RAPID IDENTIFICATION AND DETECTION OF FLAVONOID COMPOUNDS FROM BAMBOO LEAVES BY LC-(ESI)-IT-TOF/MS

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This paper provides an applicable approach to identifying flavonoid compounds from bamboo leaves extracts, based on the use of the powerful Liquid Chromatography Electrospray Ionization source in combination with hybrid Ion Trap and high-resolution Time-of-flight Mass Spectrometry (LC-(ESI)-IT-TOF/MS). The strategy involves four procedural steps including searching flavonoid components based on an ultraviolet spectrum scan, getting the accurate mass of flavonoid components parent ion, retrieving the corresponding formula by software, and speculating as to the chemical structure according to mass spectrum decomposition rules. The presently developed methodology has been well proven to be useful and valuable by successful application to the identification of flavonoid components from *Dendrocalamopsis oldham* leaves. All of the 13 flavonoid components detected have been successfully identified by this approach, except that it failed to confirm 3 flavonoid component chemical structures. The calibration curves of two flavonoid components (orientin and vitexin) that had been identified in bamboo leaves showed a good linear fit ($R^2 \geqslant 0.9998$) in the concentration range of 6.25 to 200 mg/L. The limits of detection (LOD) were less 0.02 mg/L (S/N=3), and the estimated limits of quantification (LOQ) were less 0.06 mg/L (S/N=10). Intra- and inter-day relative standard deviations were less than 1.04 and 1.82%, respectively.

Keywords: LC-(ESI)-IT-TOF/MS; Flavonoid compounds; Bamboo leaves; Structure characterization

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INTRODUCTION

Bamboo is one of the most valuable naturally-occurring plants worldwide, because its different edible parts contain important nutrients. Bamboo leaves have been used in traditional Chinese medicine for treating fever and for detoxification for over 1000 years (Zhang and Ding 1996). In addition, the antioxidants contained in bamboo leaves have been included in China's national standards (GB-2760) as a kind of food antioxidant. Published research data show different pharmacological activities of bamboo grass, such as spontaneous motor activity (Nagasawa and Hatorri 2001) and anti-tumor activity (Tsunoda et al. 1998). Many papers have also indicated that bamboo-leaf extract has multiple biological effects, such as free radical scavenging, anti-oxidation, anti-aging, and the prevention of cardiovascular diseases (Zhang and Ding 1996; Zhang and Ding 1997; Tang and Ding 2000).

Flavonoids, which are present in the food we eat, have generated particular interest with regard to human health effects, including, for example, antioxidant activities (Pannala et al. 2001; Heim et al. 2002; Bahorun et al. 2004), protection from cardiovascular diseases (Sesso et al. 2003; Wilmsen et al. 2005), and cancer prevention (Kosmider and Osiecka 2004). One of the main functional components in bamboo leaves extraction was reported to be flavonoids (Lu et al. 2005; Zhang et al. 2005). The ability to rapidly identify the complicated and unpredictable flavonoid compounds from bamboo leaves should be very helpful for the development of bamboo-based functional foods.

It has been well acknowledged that for natural plants as foods and medicinal applications, the identification of their chemical components is of great significance to their quality control and to reveal the secrets underlying their effectiveness. Accordingly, qualitative and quantitative determinations of components contained in natural plants have now become a very hot issue. However, the rapid and reliable identification of chemical components contained in natural plants remains still a great challenge, despite recent advances in various analytical technologies. Although the previously reported methodologies could successfully identify up to dozens of components from natural plants products (Wang et al. 2004; Chen et al. 2005; Zhu et al. 2007), most of them have been limited to target components and depended largely on the use of reference compounds and/or the comparisons with the literature data. Considering that the reference compounds are always difficult to obtain and most components contained in natural plants are unknown, the previously reported methods are apparently insufficient for the global detection and identification of the complicated components in natural plants products. Therefore, developing more powerful analytical tools and methods for the characterizations of the chemical constituents in natural plants (especially in bamboo leaves) is of great concern.

