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Screening and identification of various components in *Thalictrum fortunei* using a combination of liquid chromatography/time-of-flight tandem mass spectrometry

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An approach for screening and identification of various components in a traditional Chinese medicine (TCM), using a combination of LC/TOF-MS technique was described in this paper. The chemical profile of *Thalictrum fortunei*, well-known in TCM, was studied using the established method. The possibilities of screening and identifying non-target components inside TCM with modern data acquisition methods of acceleration time of flight mass spectrometers, such as data-dependent MS to MS/MS switching were investigated. As a result, 27 components were identified. This study was aimed to screen and identify the main components of *T. fortunei* using LC/TOF-MS, expecting to provide a rapid, sensitive, economical and systematical method for the identification and further quality evaluation of TCM preparation.

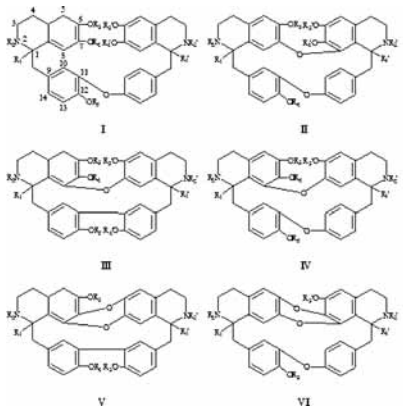
1. Introduction

In recent years, TCM has gained increasing popularity worldwide for their complementary therapeutic effects to the Western drugs but with a minimum of side effects (Zhang et al. 2006; Shi et al. 2007). The effects of TCM are, of course, brought about by its chemical constituents; thus, the chemical analysis of TCM is important because it helps to understand which ingredients are the real bioactive ones for certain therapeutic effects, and then to establish scientific and rational quality control methods. Each traditional Chinese herb comprises hundreds of different constituents, therefore, systematical and comprehensive analysis of TCM is a challenging task.

The classical chemical research methods for identification of constituents of TCM are time consuming and expensive. No matter the conventional approaches such as LC, NMR or the applications of hyphenated techniques such as GC-MS, CE-MS, *etc*, the applications of these methodologies are greatly limited by the time-consuming periods and the lack of appropriate standards. In addition, the effectiveness of a TCM could not be evaluated relying on only a few compounds. So, a combinative and powerful methodology which could offer higher quality structural information and comprehensive components inside is therefore required for the extensive characterization of TCM systems.

HPLC/ElectroSpray Ionization (ESI) MS had been shown to be a useful analytical tool for the identification of compounds in TCM prescriptions (Montoro et al. 2010; Fan et al. 2006; Boss et al. 1999). And, to solve the problem of uncertainties of unknown compounds existing in the identification and elucidation, a more powerful methodology, Time-of-Flight Mass Spectrometry (TOF-MS), has been developed for the precise and sensitive analysis (Zhou et al. 2009; Bobeldijka et al. 2001). Benefit from the increased resolving power, accurate mass measurement and high full-scan capability, TOF-MS can provide the elemental compositions of the compounds with low limited accuracy (routinely within 5 ppm). Currently, this strategy has been successfully developed and applied in the analysis of environmental contaminants including pharmaceuticals and pesticide degradates (Ferrer et al. 2005; Thurman et al. 2005a, b). However, to the best of our knowledge, only a few studies on complex TCM systems with this technique have been reported yet (Barnes et al. 2009; Zheng et al. 2008; Qi et al. 2009).

Thalictrum fortunei, known as *Huadong Tangsongcao* in China, is widely distributed over China and southeast Asia. Benzylisoquinoline alkaloids inside *T. fortunei* have shown various and significant bioactivities, such as anti-tumor (Hirpara et al. 2000; Chen et al. 2001; Mircheva 1984), anti-imflammatory (Koh et al. 2003; Kang et al. 2003; Hwang et al. 2005), anti-viral (Miranda et al. 2002; Kashiwada et al. 2005; Serkedjieva and Velcheva 2003), anti-arrhythmia (Zhang et al. 2002), body immunoloregulatory activities (Lai 2005) (Fig. 1). These bioactivities were related to benzylisoquinoline alkaloids which were unique in the genus *Thalictrum*. To our knowledge, there is only few literature about the major benzylisoquinoline alkaloids in *T. fortunei* up to now. The aim of this work was to screen and identify the main benzylisoquinoline alkaloids of *T. fortunei* using LC/TOF-MS. The work of simultaneous quantitative determination is now being carried out in our laboratory.



