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Characterization of seventy polymethoxylated flavonoids (PMFs) in the leaves of *Murraya paniculata* by on-line high-performance liquid chromatography coupled to photodiode array detection and electrospray tandem mass spectrometry

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ABSTRACT

A sensitive HPLC-DAD–ESI-MS/MS method was established to screen and identify the polymethoxylated flavonoids (PMFs) in the leaves of *Murraya paniculata* (L.) Jack. 16 PMF standards were first to be analyzed in positive mode by the CID-MS/MS. For polymethoxylated flavones, the fragments of $[M+H-n\times 15]^+$ produced by loss of one or more methyl radicals from the protonated molecule, as well as $[M+H-16]^+$, $[M+H-28]^+$, $[M+H-29]^+$, $[M+H-31]^+$, $[M+H-33]^+$, $[M+H-43]^+$, $[M+H-44]^+$, $[M+H-46]^+$ and $[M+H-61]^+$ fragment ions were detected, which could be taken as their diagnostic characters. For polymethoxylated flavanones and chalcones, their $[M+H]^+$ ions usually underwent RDA cleavage fragmentation of the C-ring prior to the similar loss of diagnostic fragment ions as polymethoxylated flavones, which could be adopted as a shortcut to distinguish them from ordinary flavones rapidly. For the PMF glycosides, the neutral loss of the similar fragments with polymethoxylated flavones from their $[aglycone+H]^+$ could be adopted as a simple method to screen them out from complex mixture. Based on these characterizations of PMFs and the results of EIC-MS/MS experiment, 70 PMFs including 45 flavones, 17 flavanones or chalcones and 8 PMFs glycosides were screened out from the complex extract of the leaves of *M. paniculata*. Among them, 16 compounds were unambiguously identified by comparison with reference substances. The results indicated that the developed analysis method could be employed as a rapid, effective technique for structural characterization of PMFs.

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1. Introduction

Murraya paniculata (L.) Jack (Qianlixiang) is one of the two *Murraya* species of Murrayae folium et cacumen officially listed in the Chinese Pharmacopoeia [1]. As an important traditional Chinese medicines (TCMs), it showed many strong bioactivities, such as febrifuge, astringent, antidysenteric, tonic, toothache remedy, antidiarrheic and stimulant, and so on [2,3]. Previous studies indicated that the polymethoxylated flavonoids (PMFs) were considered to be the representative constituents of the plant [4].

PMFs, one special group of flavonoids, possess many important biological properties such as anti-allergic, anti-oxidant, anti-bacterial, anti-proliferative and anti-inflammatory activities [5–10]. Therefore, it is of great importance to screen out PMFs from *M. paniculata*, which can give wide outlook on the applications of the Chinese herb.

In phytochemical investigations, bioactive constituents including PMFs were usually elucidated only by way of being extracted, isolated and purified into adequate amounts for nuclear magnetic resonance (NMR) or even two-dimensional nuclear magnetic resonance (2D-NMR). These methods are time-consuming, laborious and expensive. Moreover, some minor and unstable constituents are only present in raw plant materials and may be of trace amount to be separated and purified for further structural identification. Therefore, the development of a sensitive and rapid characterization of compounds in TCMs is of great significance.

Early reported methods for analysis of PMFs were based on high-performance liquid chromatography (HPLC) separation coupled with ultraviolet (UV) detection [11,12]. However, some constituents could not be detected owing to low abundance, coelution and high background of HPLC. Therefore, high-resolution

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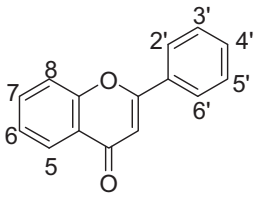
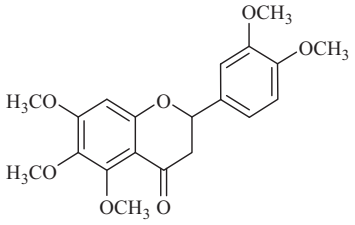
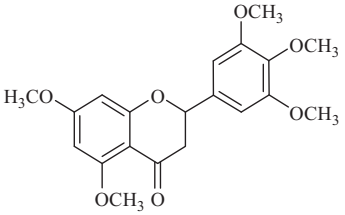


Compounds	-OH[a]	-OCH_3[a]	Identification
P-3	-	5, 7, 3', 4'	5, 7, 3', 4'-tetramethoxyflavone
P-4	-	5, 7, 8, 3', 4', 5'	5, 7, 8, 3', 4', 5'-hexamethoxyflavone
P-5	-	5, 7, 3', 4', 5'	5, 7, 3', 4', 5'-pentamethoxyflavone
P-7	-	5, 6, 7, 3', 4', 5'	5, 6, 7, 3', 4',5'-hexamethoxyflavone
P-8	5, 3'	7, 8, 4', 5'	5, 3'-dihydroxy-7, 8, 4', 5'-tetramethoxyflavone
P-9	-	5, 6, 7, 8, 3', 4', 5'	5'-methoxynobiletin
P-10	5	6, 7 ,3', 4'	5-hydroxy-6, 7 ,3', 4'-tetramethoxyflavone
P-11	5	7, 3', 4', 5'	5-hydroxy-7, 3', 4', 5'-tetrapentamethoxyflavone
P-12	5	6, 7, 3', 4', 5'	5-hydroxy-6, 7, 3', 4', 5'-pentamethoxyflavone
P-13	5	6, 7, 8, 3', 4'	5-desmethylnobiletin
P-14	5	7, 3', 4'	5-hydroxy-7, 3', 4'-trimethoxyflavone
P-15	5	6, 7, 8, 3', 4', 5'	gargenin A

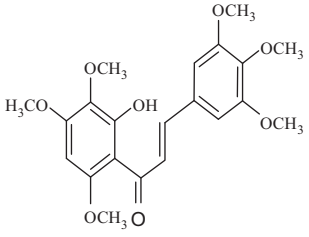
a: The positions of the substituent groups



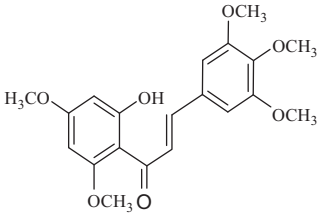
P-1 5,6,7,3',4'-pentamethoxyflavanone



P-6 5,7,3',4',5'-pentamethoxyflavanone



P-2 6'-hydroxy-3,4,5,2',3',4'-hexamethoxychalcone



P-16 6'-hydroxy-3,4,5,2',4'-pentamethoxychalcone

Fig. 1. Structures of sixteen PMFs reference standards isolated from *Murraya paniculata.*

chromatographic methods coupled to highly sensitive and selective detectors are needed. MS, especially coupled to soft ionization-source such as electrospray ionization (ESI), has turned the possibility of coupling with HPLC instrument into reality and provided rich information including molecular mass and structural information on-line. Recently, HPLC–ESI-MS and HPLC–ESI-MS/MS have been becoming a very powerful approach for the rapid identification of constituents in botanic extracts and crude material of TCMs [13–19].

There have been few studies dealing with the systematic analysis of PMFs in *M. paniculata* till now. Therefore, in the purpose of selective phytochemical screening and structural characterization of PMFs in *M. paniculata*, a developed HPLC-DAD–ESI-MS/MS method is described in this paper.

2. Experimental

2.1. Chemicals and materials

Sixteen PMF reference compounds were previously extracted, isolated and identified from *M. paniculata* in our laboratory. Their structures (shown in Fig. 1) were fully elucidated by comparison of their spectra data (ESI-MS and ^1H, ^{13}C NMR) with those published literature values [20–25]. The purities of the sixteen compounds were determined to be higher than 98% by HPLC–UV.

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was purchased from Sigma Aldrich (St. Louis, MO, USA). Ultrapure water (Wahaha, Hangzhou, China) was used throughout the experiment. The

Table 1
Characterizations of sixteen PMFs standards in *Murraya paniculata* by CID-MS/MS.