The coupling of High-Performance Liquid Chromatography (HPLC) and Electrospray-Ionization Mass Spectrometry (ESI/MS) combines efficiently the separation capability of HPLC and the great power of structural characterization of Mass Spectrometry (MS). It provides a powerful approach to characterize and identify a variety of polar and thermally labile compounds such as flavonoids in crude extracts of plants (Wang et al. 2004; Schutz and others 2005; Tai et al. 2006; Guo et al. 2007; Ren et al. 2007; Zheng et al. 2007; Avul et al. 2008; Liu et al. 2008). Various hyphenated and hybrid mass spectrometers have now become widely adopted, and, among them, Time-Of-Flight (TOF) MS and its hybrid or in combination use with Tandem Mass Spectrometry (MS/MS) have proven to be very powerful tools for the structural characterization of natural compounds in view of their complementary capacity to provide multistage fragmentations and accurate mass measurements for precise elemental compositions of molecular ions and subsequent product ions (Schutz et al. 2005; Ferrer et al. 2004; Yokosuka et al. 2006; Zhu et al. 2007). Recently, Liquid Chromatography Electrospray Ionization source in combination with hybrid Ion Trap and high-resolution Time-of-flight Mass Spectrometry (LC-(ESI)-IT-TOF/MS) has been successful in the identification and characterization of compounds in various fields (Li et al. 2007; Gao et al. 2008).

This study was thus aimed at developing a generally applicable approach and methodology for the identification and detection of flavonoid components from bamboo leaves based on LC-(ESI)-IT-TOF/MS analysis and an original developed strategy. Such

a strategy was mainly proposed from an idea that the bamboo leaves flavonoid components' accurate mass can usually be determined, and a certain chemical structure of flavonoid components could be speculated on the basis of components' fragmentation schemes. Following this idea, a strategy involving four procedural steps, including searching for flavonoid components based on ultraviolet spectrum scan, getting the accurate mass of flavonoid components parent ion, retrieving the corresponding formula by software, and speculating as to the chemical structure according to mass spectrum decomposition rules. The prominent advantage of this strategy can be simply described as transforming the complete "nontarget identification" to "semitarget identification".

Such a novel approach has been successfully applied to the identification of flavonoid components in *Dendrocalamopsis oldham* leaves. Using the presently developed approach and methodology, we used the strategy for the first time to identify and detect 13 flavonoid components from *Dendrocalamopsis oldham* leaves.

EXPERIMENTAL

Reagents

Methanol and acetonitrile were all supplied by Fisher (HPLC grade, Fisher Scientific International Inc, New Jersey, USA). Formic acid, analytical grade, was obtained from Beijing Hongxing chemical plant (Beijing, China). Ultra high quality water obtained with a Millipore apparatus (Milli-Q Academic, Molsheim, France) was used throughout the whole trial period. Methanol, acetonitrile, and ultra-high quality water were filtered through a 0.22 μm membrane filter to eliminate particulate impurities. Orientin and vitexin standard samples were bought from National Institutes for Food and Drug Control (Beijing, China).

Bamboo Leaves Material

Fresh bamboo leaves from *Dendrocalamopsis oldham* were collected during the spring season in Nanping City, Fujian Province, China. The leaves samples were sent to Xishuangbanna Tropical Botanical Garden of the Chinese Academy of Science in Yunnan Province for species confirmation.

Preparation of Flavonoid Compounds Extraction from Bamboo Leaves

The fresh bamboo leaves were air-dried, cut into small pieces ranging in size from 0.5 cm to 1.0 cm, and then used to prepare flavonoids of bamboo leaves' extracts. The coarse pieces of bamboo leaves (20 g) were soaked in 200 mL of an aqueous solution of methanol (methanol/water 7:3) for 2 h at room temperature (24 ± 2°C), and refluxed for about 3 h in a round flask. The aqueous solvent was cooled to room temperature and poured out of the round flask, leaving the residue of the bamboo leaves behind. The aqueous solvent was centrifuged at 4000 g for 15 min at room temperature. The supernatant was filtered with a 0.22 μm membrane filter, and a volume of 5 μL supernatant was injected into the LC-(ESI)-IT-TOF/MS for analysis.