Compound	Type of benzylisoquinoline alkaloids
berbamunine	I
thaligrisine	I
aromoline	II
daphnandrine	II
funiferine	III
liliagcine	III
berbamine	IV
fangeluinoline	IV
taliacorinine	V
limacine	V
trilobine	VI
apateline	VI

Fig. 1: Main benzylisoquinoline alkaloids in *T. fortunei*

2. Investigations, results and discussion

2.1. HPLC separation

A representative HPLC chromatogram of *T. fortunei* is shown in Fig. 2a. The proposed method was therefore acceptable as well as adequate for further MS and MS^2 analysis.

2.2. Procedure for the identification of multi components

The base peak chromatograms (BPC) of *T. fortunei* obtained by LC/TOF-MS in positive ion mode are presented in Fig. 2b. The accurate mass spectrum of each peak in the HPLC or MS chromatogram and the empirical formulae corresponding to the probable existent compounds were obtained subsequently, and then the screening, identification and further confirmation of multi components were then performed by detailed studies of their MS and MS^2 spectral data combined with the published literatures.

Figures 3 and 4 show the MS and MS^2 spectra of peak 19, thalidasine. The MS spectra in the positive mode exhibited two abundant parent ions $[M+H]^+$ at *m/z* 653.3227 (Calcd. 653.3221) and $[M+Na]^+$ at *m/z* 675.3049 (calcd. 675.3041). According to these two molecular weights, the molecular formula could be deduced as $C_{39}H_{44}N_2O_7$, which was accordant with thalidasine. Figure 5 shows the hypothetic fragmentation pathway according to the data of thalidasine in MS^2. The MS^2 spectra of *m/z* 653 exhibited several fragment ions at *m/z* 637, 621, 515, 431, 411, 394, 379, 213 and 190. The fragment ion at *m/z* 637 could be attributed to the lost of CH_3, though it was still not clear for sure which CH_3 had been lost, according to the structure rigidity, 4-OCH_3 would be more likely cleaved for the formation of conjugated system of benzene ring and carbonyl group to stabilize the carbocation. Then the neutral lost of 16 could be attributed to the O group which could form a much more stable conjugated system, for the carbonium ion was on the benzene ring and could be stabilized by the ring's abundant electron atmosphere. This fragmentation pathway is very common when a benzene compound is crashed by argon in the collision cell of mass-spectrometry. Then, some other rearrangement and cleavage, such as α and β cleavage, i and rH_A rearrangement, could be deduced out among the total fragment pathway according to the MS^2 data and some references. These fragmentation was common for the benzylisoquinoline alkaloids in *T. fortunei*. According to these rules, some other benzylisoquinoline alkaloids were also deduced.

2.3. Results

In this work, a reliable and powerful analytical method by using LC/TOF-MS for rapid screening and identification of multi components in *T. fortunei* was established. As a result, 27 components were identified. According to the literature, most of the identified compounds possess pharmacological activities. So the 27 components identified represent the main pharma-