Peak	t_R[a]	$[M+H]^+$ (m/z)	MS^2(m/z)			MS^3(m/z)		
			P-ion (%)[b]	Loss[c]	Radical/neutral loss	P-ion (%)[b]	Loss[c]	Radical/neutral loss
P-1	24.64	375	211[f](100)	RDA	$^{1,3}A^+$[d]	196[f](100)	15	$CH_3^\bullet$
			191(17.5)	RDA	$^{1,4}B^+$[d]	183(29.3)	28	CO
			357(13.1)	18	H_2O	178(29.1)	33	$H_2O + CH_3^\bullet$
						150(21.9)	61	$CO + H_2O + CH_3^\bullet$
P-2	27.37	405	221[f](100)	RDA	$^xB^+$[e]	193[f](100)	28	CO
			387(25.5)	18	H_2O	190(63.0)	31	$CH_4 + CH_3^\bullet$
			211(17.4)	RDA	$^yA^+$[e]	191(38.6)	30	$2CH_3^\bullet$
						206(35.6)	15	$CH_3^\bullet$
P-3	32.91	343	328[f](100)	15	$CH_3^\bullet$	299[f](100)	29	$HCO^\bullet$
			327(53.3)	16	CH_4	312(67.1)	16	CH_4
			299(5.1)	44	CO_2			
P-4	33.68	403	373[f](100)	30	$2CH_3^\bullet$	345[f](100)	28	CO
			388(72.4)	15	$CH_3^\bullet$	358(25.5)	15	$CH_3^\bullet$
						344(10.1)	29	$HCO^\bullet$
P-5	39.12	373	313[f](100)	60	$4CH_3^\bullet$	284[f](100)	29	$HCO^\bullet$
			358(56.7)	15	$CH_3^\bullet$	283(12.5)	30	$2CH_3^\bullet$
			312(39.1)	61	$CO + H_2O + CH_3^\bullet$	285(11.4)	28	CO
			343(37.2)	30	$2CH_3^\bullet$	297(10.5)	16	CH_4
P-6	40.42	375	221[f](100)	RDA	$^xB^+$[e]	193(100)	28	CO
						190(56.9)	31	$CH_4 + CH_3^\bullet$
						191(34.6)	30	$2CH_3^\bullet$
P-7	43.55	403	388[f](100)	15	$CH_3^\bullet$	327[f](100)	61	$CO + H_2O + CH_3^\bullet$
			385(75.0)	18	H_2O	357(19.3)	31	$CH_4 + CH_3^\bullet$
			343(71.0)	60	$4CH_3^\bullet$	328(9.2)	60	$4CH_3^\bullet$
			373(39.8)	30	$2CH_3^\bullet$			
P-8	51.20	375	360[f](100)	15	$CH_3^\bullet$	345[f](100)	15	$CH_3^\bullet$
			345(90.4)	30	$2CH_3^\bullet$	327(31.3)	33	$H_2O + CH_3^{\bullet\bullet}$
			346(19.0)	29	$HCO^\bullet$	342(14.1)	18	H_2O
P-9	55.91	433	418[f](100)	15	$CH_3^\bullet$	403[f](100)	15	$CH_3^\bullet$
			404(83.1)	29	$HCO^\bullet$			
			403(79.0)	30	$2CH_3^\bullet$			
			405(8.6)	28	CO			
P-10	60.35	359	344[f](100)	15	$CH_3^\bullet$	326[f](100)	18	H_2O
			328(67.2)	31	$CH_4 + CH_3^\bullet$	328(4.5)	16	CH_4
			326(55.1)	33	$H_2O + CH_3^\bullet$			
P-11	67.18	359	344[f](100)	15	$CH_3^\bullet$	315[f](100)	29	$HCO^\bullet$
						326(42.3)	18	H_2O
						328(41.4)	16	CH_4
P-12	69.83	389	374[f](100)	15	$CH_3^\bullet$	356[f](100)	18	H_2O
			358(93.9)	31	$CH_4 + CH_3^\bullet$	328(5.4)	46	$CO + H_2O$
			359(20.4)	30	$2CH_3^\bullet$			
P-13	73.31	389	359[f](100)	30	$2CH_3^\bullet$	344[f](100)	15	$CH_3^\bullet$
			374(88.9)	15	$CH_3^\bullet$	341(88.0)	18	H_2O
						343(46.0)	16	CH_4
						331(29.0)	28	CO
P-14	77.51	329	313[f](100)	16	CH_4	285[f](100)	28	CO
			314(74.8)	15	$CH_3^\bullet$	283(22.5)	30	$2CH_3^\bullet$
						298(20.7)	15	$CH_3^\bullet$
						268(20.3)	45	$3CH_3^\bullet$
P-15	80.80	419	389[f](100)	30	$2CH_3^\bullet$	356[f](100)	33	$H_2O + CH_3^\bullet$
			404(66.3)	15	$CH_3^\bullet$	371(89.7)	18	H_2O
						361(85.1)	28	CO
						359(79.5)	30	$2CH_3^\bullet$
						374(79.1)	15	$CH_3^\bullet$
P-16	90.49	375	221[f](100)		$^xB^+$[e]	193[f](100)	28	CO
			181(8.9)		$^yA^+$[e]	190(51.2)	31	$CH_4 + CH_3^\bullet$
						191(36.8)	30	$2CH_3^\bullet$
						206(26.4)	15	CH3•

[a] t_R, retention time.
[b] P-ion (%), the product ions and the relative intensity.
[c] Loss (Da).
[d] $^{1,3}A^+$, $^{1,3}B^+$ stand for the fragment ions from the RDA cleavage from 1,3-position on the C-ring of flavanones.
[e] $^yA^+$, $^xB^+$ stand for the fragment ions from the RDA cleavage from the C-ring of chalcones.
[f] Precursor-ion for next stage MS.

0.22 μm membranes used in the experiment were purchased from Xinjinghua Co. (Shanghai, China).

Material of *M. paniculata* was purchased from China Resources Sanjiu Medical & Pharmaceutical Co., Ltd. (Shenzhen city, China). The material was used for a famous TCM preparation named Sanjiuweitai, which has been used worldwide for superficial gastritis and erosive gastritis. And it was authenticated by Professor Peng-fei Tu. The voucher specimen was deposited at the Department of Natural Medicines, Peking University, China.

2.2. Sample preparation for analysis

Powdered dried leaves of *M. paniculata* were dried at 40 °C in the oven for 2 h before the analysis. The sample was weighed