LC-(ESI)-IT-TOF/MS Apparatus and Analysis

LC experiments were conducted using a Shimadzu (Kyoto, Japan) HPLC system consisting of an LC-20AB binary pump, an SIL-20AC autosampler, a CTO-20AC column oven, and an SPD-M20A PDA. Chromatographic separation of analytes was achieved using a 4.6 mm×250 mm Dikma Diamonsil C18 analytical column (Dima Co., Ltd., Orlando, FL). The column and autosampler tray temperatures were set at 35°C and 4°C, respectively. The mass detection was carried out using a Shimadzu Ion Trap/Time-Of-Flight hybrid mass spectrometry (IT-TOF/MS) (Shimadzu, Kyoto, Japan), equipped with an electrospray ionization source. In automatic mode, all ions were firstly accumulated in octopole and then rapidly pulsed into IT for MS^n analyses according to the criteria settings. All ions produced were finally introduced into the TOF instrument for accurate mass determinations. Data acquisition and analysis were performed with LC Solution 3.2 software (Shimadzu, Kyoto, Japan), including a Formula Predictor 4.2 software (Shimadzu, Kyoto, Japan) to predict the chemical formula.

A volume of 5 μL bamboo leaves extraction solution was injected into the LC-(ESI)-IT-TOF/MS for analysis. A mobile phase composed of eluent A (0.1% formic acid in in a mixture of 60% acetonitrile and 40% methanol, v/v) and B (0.1% formic acid in water, v/v) with a gradient elution was employed for the separation. The mobile phase was consecutively programmed as follows: an isocratic elution of 75% B for the first 12 min, followed by a linear gradient elution of 75-50% B from 12 to 25 min, 50-0% B from 25 to 35 min, and 0-75% B from 35 to 40 min. After holding the solvent composition of 75% B for the next 5 min, the column was returned to its starting conditions. The flow-rate was 0.8 mL/min with a post-column splitting into two flows, one for UV detection which was set at 340 nm and the other for MS detection.

The optimized MS conditions were as follows: negative ion mode; electrospray voltage, -3.5 kV; CDL temperature, 200°C; Block Heater temperature, 200°C; nebulizing gas (N_2), 1.5 L/min; and drying gas (N_2) pressure, 0.1 MPa. Mass spectra were acquired in the range of m/z 200 to 1500 for MS^1, 100 to 1500 for MS^2, and 50 to 1500 for MS^3. The MS^n data were collected in an automatic mode, and the software could automatically select precursor ions for MS^n analysis according to criteria settings (such as ion intensity). Argon was used as the collision gas and the collision energy was set at 50% both for MS^2 and MS^3. Prior to data acquisition, the instrument was calibrated with sodium trifluoroacetic acid clusters against the entire mass range (m/z 50 to 5000) specified for the instrument.

Peak Selections and Data Processing

It has been found in the preliminary study that the peaks with intensity below 100,000 gave few fragments; therefore, only the peaks detected with intensity over 100,000 were selected for identifications. The chemical formulae for all parent and fragment ions of the selected peaks were calculated from the accurate mass using a formula predictor by setting the parameters as follows: C [0-60], H[0-120], O [0-30], double bond equivalent (DBE)[0-20], and H/C ratio [0-3]. Other elements such as P, S, Cl, and Br were not considered, since they are rarely present in bamboo leaves components.