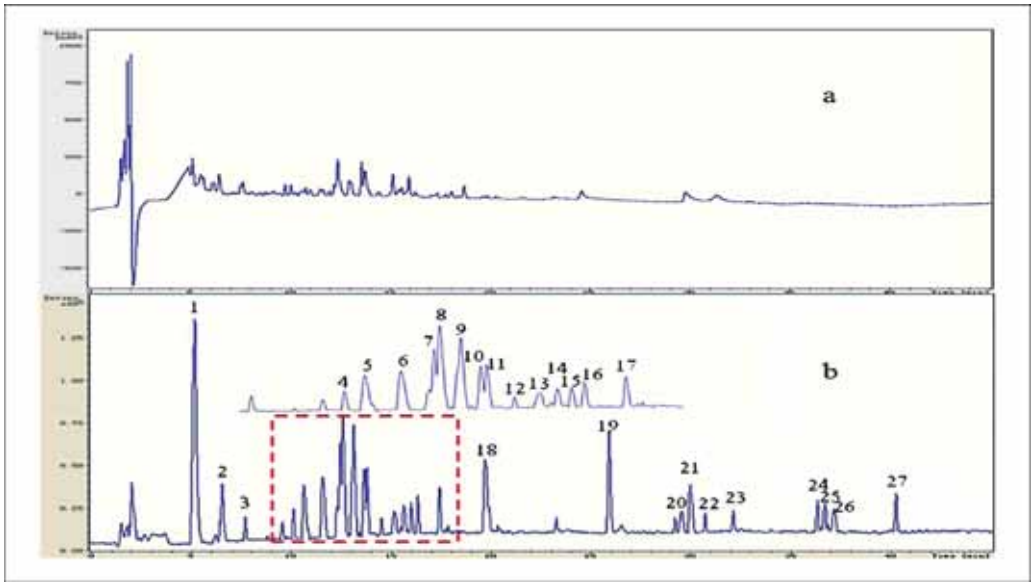


Fig. 2: Chromatograms of multi compounds in *T. fortunei* a UV chromatogram at 278 nm; b BPC chromatogram in positive mode

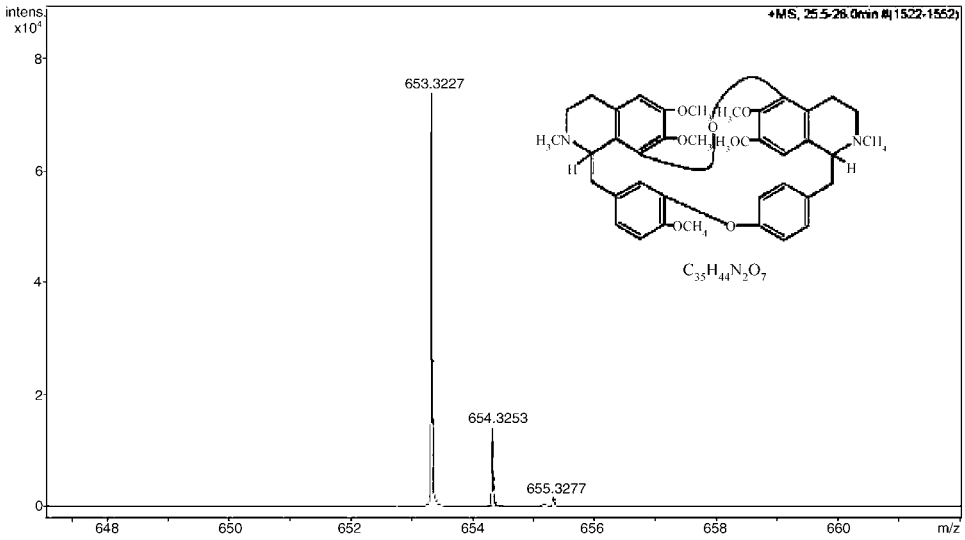


Fig. 3: TOF-MS data of thalidasine

cological substances and the chemical profile of *T. fortunei*. On the whole, the LC/TOF-MS has a powerful capability for screening and identification of multi components. This method could identify multi components in TCM without long-time-consuming isolation and purification period, just relying on the abundant MS and MS/MS data acquired by LC/TOF-MS, the comprehensive investigation of the previous literatures and a much smaller amount of relative standards. It would provide a rapid, sensitive, economical and systematical method for the improvement of quality control in TCM.

3. Experimental

3.1. Chemicals and reagents

HPLC grade methanol was purchased from Caledon Laboratories LTD. (Georgetown Ont., Canada). Ultrapure water was self-made in our laboratory. Formic acid was of an analytical grade (Yuwang, Shandong, China). *T. fortunei* was purchased from Sichuan, China and identified by Pharmacognosist Zengxi Guo, and also been kept under controlled conditions for future identification.

