-1-[1-carboxy-2-(3,4-dihydroxyphenyl)ethyl] ester (NC7 in Fig. 6). Due to SciFinder Scholar database gives no structure of this molecular weight, peak 25 may be a new compound.

3.2.3. Assignment of diterpenoids (peaks 34, 36, 37 and 39)

The (-)ESI-Q-TOF-MS demonstrated that peak 34 (t_R = 275.1 min) gave molecular ions of $[M-H]^-$/363 and $[2\ M-H]^-$/727. The fragmentation pattern of $[M-H]^-$/363 formed daughter ions of $[M-H-H_2O]^-$/345, $[M-H-CH_2O]^-$/333, $[M-H-2H_2O]^-$/327, $[M-H-CH_2O-H_2O]^-$/315, $[M-H-3H_2O]^-$/309, $[M-H-4H_2O]^-$/291, $[M-H-CH_2O-H_2O-CO_2]^-$/271 and $[M-H-CH_2O-2H_2O]^-$/297, which evidenced the existence of hydroxy group and $-CH_2O$. Besides, the t_R and mass spectrum of the authentic standard oridonin were the same as that of peak 34 (Fig. 7). Thus peak 34 was assigned to oridonin.

The (-)ESI-Q-TOF-MS demonstrated that peak 36 (t_R = 296.3 min) gave molecular ions of $[M-H]^-$/361 and $[2\ M-H]^-$/723. The fragmentation pattern of $[M-H]^-$/361 formed daughter ions of $[M-H-H_2O]^-$/343, $[M-H-2H_2O]^-$/325, $[M-H-H_2O-CO_2]^-$/299 and $[M-H-H_2O-CO_2-CO]^-$/271, which evidenced the existence of hydroxy, carboxylic and carbonyl groups. Besides, the mass and UV spectra (λ_{max} 280 nm) of peak 36 were the same as that of ponicidin [24]. Thus peak 36 was assigned to ponicidin.

Table 4
^{1}H NMR and ^{13}C NMR of NC6 (peak 27) in D_2O.



	Position	Fragment A	Fragment B	Fragment C	Fragment D
^{1}H NMR	2	6.65 (s, 1 H)	6.46 (s, 1 H)	6.49 (s, 1 H)	6.51 (s, 1 H)
	5	6.56 (d, *J* = 8.0 Hz, 1 H)	6.56 (d, *J* = 8.0 Hz, 1 H)	6.56 (d, *J* = 8.0 Hz, 1 H)	6.59 (d, *J* = 8.0 Hz, 1 H)
	6	6.72 (d, *J* = 8.0 Hz, 1 H)	6.52 (d, *J* = 8.0 Hz, 1 H)	6.51 (d, *J* = 8.0 Hz, 1 H)	6.55 (d, *J* = 8.0 Hz, 1 H)
	7	7.64 (d, *J* = 16.1 Hz, 1 H)	3.05 (m, 2 H)	3.04 (m, 2 H)	3.03 (m, 2 H)
	8	6.36 (d, *J* = 16.1 Hz, 1 H)	5.16 (dd, *J* = 4.5 Hz, *J* = 8.9 Hz, 1 H)	5.21 (dd, *J* = 4.5 Hz, *J* = 8.9 Hz, 1 H)	5.28 (dd, *J* = 4.5 Hz, *J* = 8.9 Hz, 1 H)
^{13}C NMR	1	130.21	132.22	133.28	133.36
	2	114.91	115.24	114.81	114.85
	3	147.71	146.97	146.97	146.98
	4	143.62	144.28	144.58	145.07
	5	116.23	118.23	116.32	116.69
	6	122.31	121.64	122.19	121.39
	7	144.26	37.39	38.28	37.85
	8	117.54	73.19	74.67	74.97
	9	167.37	171.26	171.37	173.20