Strategy for Flavonoid Components Identification

The first step of this strategy is to search for the typical flavonoid components from all experimentally generated peaks in total ion chromatograms (TIC), based on an ultraviolet spectrum scan (200 to 400 nm) from PDA. In the UV wavelength range 240 nm to 400 nm there are two main absorptions in flavonoid components. Band I is 300 nm to 400 nm, whereas Band II is 240 nm to 280 nm (Mabry et al. 1970). Components with the above typical flavonoids UV spectrum characteristics were selected from the TIC peaks for the purpose of determining the parent ion accurate mass. The second step is to determine the accurate mass of those flavonoid components' parent ion. The third step is to retrieve the corresponding formula of the flavonoid component by Formula Predictor 4.2 software. To be sure of the accuracy of predicted formula, the mass difference between the measured molecular ions and the predicted formula was below 5 ppm. The fourth step is to speculate as to the chemical structures based on the flavonoid components' fragmentation schemes and mass spectrum decomposition rules. Once the aglycone type, the glycosyl type, and the glycosyl replace type have been determined, the structure of the flavonoid components can be determined. The procedure of "nontarget identification" to "semitarget identification" has been completed.

Validation of HPLC Method

The standard solutions over the range 6.5 to 200 mg/L of two flavonoid components (orientin and vitexin) that had been identified in bamboo leaves were analyzed, and peak areas of the two flavonoid components were recorded. The regression equations of peak area compared with absolute values of the two flavonoid components were obtained. Linearities were verified in triplicate. LOD were estimated from the signal-to-noise ratios (S/N) of two flavonoid component peaks of at least 3. LOQ were determined from the signal-to-noise ratios (S/N=10).

To investigate the accuracy of the method, five replicates of *Dendrocalamopsis oldham* leaves samples were extracted and analyzed independently within 24 h to evaluate intra-day variation. The sample was extracted and analyzed on each day within a 5-day period to evaluate the inter-day variation.

The data of each repetition were used to obtain RSD. All data were analyzed using the STATISTICA 6.0 software (Stat Soft, Inc., Tulsa, Okla., U.S.A.).

RESULTS AND DISCUSSION

Structural Characterization of Bamboo Leaves Flavonoid Components

The *Dendrocalamopsis oldham* leaves flavonoid components (components 1 to 13) were identified essentially on the basis of the LC-(ESI)-IT-TOF/MS analysis. All relevant data including peak number, retention time, accurate mass of deprotonated molecule ion ($[M-H]^-$), the predicted chemical formula, corresponding mass error, subsequent product ions accurate mass, ions' intensity, and flavonoid component structures are summarized in Table 1.

Table 1. Flavonoids Characterization of Bamboo Leaves Extraction from *Dendrocalamopsis oldhami* by LC-(ESI)-IT-TOF/MS

Peak No.	tR (min)	λmax by HPLC-PDA (nm)	$[M-H]^-$			Formulae	MS^2*	MS^3*	Partial indentification
			Measured (m/z)	Predicted (m/z)	Error (ppm)				
1	11.013	269,344	463.1084	463.1088	-0.86	$C_{18}H_{24}O_{14}$	231.0494(100.00)	137.0489(9.99) 185.0435(100.00) 199.0238(9.07) 203.0404(9.07)	Not indentified
2	13.8620	251sh,270, 304sh,338	609.1460	609.1456	0.66	$C_{27}H_{30}O_{16}$	309.0388(45.03) 357.0620(11.49) 429.0787(100.00) 489.1063(67.40) 369.0679(24.05) 339.0517(6.98)	309.0392(100.00) 351.0477(44.32)	Not indentified
3	14.247	248sh,270, 298sh,334	579.1360	579.1350	1.73	$C_{26}H_{28}O_{15}$	309.0412(14.70) 357.0666(51.51) 429.0815(100.00) 459.0955(57.96)	309.0391(100.00) 351.0478(29.05)	O-pentosyl 8-C-hexosyl luteolin
4	14.483	247sh,270, 299sh,346	579.1358	579.1350	1.38	$C_{26}H_{28}O_{15}$	309.0404(39.52) 357.0607(54.17) 429.0810(100.00) 459.0917(90.74)	309.0408(100.00) 351.0515(39.23)	O-pentosyl 6-C-hexosyl luteolin
5	16.305	244sh,269, 299sh,347	447.0932	447.0927	1.12	$C_{21}H_{20}O_{11}$	327.0492(100.00) 357.0603(82.37) 429.0797(14.81)	284.0374(5.48) 299.0589(100.00)	6-C-glucosyl luteolin (isoorientin)
6	17.010	262sh,289, 298sh,339	447.0918	447.0927	-2.01	$C_{21}H_{20}O_{11}$	327.0499(100.00) 357.0617(20.40) 369.0679(6.84)	284.0347(15.68) 299.0568(100.00)	8-C-glucosyl luteolin (orientin)
7	20.565	249sh,271, 304sh,334	563.1416	563.1401	2.66	$C_{26}H_{28}O_{14}$	311.0563(0.94) 413.0882(100.00) 443.0945(2.37)	293.0454(100.00)	O-pentosyl 8-C-hexosyl apigenin