3.2. Sample preparation

T. fortunei (5 g) was pulverized, passed through a 0.45 mm sieve, then 10 ml methanol with 0.5% formic acid was added into the powder for 30-min ultrasonic batch at room temperature. The extract was then evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved to a 5 ml volumetric flask with methanol. The solution was ready for chromatographic analysis after passing through a 0.45 μm membrane filter.

3.3. HPLC conditions

An Agilent 1200 series LC system was employed in this research, which consisted of a G1376A Cap Pump, a G1379B Degasser, a G1365B Multi-Wave Detector, a G1376B Autosampler and a Hystar PP work station.

The analysis of the alkaloids was carried out on a Agilent Extend C18 (250 × 4.6 mm, 5 μm, Agilent, USA), which was protected by a RP18 guard column.

The solvents used for HPLC separation were buffer solution (A, containing 0.3% formic acid) and acetonitrile (B) at a flow rate of 1.0 ml min^{-1}. The mobile phase was as the following: the proportion of B was increased from 15 to 55% in the first 25 min, then increased to 70% in 10 min and to 90% in 5 min, which was then maintained for 5 min. The column temperature was 35 °C and the sample injection volume was 10 μL.

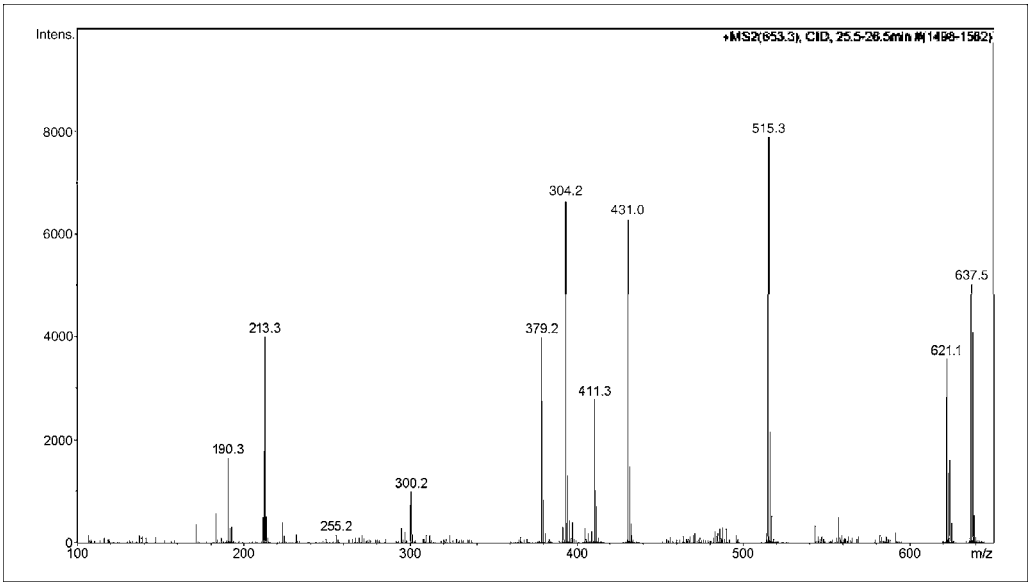


Fig. 4: MS^2 data of thalidasine

Table: Components identified in *T. fortunei* by LC/TOF-MS and MS^2

No	t_R (min)	Formula	Calcd. $[M+H]^+$	Observed $[M+H]^+$	Compound	Daughter ion
1	5.2	$C_{20}H_{18}NO_4$	336.1230	336.1231	Berberine	321.1,306.1, 292.2, 278.2, 263.1
2	6.5	$C_7H_6O_4$	155.0339	155.0330	Protocatechuic acid	137.1, 109.0, 97.2, 81.1
3	7.7	$C_9H_8O_3$	165.0546	165.0541	Coumaric acid	147.1, 121.0, 93.2, 65.2
4	10.1	$C_{20}H_{19}NO_4$	338.1387	338.1385	Jatrorrhizine	336.2, 306.1, 292.1, 278.2, 206.1, 135.2, 107.2
5	10.6	$C_{14}H_{19}NO_8$	330.1183	330.1181	Thalictoside	265.0, 219.1, 121.2, 106.3, 93.1
6	11.6	$C_{30}H_{33}NO_8$	536.2279	536.2279	Thaliadine	520.2, 504.5
7	12.4	$C_{13}H_{15}NO_4$	250.1074	250.1071	Thalactamine	234.1, 219.2, 103.2, 93.1
8	12.6	$C_{20}H_{17}NO_5$	352.1179	352.1173	Oxyberberine	336.2, 322.2, 308.3, 292.1, 175.5
9	13.1	$C_{21}H_{25}NO_5$	372.1805	372.1801	Acutifolidine	356.1, 340.1, 328.1, 313.1 180.1
10	13.7	$C_{12}H_{15}NO_3$	222.1125	222.1120	N-Methylcorydaldine	178.1, 150.1, 107.2, 58.2