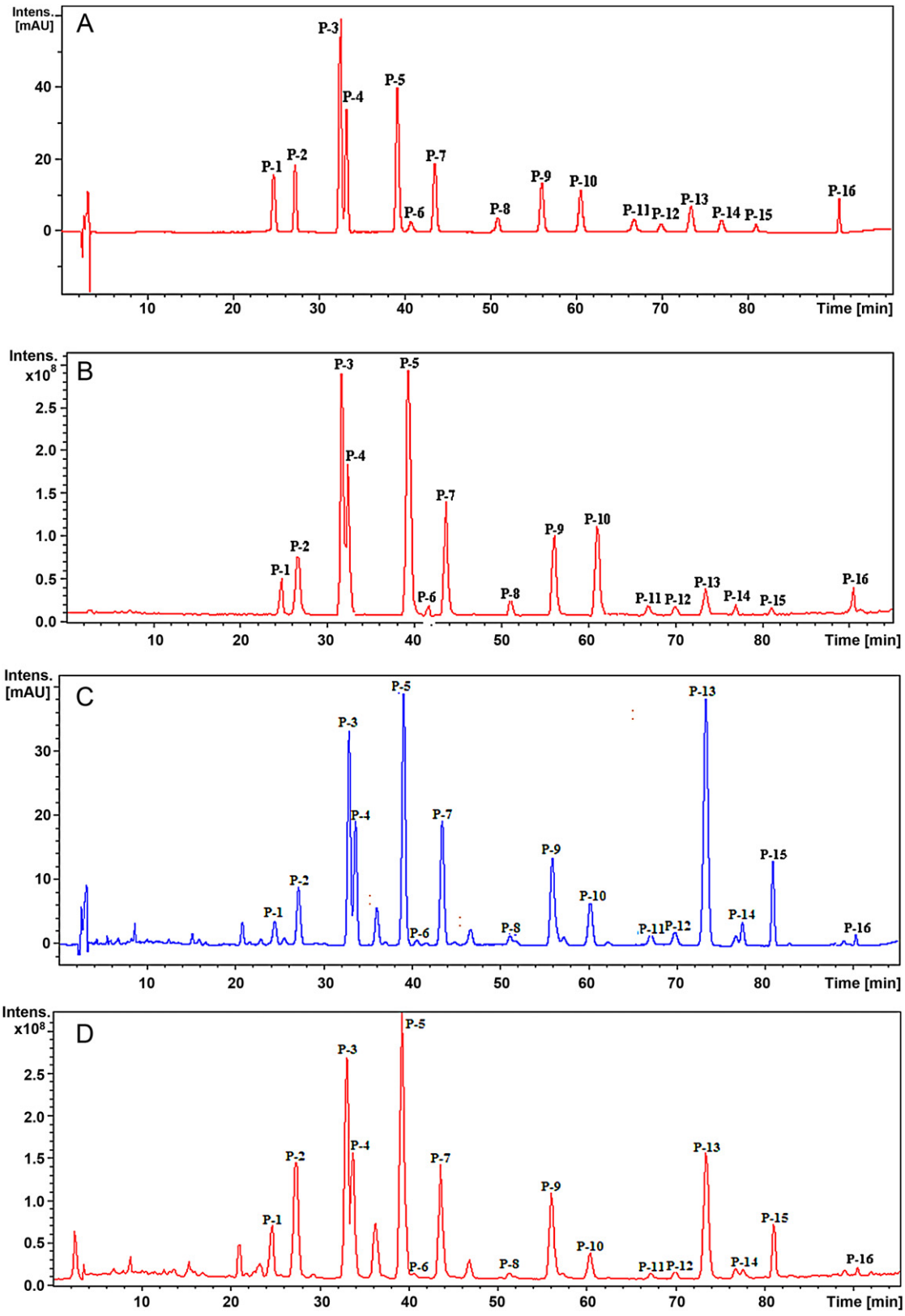


Fig. 2. HPLC-DAD–MS/MS analysis of extract of *Murraya paniculata*. (A) HPLC-DAD chromatogram of the reference standards at 330 nm; (B) the ESI-MS total ion chromatogram (TIC) of the reference standards in positive mode; (C) HPLC-DAD chromatogram of the extract at 330 nm; (D) the ESI-MS total ion chromatogram (TIC) of the extract in positive mode.

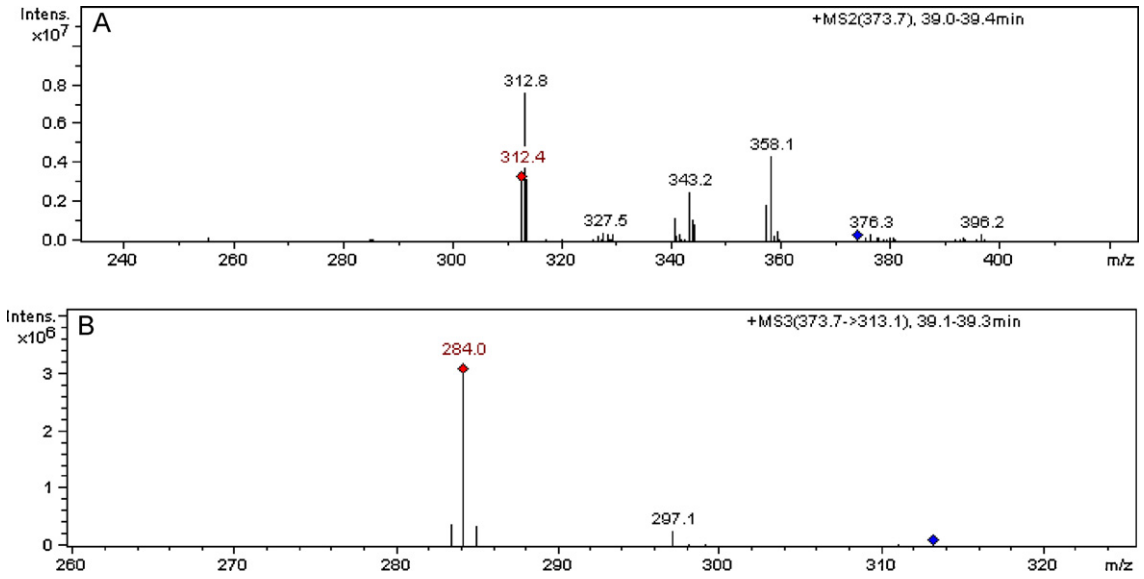


Fig. 3. MS^n spectra of **P-5**: (a) MS^2 spectrum (precursor-ion was *m*/*z* 405); (b) MS^3 spectrum (precursor-ion was *m*/*z* 221).

accurately (0.3 g) and placed into a 50 mL flask containing 5 mL of methanol/water (70:30, *v*/*v*), then the mixture was extracted in ultrasonic bath (Eima Ultrasonics Corp., Germany) at room temperature for 0.5 h. The methanol solution was filtered through a 0.22 μm membrane, then an aliquot of 10 μL of the filtrate was injected into the HPLC–MS system for analysis.

2.3. HPLC-DAD–ESI-MS/MS analysis

The HPLC-DAD analysis was carried out on an Agilent 1200 Series liquid chromatograph system (Agilent Technologies, USA), equipped with a binary pump, an auto sampler, a photo-diode array detector and a column temperature controller. The analytical column was an Agilent-Zorbax Eclipse Plus C_{18} (5 μm, 250 mm × 4.6 mm i.d.) with the oven temperature maintained at 25 °C. 0.1% formic acid aqueous solution (*v*/*v*, solvent A) and acetonitrile (solvent B) were used as mobile phase for the LC separation. The elution conditions applied with a linear gradient of as follows: 0–5 min, 20–28% B; 5–70 min, 28–42% B; 70–90 min, 42–64% B; 90–95 min, 64–100% B. The flow rate was at 1.0 mL/min and peaks were detected at 330 nm.

For ESI-MS/MS analysis, a 6320 Ion Trap Mass spectrometer (Santa Clara, CA, USA) was connected on the same Agilent 1200 HPLC instrument via an electrospray ionization (ESI) interface. The HPLC effluent was introduced into the ESI source in a post-column splitting ratio of 1:4. The ESI-MS was performed in positive ionization mode with source settings as follows: nebulizer gas pressure of 30.00 psi; dry gas flow rate of 12.00 L/min; electrospray voltage of the ion source of 3000 V; capillary temperature of 350 °C; capillary exit of 121.0 V; skimmer of 40.0 V; compound stability of 50%; trap drive level of 100%; target mass of *m*/*z* 400; scan range of *m*/*z* 100–700; AutoMS(n) operation mode; collision energy of 1 V; SmartFrag Start Ampl of 30%, SmartFrag End Ampl of 200%. A data-dependent program was used in the HPLC–ESI-MS^n analysis so that the protonated or deprotonated ions could be selected for further MS^n analysis. Nitrogen (>99.99%) and He (>99.99%) were used as sheath and damping gas, respectively. The Agilent 6300 Series Trap Control workstation (Version 6.1) was used for the data processing.