8	21.395	253sh,270, 294sh,322	431.0974	431.0978	-0.93	$C_{21}H_{20}O_{10}$	283.0650(2.57) 311.0564(100.00) 341.0669(2.57)	191.0306(2.02) 283.0596(100.00)	8-C-Glucosyl apigenin (vitexin)
9	22.373	247sh,269, 296sh,338	431.0984	431.0978	1.39	$C_{21}H_{20}O_{10}$	283.0650(0.80) 311.0564(100.00) 341.0669(26.67)	239.0707(1.85) 283.0600(100.00)	6-C-Glucosyl apigenin (isovitexin)
10	23.495	300sh,342	447.0918	447.0927	-2.01	$C_{21}H_{20}O_{11}$	285.0395(100.00)	133.0309(8.93) 151.0063(41.02) 175.0409(100.00) 199.0415(71.36)	O-hexosyl luteolin
11	25.653	250sh,268, 297sh,333	577.1578	577.1557	3.64	$C_{27}H_{30}O_{14}$	269.0438(100.00)	117.0367(100.00) 159.0444(31.12) 197.0633(42.90)	O-deoxyhexose-hexosyl apigenin
12	26.283	268sh,274,	577.2493	577.2496	-0.52	$C_{26}H_{42}O_{14}$	531.2396(100.00) 341.1109(31.60)	——	Not indentified
13	27.973	301sh,328	491.1192	491.0090	0.41	$C_{23}H_{24}O_{12}$	329.0666(100.00) 476.0952(31.55)	314.0465(100.00)	O-hexosyl tricin

sh: shoulder.
*The base peak of MS^2 was the precursor ions of MS^3.

On-line UV spectra of the compounds are typical of flavonoids according to Mabry et al. (1970). In fact, these compounds exhibited two major absorption bands in the UV region, band I absorption occurring in the 330 to 351 nm range and band II in the 254 to 272 nm range. For predicting the chemical formula, a generally acceptable mass error at 5 ppm was considered for all peaks. Data from Table 1 shows that the mass differences between the measured molecular ions and the predicted ones were all below 5 ppm. The HPLC-PDA profile of the *Dendrocalamopsis oldham* leaves extraction recorded at 340 nm is displayed in Fig. 1.