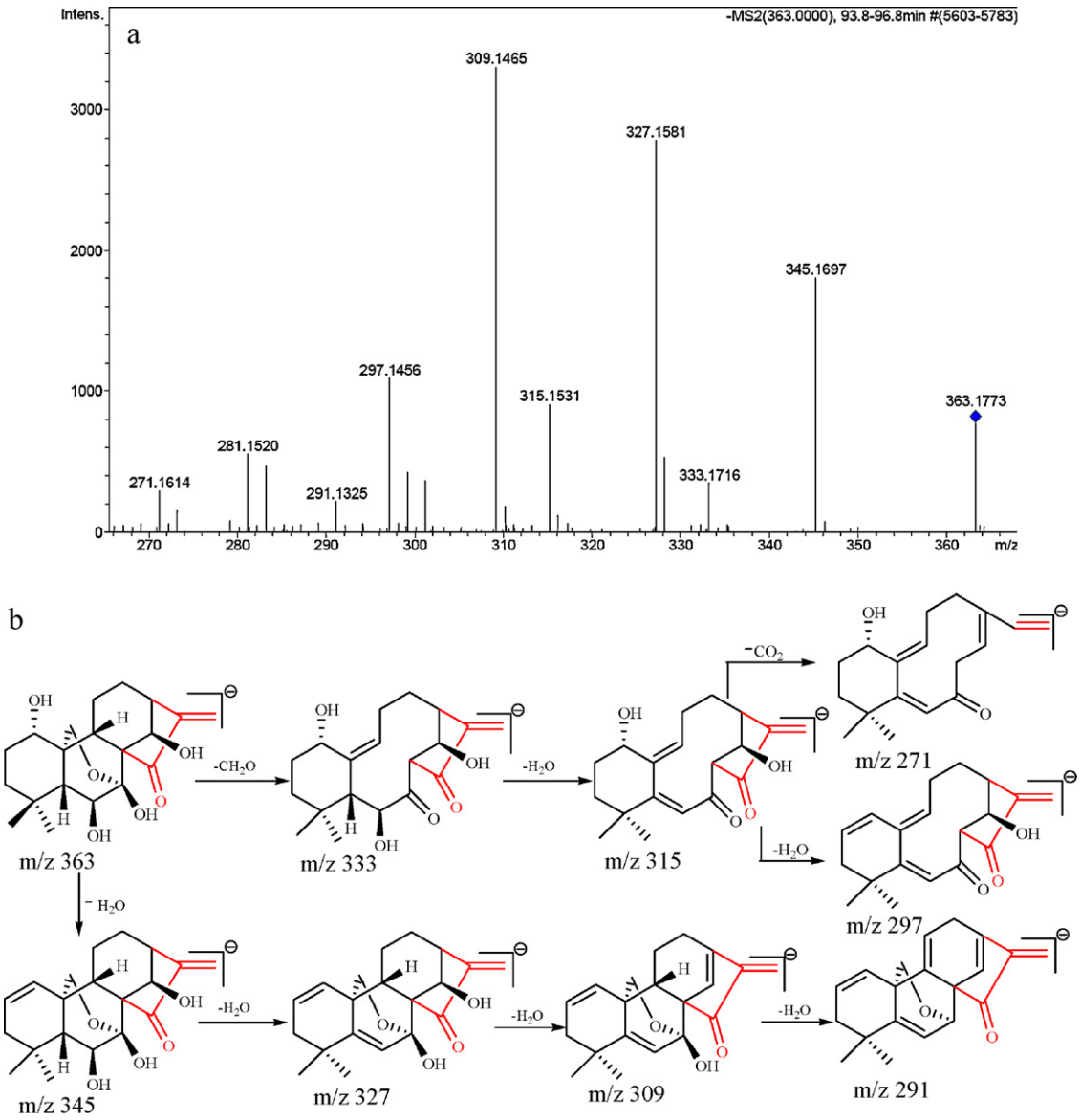


Fig. 7. MS^2 spectrum (a) and proposed MS fragmentation pathway (b) for peak 34.

The (-)ESI-Q-TOF-MS demonstrated that peak 37 (t_R = 309.2 min) gave molecular ions of $[M-H]^-$/407 and [2 M−H]$^-$/815. The fragmentation pattern of $[M-H]^-$/407 formed daughter ions of $[M-H-CH_3COOH]^-$/347, $[M-H-CH_3COOH-H_2O]^-$/329 and $[M-H-CH_3COOH-2H_2O]^-$/311, which evidenced the existence of acetyl and hydroxy groups. Besides, the mass spectrum of peak 37 was the same as that of lasiokaurinol [24]. Thus peak 37 was assigned to lasiokaurinol.

The (-)ESI-Q-TOF-MS demonstrated that peak 39 (t_R = 362.8 min) gave molecular ions of $[M-H]^-$/347. The fragmentation pattern of $[M-H]^-$/347 formed daughter ions of $[M-H-H_2O]^-$/329, $[M-H-2H_2O]^-$/311, $[M-H-H_2O-C]^-$/317, $[M-H-2H_2O-C]^-$/299 and $[M-H-2H_2O-C-CH_2]^-$/285, which evidenced the existence of hydroxyl and methylene groups. Besides, the mass spectrum of peak 39 was the same as that of effusanin [24]. Thus peak 39 was assigned to effusanin.

3.3. The NMR data of NC5 (peak 26) and NC6 (peak 27)

NC5 (120 min) and NC6 (145 min) were isolated with Preparative RP-HPLC. NC5 was obtained as colorless powders in 0.26% yield (7 mg). NC6 was obtained as colorless powders in 0.20% yield (5 mg). Their ^{1}H NMR and ^{13}C NMR spectra were recorded on a spectrometer (BRUKER 500, Ultra Shield^{TM}). The samples were dissolved in D_2O and analyzed at room temperature. The ^{1}H NMR and ^{13}C NMR data are shown in Tables 3 and 4, respectively.