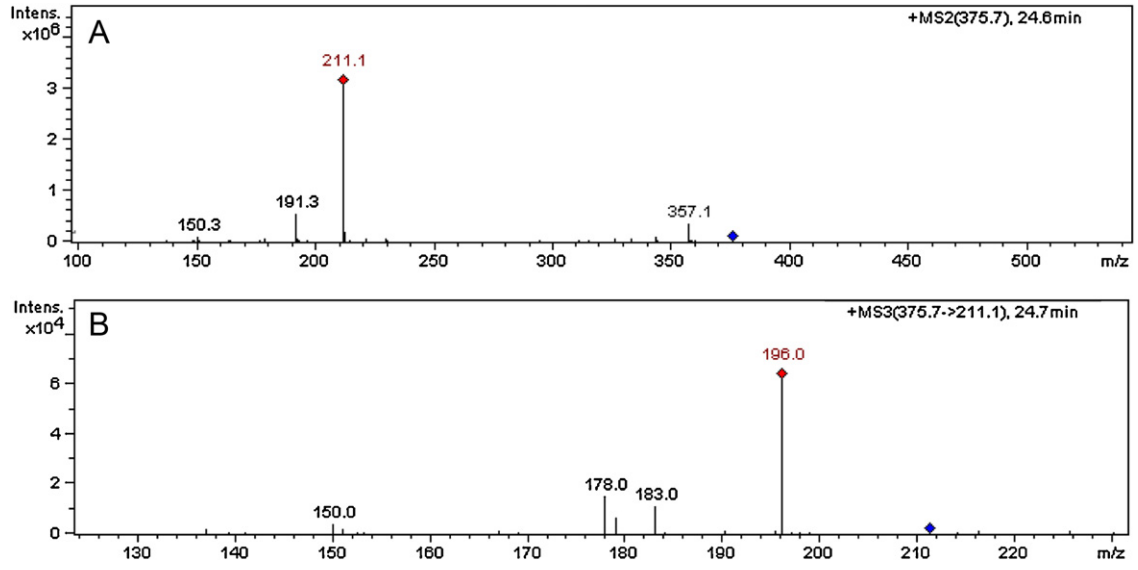


Fig. 4. MS^n spectra of **P-1**: (a) MS^2 spectrum (precursor-ion was *m*/*z* 375); (b) MS^3 spectrum (precursor-ion was *m*/*z* 211).

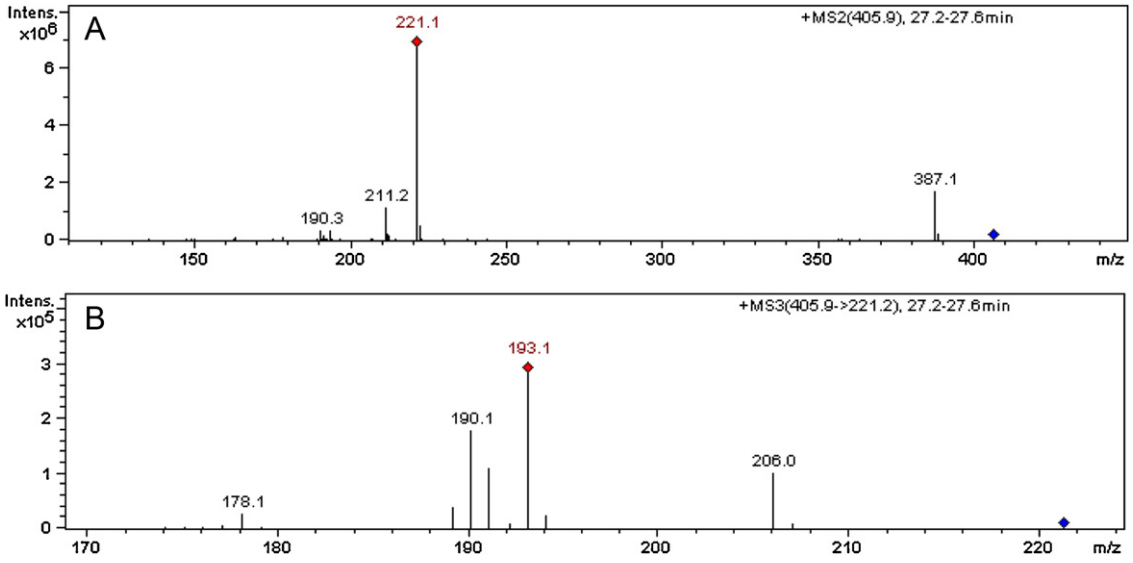


Fig. 5. MS^n spectra of **P-2**: (a) MS^2 spectrum (precursor-ion was *m*/*z* 405); (b) MS^3 spectrum (precursor-ion was *m*/*z* 221).

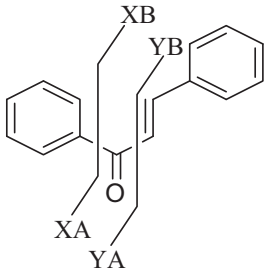


Fig. 6. Proposed MS fragmentation pathway for chalcones derivatives.

3. Results and discussion

3.1. Optimization of HPLC conditions

In order to obtain satisfactory extraction efficiency for all the PMFs, extraction conditions, including extraction methods (ultrasonication, refluxing and standing overnight), extraction solvents (30%, 50%, 70%, 100% methanol) and extraction time (15, 30 and 45 min) were assessed based on single factor experiments. The best extraction efficiency was obtained by ultrasonication extraction with 70% ethanol for 30 min. Meanwhile, it was found that the choice of detection at 330 nm could provide an optimum S/N for most of PMFs compounds. Because the ingredients in the sample could not be separated with isocratic elution, gradient elution was carried out. The different HPLC parameters including mobile phases (methanol/water and acetonitrile/water), the concentration of formic acid in water (0.05%, 0.1% and 0.3%), category of RP-ODS columns (Agilent Zorbax Eclipse SB C_{18} column, 250 mm × 4.6 mm i.d., 5 μm; Agilent-Zorbax Eclipse Plus C_{18}, 250 mm × 4.6 mm i.d., 5 μm and Waters Symmetry Shield C_{18} column, 250 × 4.6 mm i.d., 5 μm), column temperature (20, 25 and 30 °C) and flow rate (0.8, 1.0 and 1.2 mL/min) were examined. The addition of formic acid was advantageous to obtain the best resolution of adjacent peaks during chromatographic separation (shown in Fig. 2).

3.2. Optimization of ESI-MS/MS conditions

In order to achieve optimum conditions to identify PMFs in *M. paniculata* as much as possible, all factors related to MS performance including ionization mode, nebulizer gas pressure, electrospray voltage of the ion source and collision energy have been experimented. The results showed that ESI in positive ion mode was more sensitive to PMFs than in negative ion mode. The major constituents were well detected (shown in Fig. 2), and most of the investigated compounds exhibited quasi-molecular ions $[M+H]^+$ and product-ions with rich structural information in the positive mode of CID-MS/MS.

3.3. HPLC-DAD–MS/MS analysis of authentic compounds

In order to identify structures of the constituents in *M. paniculata*, sixteen reference compounds were analyzed by HPLC-DAD–ESI-MS/MS techniques. According to their chemical structures, UV absorption maxima and dominant fragmentation pathways, the authentic compounds could be classified into three groups including polymethoxylated flavones, flavanones and chalcones. In the full scan mass spectra, most of PMF standards

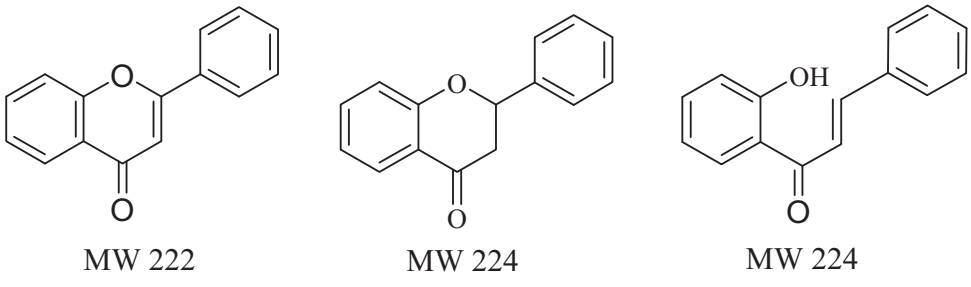


Fig. 7. Basic structures of the aglycones of flavones, flavanones and chalcones.