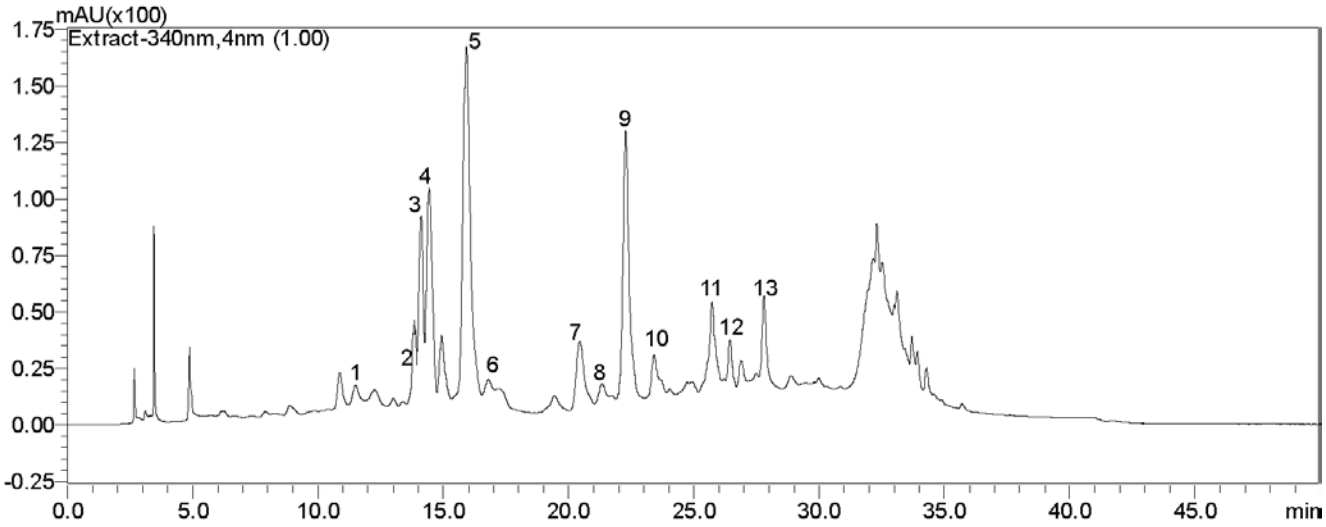

Fig. 1. HPLC-PDA profile of the bamboo leaves extraction from *Dendrocalamopsis oldham* leaves recorded at 340 nm

In Table 1, all deprotonated molecule ions were subjected to up to MS^3 fragmentations. Although this instrument possesses the capacity of performing up to MS^{10} fragmentations, we conducted only up to MS^3 fragmentations in view that the obtained information is sufficient for structural characterization, whereas fragmentation over MS^3 is extremely time-consuming for multiple component analysis.

Mono-C-glycosylflavones

The compounds with the substitution pattern mono-C-glycosylflavones were identified according to the fragmentation proposed by Ferreres et al. (2003). There were four kinds of mono-C-glycosylflavones, as shown in Table 1. Compound 5 exhibited a typical luteolin derivative UV spectrum and a pseudomolecular ion at m/z 447 $[(M-H)]^-$. The typical fragmentation pattern of a C-glycoside, representing the major of fragments, was observed at m/z 357 $[(M-H)-90]^-$ and 327 $[(M-H)-120]^-$. The presence of the ion at m/z 327 $[(M-H)-120]^-$ and the absence of the fragment $[(M-H)-60]^-$ indicated a hexose as the sugar of the C-glycosylation. The higher abundance of fragment at m/z 357 $[(M-H)^- 90]^-$ suggested that the mono-C-glycosylation should be in position 6. Regarding glucose as the most representative of hexose in nature, compound 5 was identified as 6-C-glucosyl luteolin. Comparison with a standard compound confirmed the identity of that compound as isoorientin.

Like compound 5, compound 6 had a typical luteolin derivative UV spectrum, a pseudomolecular ion, and major fragments. However, the ion intensity at m/z 357 [(M-H)-90]$^-$ of compound 6 (20.40) was only one-fourth that of compound 5 (82.37). In mono-C-glycosylflavones, the presence of the sugar units commonly linked at positions 6 and 8, the fragmentation being for the C-6 sugar residue was more extensive than at position 8 (Ferreres et al. 2003). Consequently, the compound 6 could be related to 8-C-glucosyl luteolin (orientin).

Compounds 8 and 9 are both apigenin derivatives because they exhibit characteristic UV spectra. MS analysis showed a pseudomolecular ion at m/z 431 [M-H]$^-$ and an MS^2 fragmentation pattern of an mono-C-glycoside. The fragments at m/z 341[(M-H)-90]$^-$ and 311[(M-H)-120]$^-$ indicated the presence of a C-glucosyl unit. However, the peak intensity of 341 [(M-H)-90]$^-$ in compound 9 was as much ten times as great as compound 8. The abundance of the fragments in compound 9 related to the glucosyl unit suggested a 6-C-glucosylation. Therefore, compound 9 had the structure of a 6-C-glucosyl apigenin (isovitexin). Meanwhile, comparison with a standard compound showed that compound 8 could be related to a 8-C-glucosyl apigenin (vitexin).