11	13.8	$C_{42}H_{48}N_2O_{10}$	741.3382	741.3387	Thalmelatidine	709.1, 693.3, 533.3, 518.4, 502.2, 384.3, 206.3
12	14.5	$C_{42}H_{50}N_2O_{10}$	743.3538	743.3541	Thalmineline	520.3, 370.3, 354.2, 236.3
13	15.2	$C_{20}H_{19}NO_5$	354.1336	354.1333	Thalimicrinone	338.1, 322.1, 310.2, 135.2, 107.2, 92.1, 77.3
14	15.6	$C_{42}H_{50}N_2O_9$	727.3589	727.3594	Adiantifoline	521.2, 519.2, 370.3, 354.2, 206.1
15	16.0	$C_{39}H_{44}N_2O_8$	669.3170	669.3175	Thalistine	621.2, 515.3, 394.2, 365.2, 227.3
16	16.3	$C_{20}H_{23}NO_4$	342.1700	342.1694	Magnoflorinechloride	295.2, 236.1, 206.2, 148.1, 58.1
17	17.4	$C_{39}H_{44}N_2O_8$	669.3170	669.3174	Thalmirabine	609.3, 431.2, 227.3, 206.3, 190.2, 175.1
18	19.7	$C_{20}H_{21}NO_4$	340.1543	340.1541	Nantenine	324.1, 308.2, 296.2, 281.3, 265.1
19	25.9	$C_{39}H_{44}N_2O_7$	653.3221	653.3227	Thalidasine	637.5, 621.1, 515.3, 431.0, 394.2, 379.2, 213.3
20	29.5	$C_{40}H_{46}N_2O_8$	683.3327	683.3331	Thalifarentine	520.3, 431.0, 394.2, 370.3, 354.2, 236.3
21	30.0	$C_{21}H_{21}NO_4$	352.1543	352.1541	Thaliglucine	293.2, 250.1, 134.1, 58.1
22	30.8	$C_{35}H_{34}N_2O_6$	579.2490	579.2494	Cocsulinine	515.3, 394.2, 342.2, 283.1, 58.0
23	32.1	$C_{38}H_{42}N_2O_7$	639.3064	639.3069	Thalifoetidine	623.3, 607.2, 417.2, 213.3, 206.3, 190.2, 175.1
24	36.1	$C_{38}H_{42}N_2O_6$	623.3116	623.3117	*O*-Methylthalicberine	395.2, 213.3, 206.3, 199.3, 175.2
25	36.6	$C_{17}H_{34}O_2$	271.2632	271.2625	Methyl hexadecanoate	239.1, 227.1, 213.1, 199.1, 185.2, 171.2, 157.1, 87.1, 73.1, 59.2
26	37.2	$C_{19}H_{34}O_2$	295.2632	295.2627	Methyl linoleate	263.2, 157.2, 143.2, 137.1, 129.1, 115.1, 111.2, 87.1, 73.2, 59.2
27	40.1	$C_{38}H_{44}N_2O_6$	625.3272	625.3728	Thalibrine	518.2, 370.1, 354.1, 220.2,157.1

3.4. LC/TOF-MS

The HPLC system was coupled to an Bruker micrOTOF*Q* 125 (Bruker Ltd., USA) equipped with an electrospray interface. The electrospray source includes dual nebulizers—one nebulizer for the LC eluent and the other for the internal reference solution. The reference standard was sodium formiate, introduced into the TOF-MS with a automated calibrant delivery system (CDS), which would be used as the internal standard for acute mass weight calibration. Accurate mass measurements of the components were obtained with this CDS and thus achieved with this on-line prompt calibration.