Table 2
Chemical formula and mass of all possible polymethoxylated flavones.

Substituents	–	OH	2OH	3OH	4OH	5OH
$2OCH_3$	$C_{17}H_{14}O_4$ 282	$C_{17}H_{14}O_5$ 298	$C_{17}H_{14}O_6$ 314	$C_{17}H_{14}O_7$ 330	$C_{17}H_{14}O_8$ 346	$C_{17}H_{14}O_9$ 362
$3OCH_3$	$C_{18}H_{16}O_5$ 312	$C_{18}H_{16}O_6$ 328	$C_{18}H_{16}O_7$ 344	$C_{18}H_{16}O_8$ 360	$C_{18}H_{16}O_9$ 376	
$4OCH_3$	$C_{19}H_{18}O_6$ 342	$C_{19}H_{18}O_7$ 358	$C_{19}H_{18}O_8$ 374	$C_{19}H_{18}O_9$ 390		
$5OCH_3$	$C_{20}H_{20}O_7$ 372	$C_{20}H_{20}O_8$ 388	$C_{20}H_{20}O_9$ 404			
$6OCH_3$	$C_{21}H_{22}O_8$ 402	$C_{21}H_{22}O_9$ 418				
$7OCH_3$	$C_{22}H_{24}O_9$ 432					

exhibited $[M+H]^+$ ions of sufficient abundance that could be subsequently isolated automatically and subjected to CID-MS/MS analysis (shown in Table 1). The proposed fragmentation patterns were helpful to clarify the structural identification of constituents in *M. paniculata*. The nomenclature commonly used for mass fragments of flavonoids was adopted in this work [26].

3.3.1. CID-MS/MS for polymethoxylated flavones

In the CID-MS/MS experiment, twelve polymethoxylated flavones standards were subsequently analyzed first. Comparing the product-ion spectra of the standards (shown in Fig. 3), some characterized dissociation pathways could be summarized for further characterization of the other polymethoxylated flavones.

First, most of the $[M+H]^+$ ions of standards except **P-14** could lose one to four methyl radicals ($CH_3^\bullet$) in their MS/MS spectra, and formed the base peaks of $[M+H-15]^+$, $[M+H-30]^+$, $[M+H-45]^+$ or $[M+H-60]^+$. However, compound **P-14** also eliminated one methyl radical and yielded the $[M+H-15]^+$ ion as the secondary peak of MS/MS spectrum. This fragmentation pathway can be taken as the major diagnostic characteristic for polymethoxylated flavones.

Second, the other dissociation pathways by loss of 16(CH_4), 18(H_2O), 28(CO), 29($HCO^\bullet$), 31($CH_4+CH_3^\bullet$), 33($H_2O+CH_3^\bullet$), 43($CO+CH_3^\bullet$), 44(CO_2), 46(H_2O+CO), 60($4CH_3^\bullet$) and 61($CO+H_2O+CH_3^\bullet$) were also frequently detected as diagnostic fragments in their MS/MS and MS/MS/MS spectra. These main product-ions mentioned above could form the characteristic ESI-MS^n "fingerprint" of PMFs, which could be used to screen out the polymethoxylated flavones from the complex extract of TCMs rapidly. The "fingerprint" set up in the study was highly similar with the one that was achieved by APCI-MS/MS [27]. Some diagnostic fragments such as 18, 28 and 44 detected in the product-ion spectra were frequently reported in characterization of ordinary flavonoids [28], too.

Last, phenomena of the neutral loss of CH_4 were seen in their MS/MS spectra of several reference standards, such as **P-3**, **P-10**, **P-12** and **P-14**. The intensity of these ions owing to the loss of CH_4 was so strong that they usually were the secondary peak or even the base peak (**P-14**) in MS/MS spectra, indicating this fragmentation pathway could occur easily. The common characteristic of these compounds was that they all had two neighboring methoxyl groups on A-ring or/and B-ring. Meanwhile, this kind of dissociation pattern was found in the MS/MS/MS spectra of several other compounds with three neighboring methoxyl groups including **P-7**, **P-11** and **P-13**. However, the relative intensities of their product-ions were often very low, demonstrating the fragmentation pattern was just subordinate reaction for them. Therefore, it was interesting to deduce that PMFs with two ortho-methoxyl groups were easier to neutral eliminate CH_4 than those with three ortho-methoxyl groups, which could be employed to judge the number of ortho-methoxyl groups on A-ring and B-ring of polymethoxylated flavones preliminarily.

3.3.2. CID-MS/MS for polymethoxylated flavanones

There were only two polymethoxylated flavanone derivatives, **P-1** and **P-6**, isolated from *M. paniculata* owing to the low abundance of flavanones in the medical plant. In CID-MS/MS experiment, their fragmentation pathways of MS spectra were similar with each other. Take **P-1** for example, it gave the $[M+H]^+$ ion at *m/z* 375, which further generated the prominent ion at *m/z* 211 as base peak in MS/MS spectrum (shown in Fig. 4). It could be deduced after analysis carefully that its dominating fragmentation pathway was RDA cleavage from the 1,3-position of C-ring. Meanwhile, the minor ion at *m/z* 191 was also detected, owing to the RDA fragmentation from the 1,4-position of C-ring. The loss of 15($CH_3^\bullet$), 18(H_2O), 28(CO), 33($H_2O+CH_3^\bullet$) and 61($CO+H_2O+CH_3^\bullet$) from the base peak at *m/z* 211, could be also detected as minor fragmentation ions in the CID-MS/MS spectrum. This kind of fragmentation pathway that the $[M+H]^+$ ion underwent RDA reaction prior to the neutral loss of $CH_3^\bullet$, H_2O, CO, etc., was different noticeably from ordinary flavanones. Therefore, it could be as a shortcut to distinguish polymethoxylated flavanones from ordinary flavones rapidly.

Table 3
Chemical formula and mass of all possible polymethoxylated flavanones or chalcones.

Substituents	–	OH	2OH	3OH	4OH	5OH
$2OCH_3$	$C_{17}H_{16}O_4$ 284	$C_{17}H_{16}O_5$ 300	$C_{17}H_{16}O_6$ 316	$C_{17}H_{16}O_7$ 332	$C_{17}H_{16}O_8$ 348	$C_{17}H_{16}O_9$ 364
$3OCH_3$	$C_{18}H_{18}O_5$ 314	$C_{18}H_{18}O_6$ 330	$C_{18}H_{18}O_7$ 346	$C_{18}H_{18}O_8$ 362	$C_{18}H_{18}O_9$ 378	
$4OCH_3$	$C_{19}H_{20}O_6$ 344	$C_{19}H_{20}O_7$ 360	$C_{19}H_{20}O_8$ 376	$C_{19}H_{20}O_9$ 392		
$5OCH_3$	$C_{20}H_{22}O_7$ 374	$C_{20}H_{22}O_8$ 390	$C_{20}H_{22}O_9$ 406			
$6OCH_3$	$C_{21}H_{24}O_8$ 404	$C_{21}H_{24}O_9$ 420				
$7OCH_3$	$C_{22}H_{26}O_9$ 434					

Table 4
Characterization of PMFs in *Murraya paniculata* by HPLC-DAD–ESI-MS/MS.