O,C-diglycosylflavones

In an O,C-diglycosides moiety the aglycone ion is not detected. Only the precursor [M-H]- fragments, as well as the ion of the interglycosidic linkage cleavage, occur (Cuyckens and Claeys 2004). The mass spectra were essentially interpreted according to the fragmentation scheme proposed in Fig. 2 (Figueirinha et al. 2008).

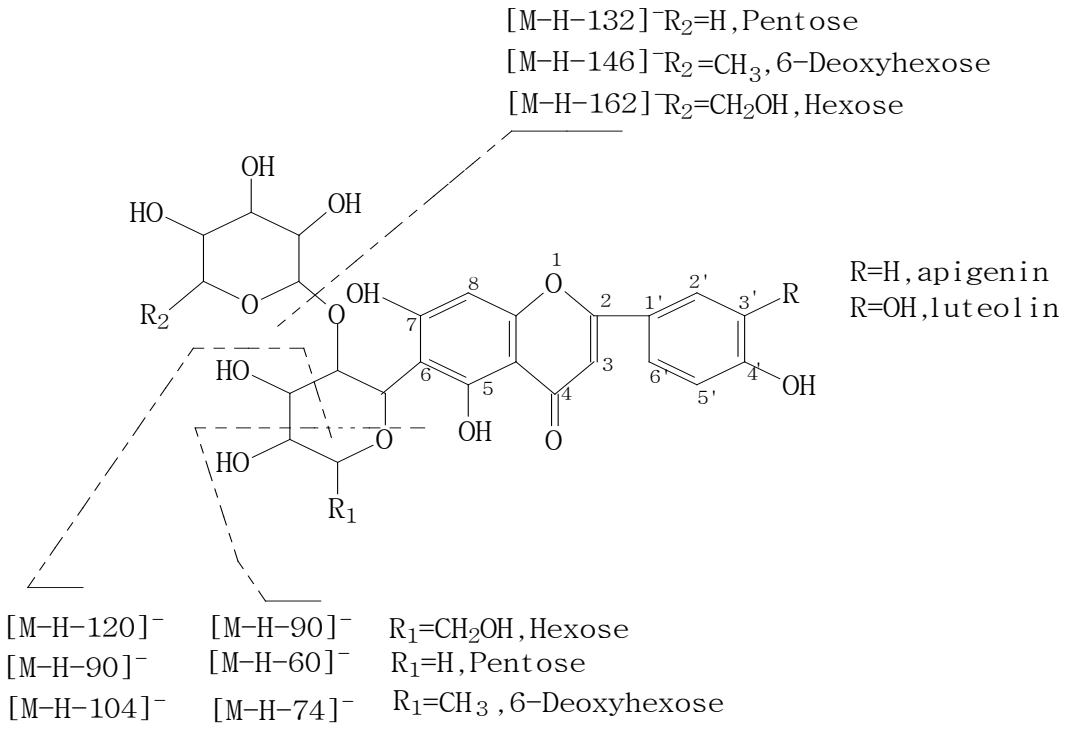

Fig. 2. Fragmentation pattern of the sugar units of O,C-diglycosylflavones

Compound 3 presented the same typical luteolin derivative UV spectrum, a pseudomolecular ion, and major fragments, as compound 4. Both of them that had a pseudomolecular ion at m/z 579 $[M-H]^-$, revealing a luteolin glycoside with a hexose and pentose. An MS^2 fragmentation pattern typical of the O,C-diglycosylflavones, together with the presence of ion at m/z 459 $[(M-H)-120]^-$, reflected the existence of a hexose directly linked to the aglycone. The presence of ion at m/z 357 $[(M-H)-90-132]^-$, resulting from the loss of a pentose unit by the cleavage of a interglycosidic linkage, was also verified. This suggested an O-pentosyl C-hexosyl luteolin structure. A fragment at m/z 459 $[(M-H)-120]^-$ with higher intensity (90.74) suggested that compound 3 was an O-pentosyl 8-C-hexosyl luteolin structure, while compound 4 was an O-pentosyl 6-C-hexosyl luteolin structure (Figueirinha et al. 2008).