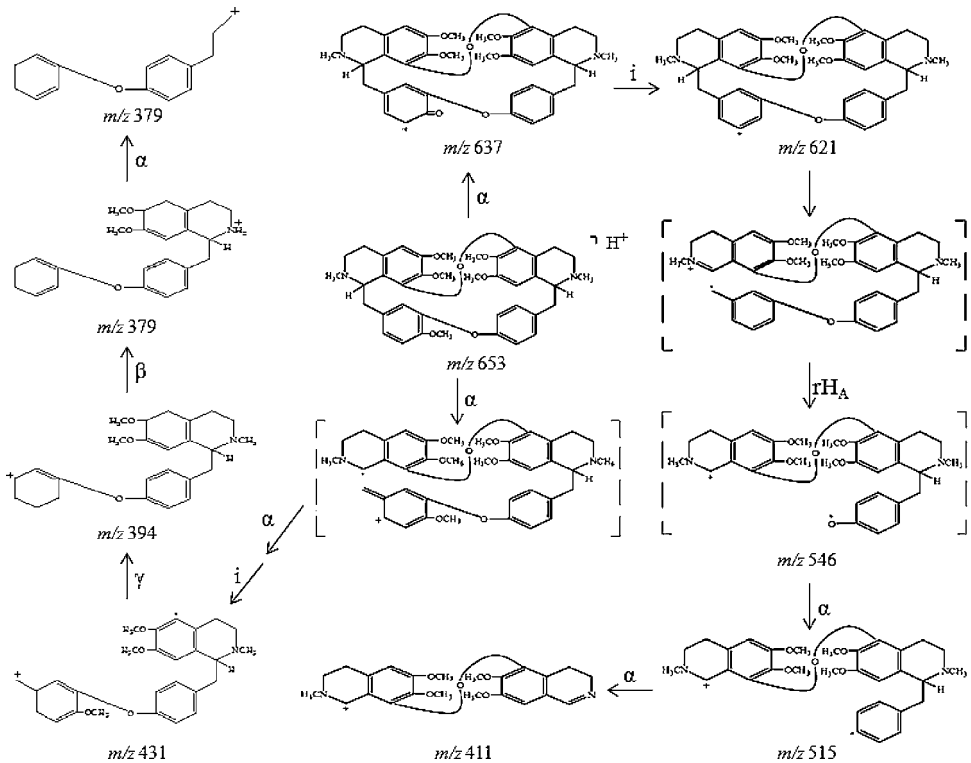


Fig. 5: Possible fragmentation pathway of thalidasine

The HPLC conditions for the LC/TOF-MS analysis were the same as for the HPLC method, except for that one-second of the eluent was introduced into the TOF-MS system with a split valve. TOF-MS analysis was performed in both positive (ESI +) and negative (ESI −) ion mode under the following operation parameters: capillary voltage 4000 V; drying gas 4 L/min; nebulizer 1.0 psi; gas temp 250 °C; fragmentor voltage 175V(ESI +) and 190 V (ESI −); skimmer voltage 60 V; octopole dc1 33.3V(ESI +) and − 40.0 V (ESI −); octopole RF 250 V. The full-scan carried out by LC/TOF-MS was recorded across the mass range 50–1500 *m*/*z*.

The elemental composition of every peak was calculated by TOF software. Considering the possible elemental composition of potential components existing in *T. fortunei*, the number and types of the expected atoms were set as follows: carbons ≤ 30, hydrogens ≤ 50, oxygens ≤ 20, nitrogens ≤ 5. The doublebond equivalent (rdb) parameter was set from 0 to 20 and the option of electron state was selected as "even". The accuracy error threshold was fixed at 5 ppm for a strict criterion.

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