No.	t_R[a]	$[M+H]^+$ (m/z)	MS^2(m/z) P-ion (%, loss)[b]	MS^3(m/z) P-ion (%, loss)[b]
1	10.55	315	300[d](100, 15)	272(100, 28), 257(23.2, 43), 285(11.3, 15)
2	56.70	315	300[d](100, 15)	272(100, 28)
3	15.32	329	314[d](100, 15)	298(100, 16), 299(42.8, 15), 285(39.0, 29)
4	20.81	329	314[d](100, 15)	286(100, 28), 285(39.1, 29)
5	21.85	329	314[d](100, 15)	286(100, 28), 285(36.8, 29), 271(14.3, 43)
6[c]	77.41	329	313[d](100, 16), 314(74.8, 15)	285(100, 28), 283(22.5, 30)
7	25.61	331	316[d](100, 15)	301(100, 15), 273(25.8, 43),
8	37.15	331	316[d](100, 15)	288(100, 28), 287(12.4, 29)
9[c]	32.99	343	328[d](100, 15), 327(53.3, 16), 299(5.4, 44)	299(100, 29), 312(67.1, 16)
10	13.93	345	330[d](100, 15)	302(100, 28), 287(29.1, 43)
11	36.74	345	181[d](100, RDA), 191(77.7, RDA)	125(100, 56), 166(84.3, 15)
12	40.65	345	330[d](100, 15), 312(77.1, 33)	312(100, 18), 315(13.6, 15)
13	44.96	345	330[d](100, 15), 329(14.7, 16)	301(100, 29), 302(58.3, 28), 314(41.8, 16)
14	45.37	345	330[d](100, 15), 301(10.7, 44), 329(10.5, 16)	301(100, 29), 312(97.6, 18), 302(91.6, 28), 314(91.0, 16)
15	62.38	345	330[d](100, 15), 329(76.5, 16), 284(16.9, 61)	284(100, 46), 301(38.9, 29,), 299(21.5, 31)
16	88.01	345	181[d](100, RDA), 191(64.5, RDA)	151(100, 30), 125(94.5, 56)
17	13.12	359	344[d](100, 15)	299(100, 45), 329(40.0, 15)
18	16.82	359	329[d](100, 30), 344(99.0, 15)	301(100, 28), 314(13.7, 15)
19	19.77	359	344[d](100, 15), 343(94.4, 16), 298(25.1, 61)	328(100, 16), 315(98.8, 29), 298(40.0, 46), 329(30.7, 15)
20[c]	60.48	359	344[d](100, 15), 328(67.2, 31), 326(55.3, 33)	326(100, 18), 328(5.8, 16)
21[c]	67.07	359	344[d](100, 15)	315(100, 29), 326(41.2, 18)
22	82.83	359	298[d](100, 61), 329(21.5, 30), 299(18.5, 60)	270(100, 28), 269(69.9, 29)
23	15.64	361	211[d](10, RDA)	153(7.5, 58)
24	26.08	361	346[d](100, 15)	313(100, 33), 331(48.9, 15), 328(20.1, 18)
25	35.85	373	358[d](100, 15), 313(40.9, 60)	312(100, 46), 329(23.4, 29), 313(19.4, 45)
26[c]	39.02	373	313[d](100, 60), 358(56.7, 15), 343(26.9, 30)	284(100, 29), 283(12.2, 30)
27[c]	24.62	375	211[d](100, RDA), 191(17.5, RDA), 357(13.1, 18)	196(100, 15), 183(29.3, 28), 178(29.1, 33)
28[c]	40.42	375	221[d](100, RDA)	193(100, 28), 190(56.8, 31), 191(34.7, 30)
29	41.72	375	360[d](100, 15), 342(75.2, 33)	342(100, 18), 345(16.7, 15)
30[c]	51.29	375	360[d](100, 15), 345(90.9, 30), 346(19.5, 29)	345(100, 15), 327(31.0, 33)
31	52.07	375	360[d](100, 15), 359(39.0, 16), 345(17.8, 30)	344(100, 16), 345(62.6, 15), 314(36.5, 46)
32	76.40	375	211[d](100, RDA), 191(23.4, RDA), 357(13.4, 18)	196(100, 15), 183(18.3, 28)
33[c]	90.49	375	221[d](100, RDA), 181(10.6, RDA)	193(100, 28), 190(51.2, 31), 191(36.8, 30)
34	16.13	389	374[d](100, 15), 359(31.8, 30)	328(100, 46), 359(70.5, 15), 356(48.8, 18) 341(46.2, 33)
35	20.02	389	374[d](100, 15), 373(30.6, 16)	359(100, 15), 343(88.6, 31), 358(58.1, 16)
36	23.16	389	374[d](100, 15)	359(100, 15), 356(86.3, 18)
37	30.41	389	374[d](100, 15), 360(63.4, 29), 359(25.8, 30)	359(100, 15), 328(13.1, 46)
38[c]	69.81	389	374[d](100, 15), 357(90.5, 32), 359(27.6, 30)	356(100, 18)
39[c]	72.99	389	359[d](100, 30), 374(88.9, 15	344(100, 15), 341(88.0, 18),343(46.0, 16)
40	75.16	389	359[d](100, 30), 374(69.2, 15), 341(50.8, 48)	331(100, 28), 341(94.1, 18), 344(76.5, 15)
41	76.89	389	374[d](100, 15)	346(100, 28), 359(28.4, 15)
42	4.42	391	149[d](100, RDA), 279(26.1, RDA)	
43	5.64	391	149[d](100, RDA), 279(24.7, RDA)	
44	13.70	391	221[d](100, RDA), 373(29.3, 18), 197(26.3, RDA)	193(100, 28), 190(65.5, 31), 191(37.0, 30)
45	16.71	391	221[d](100, RDA), 149(38.6, RDA)	193(100, 28), 190(73.5, 31), 191(48.5, 30)
46	29.95	391	375[d](100, 16), 360(63.4, 31)	359(100, 16), 360(15.5, 15)
47	75.62	391	149[d](100, RDA), 359(94.4, 32)	
48	78.7	391	149[d](100, RDA), 279(25.0, RDA)	
49	83.20	391	149[d](100, RDA), 279(38.1, RDA), 376(19.1, 15)	
50	17.34	403	372[d](100, 31), 373(65.3, 30), 387(45.9, 16)	311(100, 61), 342(67.4, 30), 357(66.8, 15)
51[c]	33.72	403	373[d](100, 30), 388(72.4, 15)	345(100, 28), 358(25.5, 15), 344(10.1, 29)
52[c]	43.61	403	388[d](100, 15), 385(74.2, 18), 343(69.2, 60)	327(100, 61), 357(18.8, 31)
53	46.41	403	388[d](100, 15), 373(53.7, 30), 374(48.1, 29)	373(100, 15)
54[c]	27.37	405	221[d](100, RDA), 387(25.3, 18), 211(17.1, RDA)	193(100, 28), 190(63.4, 31), 191(38.5, 30)
55	57.29	405	390[d](100, 15), 375(13.6, 30)	375(100, 15), 374(58.8, 16), 359(45.2, 31)
56	35.31	419	404[d](100, 15), 389(91.9, 30)	389(100, 15), 358(15.7, 46)
57[c]	80.94	419	389[d](100), 404(66.3, 15)	356(100, 33), 371(89.7, 18), 361(85.1, 28)
58	89.92	421	211[d](100, RDA), 195(34.1, RDA)	196(100, 15), 150(49.7, 61), 178(32.9, 33)
59	6.04	433	415[d](100, 18), 397(63.8, 36)	400(100, 15), 397(92.9, 18)
60[c]	55.95	433	418[d](100, 15), 404(82.8, 29), 403(78.1, 30)	403(100, 15)
61	50.32	435	241[d](100, RDA), 417(27.4, 18), 221(24.8, RDA)	226(100, 15), 208(11.9, 33)
62	85.88	435	255[d](100, RDA), 165(57.3, RDA)	240(100, 15), 225(28.6, 30)

[a] t_R, retention time.
[b] P-ion (%, loss), the product ions,the relative intensity and the loss (Da).
[c] Compounds identified by comparison with reference standards.
[d] Precursor-ion for next stage MS.