3.4. In vivo orally anti-inflammatory activity of the aqueous extract

The anti-inflammatory activity of the aqueous extract was evaluated on a xylene induced mouse ear edema model. Briefly, the suspension of the aqueous extract (average yield of the aqueous extract: 0.27 g/1 g dry leaves ± 0.0053 g, n = 3), in normal saline was administered orally at a dose of 0.32 g/kg which was equal to the single dose of the leaves per person daily. The anti-inflammatory activities of NS (blank control), 30 mg/kg of aspirin (positive control) and 0.32 g/kg of the aqueous extract are shown in Fig. 8. As seen, on a xylene induced ear edema model the anti-inflammatory activity of aspirin was significantly higher than that of NS, while the anti-inflammatory activity of the aqueous extract at this dose was significantly higher than that of aspirin at the dose of 30 mg/kg.

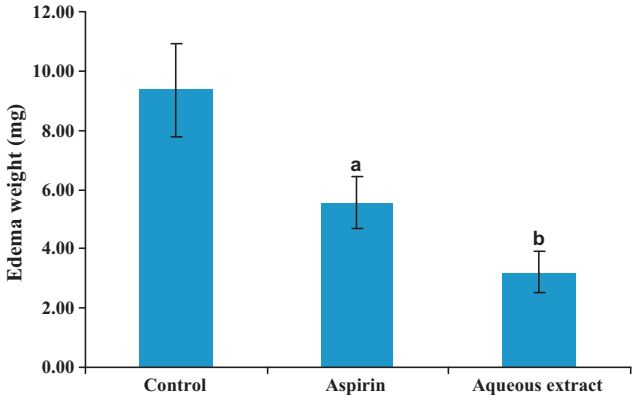


Fig. 8. Anti-inflammatory activities of the aqueous extract against xylene-induced ear edema in mice. Dose: aspirin = 30 mg/kg, aqueous extract = 0.32 g/kg, $n = 12$: (a) compared to control (NS), $p < 0.01$; (b) compared to control (NS) and aspirin, $p < 0.01$.

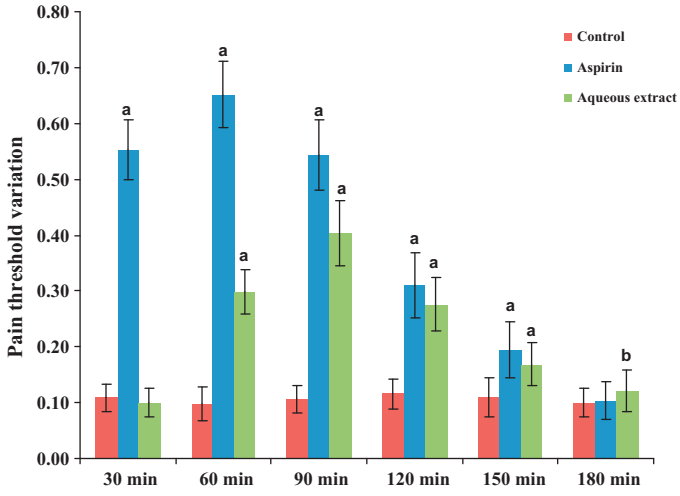


Fig. 9. The statistical analyses are carried out for the data of same time point, $n = 12$; oral dose of the aqueous extract = 0.32 g/kg; oral dose of aspirin = 30 mg/kg: (a) compared to control (NS) $p < 0.01$; (b) compared to control (NS) $p < 0.05$.

Therefore the aqueous extract of the leaves of RR is able to abate the inflammation for the peoples.

3.5. *In vivo orally analgesic activity of the aqueous extract*

On tail-flick mouse model the *in vivo* analgesic activity of NS, 30 mg/kg of aspirin and 0.32 g/kg of the aqueous extract were measured. The pain threshold variations stand for the activity and the data are shown in Fig. 9. As seen, 60 min after the oral administration the aqueous extract started to increase the pain threshold of the treated mice, 90 min after the oral administration the pain threshold reached its maximum and the analgesic action was still noticed 180 min after the oral administration. Therefore the aqueous extract of the leaves of RR is able to abate the pain for the peoples.

4. Conclusion

In conclusion, based on one single HPLC-PDA/(-)ESI-MS/MS analysis the structures of 5 nucleic acids, 21 phenolic acids and 4 diterpenoids of the 39 components of the aqueous extract of the leaves of RR could be simultaneously identified. At an oral dose of 0.32 g/kg the aqueous extract of the leaves of RR is able to effectively inhibit the inflammation and the pain of the treated mice. The inflammatory pain is accompanied by various painful responses of injury of peripheral tissue and/or inflammation produced by trauma, infection, surgery, burns, or diseases with an inflammatory component. Besides, chronic pain is generally resistant to the treatments of simple analgesics or traditional agents. Therefore chronic pain represents an unmet medical need and the oral administration of the aqueous extract of the leaves of RR could be a beneficial therapy.

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