3.3.3. CID-MS/MS for polymethoxylated chalcones

P-2 and **P-16**, two polymethoxylated chalcones standards, were analyzed by the CID-MS/MS method, too. Their dissociation pathways of MS spectra were similar with each other. Taking **P-2** for example (shown in Fig. 5), the RDA cleavage at bond X to yield the base peak ion $^{X}B^{+}$ at m/z 221 and at bond Y to yield the minor ion $^{Y}A^{+}$ at m/z 211 (shown in Fig. 6) could also be simultaneously detected in MS/MS first. The fragmentation pathway was highly similar with what happened to flavanones. This is reasonable because cyclization of 6′-hydroxychalcones to

Table 5
The MWs and structural identification of all possible PMFs detected in *Murraya paniculata.*

Peaks	Amounts	PMFs	No. of $-OCH_3$	No. of –OH	MW
1–2	2	Dihydroxy-dimethoxyflavone	2	2	314
3–6	4	Monohydroxy-trimethoxyflavone	3	1	328
7–8	2	Trihydroxy-dimethoxyflavone	2	3	330
9	1	Tetramethoxyflavone	4	0	342
10, 12–16	6	Dihydroxy-trimethoxyflavone	3	2	344
11	2	Tetramethoxyflavanone or tetramethoxychalcone	4	0	344
17–22	6	Monohydroxy-trimethoxyflavone	4	1	358
23	1	Monohydroxy-tetramethoxyflavanone or monohydroxy-tetramethoxychalcone	4	1	360
24	1	Trihydroxy-trimethoxyflavone	3	3	360
25–26	2	Pentamethoxyflavone	5	0	372
27–28, 32–33	4	Pentamethoxyflavanone or pentamethoxychalcone	5	0	374
29–31	3	Dihydroxy-tetrahydroxyflavone	4	2	374
34–41	8	Monohydroxy-pentamethoxyflavone	5	1	388
42–45, 47–49	6	Monohydroxy-pentamethoxy or monohydroxy-pentamethoxychalcone	5	1	390
46	1	Trihydroxy-tetramethoxyflavone	4	3	390
50–53	4	Hexamethoxyflavone	6	0	402
54	1	Hexamethoxychalcone	6	0	404
55	1	Dihydroxy-pentamethoxyflavone	5	2	404
56–57	2	Monohydroxy-hexamethoxyflavone	6	1	418
58	1	Monohydroxy-hexamethoxyflavanone or monohydroxy-hexamethoxychalcone	6	1	420
59–60	2	Heptamethoxyflavone	7	0	432
61–62	2	Heptamethoxyflavanone or heptamethoxychalcone	7	0	434

flavanones has been reported in a number of studies demonstrating an intramolecular equilibrium being present between a flavanone-type and a chalcone-type molecular ion [29,30]. At the same time, the loss of 15($CH_3^{\bullet}$), 16(CH_4), 18(H_2O), 28(CO), 30($2CH_3^{\bullet}$) and 31($CH_4 + CH_3^{\bullet}$) could be also detected. Thus, according to their fragmentation pathways, it was easy to tell the difference between polymethoxylated chalcones and flavones, but difficult to distinguish them from polymethoxylated flavanones. However, the differences of UV spectra between polymethoxylated chalcones and polymethoxylated flavanones provided a shortcut to classify them, because the maximum absorption of chalcones usually ranged from 330 to 370 nm, while flavanones maintained at about 320 nm.

3.4. HPLC-DAD-MS/MS analysis of the PMFs in M. paniculata

The purpose of our study was to screen out the PMFs in *M. paniculata*. PMFs have regularity in elemental composition as they have the basic aglycone structure with maximum seven substituents such as methoxyl group (OCH_3) and/or hydroxyl group (OH) on its A, B and C rings (shown in Fig. 7). The MWs of basic structures of aglycone are 222 u, 224 u and 224 u for flavones, flavanones and chalcones, respectively, which are increased by 30 u or 16 u when a methoxyl or hydroxyl was attached. Based on the numbers and the types of the substituent groups, the chemical formula and mass of every possible PMF isomer can be designated in advance (shown in Tables 2 and 3).

Because of the complex and similarity of the ingredients in *M. paniculata*, EIC-MS (extracted ion chromatogram) method was employed to analyze the PMFs in the plant (shown in Fig. 8 and Table 4).

In the study, the abundances of most of the unknown compounds especially the chalcones and flavanones were too low to afford the on-line UV absorption spectra, so it was difficult to distinguish them from flavanones and chalcones. Therefore, they were identified together in the end.

After screening the MWs with EIC-MS method, 62 PMFs including 45 flavones (12 known), 17 flavanones or chalcones (4 known) were tentatively identified (shown in Table 5). Some EIC-MS peaks were too weak to be seen clearly in the TIC (total ion chromatogram) spectra. Meanwhile, the retention times of some EIC-MS peaks were so similar that they could not be identified simultaneously in TIC spectra, either. Thus, EIC-MS method adopted in our study was confirmed to be one kind of powerful weapons to screen the ingredients preliminarily in highly complex extracts of TCMs.

Table 6
Characterization of the PMFs glycosides in *Murraya paniculata* by HPLC-DAD–ESI-MS/MS.

Peaks	t_R	$[M+H]^+$ (m/z)	MS^2(m/z)		MS^3(m/z)		Identification
			P-ion (%)	Loss	P-ion (%)[b]	Loss	
1	6.9	509	347(100)	162	332(100)	15	Dihydroxy-trihydroxyflavone-hexose
2	8.0	491	329(100)	162	314(100)	15	Monohydroxy-trihydroxyflavone-hexose
					286(3.8)	43	
3	8.8	493	331(100)	162	316(100)	15	Trihydroxy-dimethoxyflavone-hexose
4	9.8	523	361(100)	162	346(100)	15	Trihydroxy-trimethoxyflavone-hexose
					345(18.4)	16	
5	10.2	509	347(100)	162	332(100)	15	Dihydroxy-trihydroxyflavone-hexose
6	11.2	507	345(100)	162	330(100)	15	Dihydroxy-trimethoxyflavone-hexose
					299(2.6)	46	
7	11.5	553	391(100)	162	376(100)	15	Trihydroxy-tetramethoxyflavone-hexose
					330(32.6)	61	
					375(21.4)	16	
8	12.6	537	375(100)	162	360(100)	15	Dihydroxy-tetrahydroxyflavone-hexose
					343(37.1)	32	
					342(21.0)	33	

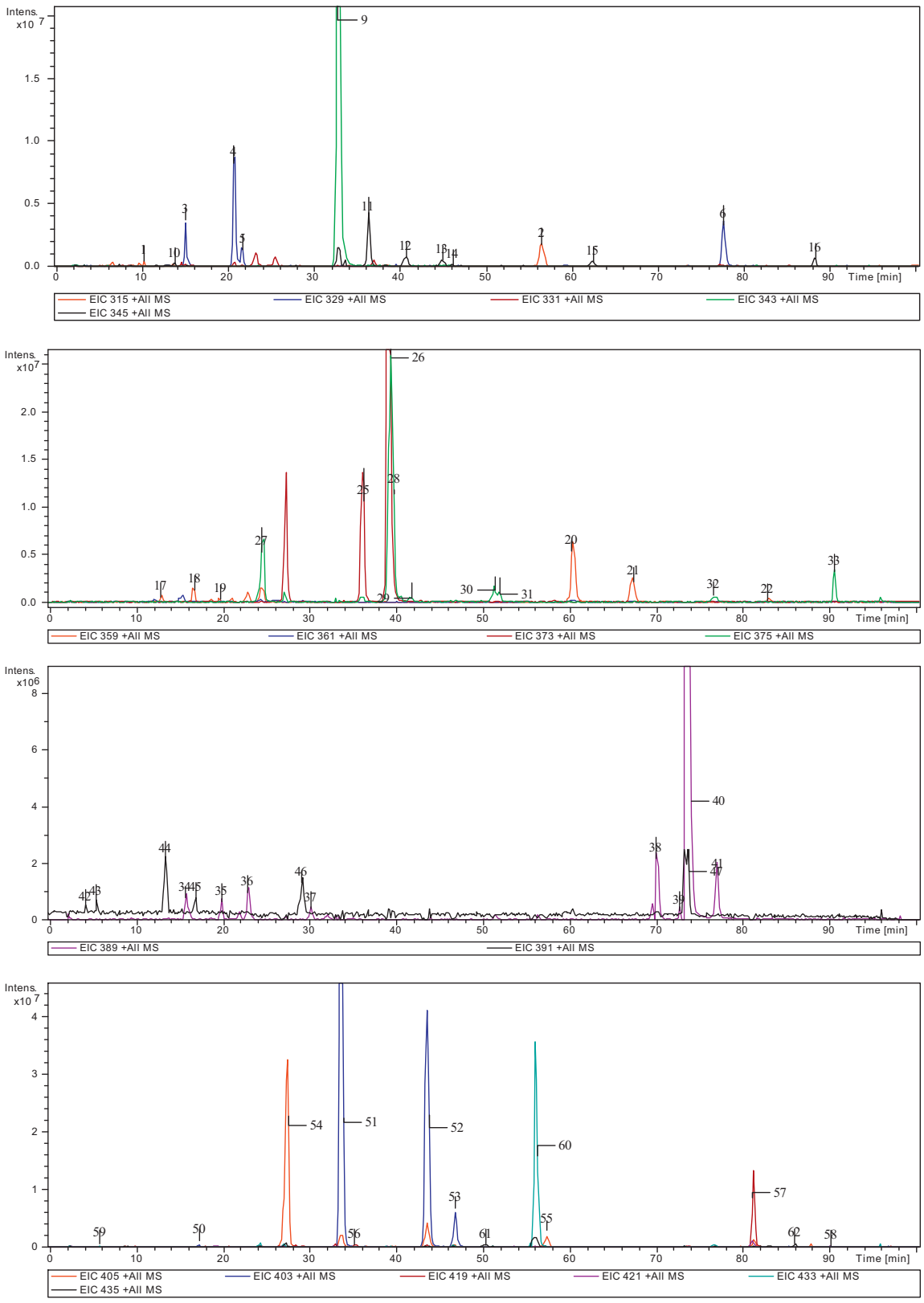


Fig. 8. The EIC-MS peaks of all possible PMFs in *Murraya paniculata*.

3.5. HPLC-DAD–ESI-MS/MS analysis of PMFs glycosides in M. paniculata

In 0–15 min of the LC–MS analysis, there were few compounds identified or tentatively characterized. From the result of full-scan of LC–MS, some compounds with the MWs between 450 and 550 had the great possibilities to be PMFs glycosides, owing to the fragmentation pathways of their [aglycone+H]$^+$ ions were similar with the diagnostic characteristics of PMFs. Therefore, EIC-MS method was employed again to identify these compounds (shown in Fig. 9).

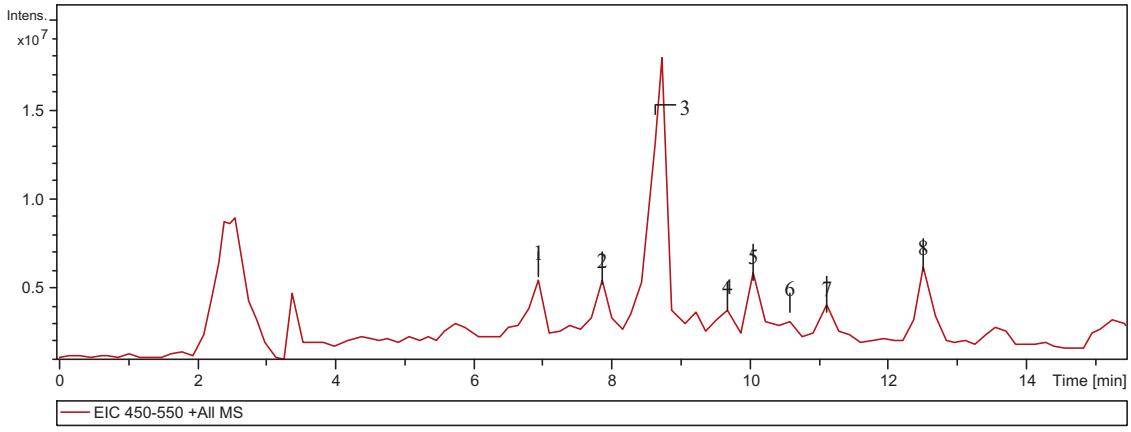


Fig. 9. The EIC-MS peaks of all possible PMFs glycosides in *Murraya paniculata*.

In their MS/MS spectra, all of the $[M+H]^+$ ions readily eliminated the sugar moiety to produce the corresponding $[aglycone+H]^+$ ions as base peak. Neutral loss scan of 162 Da revealed the presence of hexose such as glucose in their molecules. Then the $[aglycone+H]^+$ ions were selected to trace the structural information of PMFs aglycones. The neutral loss detected in their MS/MS spectra were accord with the "fingerprint" of polymethoxylated flavones, demonstrating they were PMFs glycosides presenting in *M. paniculata* probably.

The result (shown in Table 6) demonstrated that at least eight possible structures of PMFs glycosides presented in the extract of *M. paniculata*. There has been no relative report about the glycosides of PMFs from the genus *Murraya* till now. The study provided a significant clue for the phytochemical research on *M. paniculata* and the plants of genus *Murraya*.

3.6. Verification with the diagnostic characteristic of PMFs

By EIC-MS/MS, all the candidates for PMFs were preliminarily identified from the *M. paniculata* extract. However, further verification with the diagnostic characteristic of PMFs standards was still needed to perform in the end. The $[M+H]^+$ ions of polymethoxylated flavones all eliminated the mass of 15, 30 and 60 as the base peak for MS/MS spectra, except compounds 6, 22, 46, 50 and 59, all of which yielded major $[M-n{CH_3}^\bullet]^+$ ions in their mass spectra, too. Meanwhile, other diagnostic fragments loss of 16(CH_4), 18(H_2O), 28(CO), 29($HCO^\bullet$), 31($CH_4+CH_3^\bullet$), 33($H_2O+CH_3^\bullet$), 43($CO+CH_3^\bullet$), 44(CO_2), 46(H_2O+CO), 60($4CH_3^\bullet$) and 61($CO+H_2O+CH_3^\bullet$) could be also detected. For all the $[M+H]^+$ ions of polymethoxylated flavanones and chalcones, RDA fragmentation always happened as the major dissociation pathway prior to the neutral loss of the diagnostic fragments mentioned in polymethoxylated flavones. For $[aglycone+H]^+$ ions of eight PMFs glycosides detected, the fragment ions that they yielded were accord with the "fingerprint" of polymethoxylated flavones. The results were well accord with the fragmentation pathways deduced from the reference standards. Therefore, 70 compounds including 45 flavones, 17 flavanones or chalcones and 8 glycosides were preliminarily verified as PMFs among which 16 compounds were unambiguously identified by comparison with reference substances.

4. Conclusion

A sensitive HPLC-DAD–ESI-MS/MS method was established, which can be used to simultaneously identify and screen the PMFs present in the extract of *M. paniculata*. Sixteen PMFs standards including twelve flavones, two flavanones and two chalcones were analyzed by CID-MS/MS first to obtain the respective characterizations of fragment pathways, which could be adopted as the basis for further analysis the PMFs in the extract. Meanwhile, owing to regularities of PMFs in elemental composition, the EIC-MS method by MWs was employed to screen the homeomorphic PMFs from the extract. In the end, 62 PMFs were identified preliminarily and 16 of them could be unambiguously identified by comparison with reference substances. Moreover, eight PMFs glycosides were identified simultaneously from *M. paniculata* extract, which was for the first time to report the presence of PMFs glycosides in genus *Murraya*. All the results indicated that the developed HPLC-DAD–ESI-MS/MS method could be employed as a rapid, effective technique to screen and identify the PMFs from complex extract of TCMs.

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