Compound 7 showed a pseudomolecular ion at m/z 563 $[M-H]^-$ and a fragmentation pattern typical of the O,C-diglycosylflavones. MS^2 data showed a fragment at m/z 443 $[(M-H)-120]^-$, indicating the presence of a C-hexosyl unit. In the same spectrum a fragment was observed at m/z 311 $[(M-H)-90-132]^-$, corresponding to the fragmentation of a O-pentosyl unit and further proof to the presence of a C-hexosyl unit. The low abundance of fragments at m/z 311 $[(M-H)-90-132]^-$ and m/z 443 $[(M-H)-120]^-$ indicated the presence of a 8-C-hexosyl unit. Thus, the general structure of compound 7 was speculated to be O-pentosyl 8-C-hexosyl apigenin due to the absence of the aglycone (apigenin) ion.

O-glycosylflavones

Compound 10 presented a pseudomolecular ion at m/z 447 $[M-H]^-$, and the MS^2 revealed the loss of one hexose unit and the appearance of the aglycone fragment at m/z 285 $[(M-H)-162]^-$ (luteolin), due to the cleavage at the glycosidic O-linkage. Its general structure should be O-hexosyl luteolin.

Compound 11 showed a pseudomolecular ion at m/z 577 $[M-H]^-$. Moreover, the MS^2, i.e., the aglycone fragment at m/z 269 $[(M-H)-146-162]^-$ (apigenin, base peak), was observed, which implies the loss of the disaccharide deoxyhexose-hexose. This fragmentation pattern is characteristic of O-diglycosides according to Cuyckens and Claeys (2004). Therefore, compound 11 should be the O-deoxyhexose-hexosyl apigenin.

Compound 13 represented a pseudomolecular ion at m/z 491 $[M-H]^-$ and the MS^2 revealed the loss of one hexose unit and the appearance of the aglycone fragment at m/z 329 $[(M-H)-162]^-$ (tricin), due to the cleavage at the glycosidic O-linkage. Moreover, the MS^3 of the fragment observed at m/z 314 $[(M-H)-162-15]^-$ corresponded to the loss of the methyl unit. Thus, compound 13 should be the O-hexosyl tricin.

Summary of flavonoid compounds identified from Dendrocalamopsis oldham leaves

A total of 13 flavonoid components were detected from *Dendrocalamopsis oldham* bamboo leaves extraction by use of LC-(ESI)-IT-TOF/MS, and all were structurally characterized (Table 1 and Fig. 2), except that these methods failed to confirm the structures (peaks 1, 2, and 12). The identified flavonoid compounds can be structurally classified into mono-C-glycosylflavones (4 components), O,C-diglycosylflavones (3 components), and O-glycosylflavones (3 components). All of those flavonoid compounds contained three types of aglycone, that is, luteolin, apigenin, and tricin.

Validation of HPLC Method

Calibration curves were obtained for orientin and vitexin using a series of standard solutions in the concentration range of 6.25, 12.5, 25.0, 50.0, 100, and 200 mg/L. Three replicate injections of standards at each concentration were performed. Calibration curves were linear in the concentration range of 6.5 to 200 mg/L for orientin and vitexin. The regression equations of peak area (x, $\times 10^{-6}$ mv) compared with concentration values of two components (mg/L) were given: y=18.52 x+1.956 (orientin) and y=16.56 x+1.712 (vitexin), having a coefficient of determination (R^2) of 0.9999 and 0.9998, respectively.

The LOD were estimated from the signal-to-noise ratios (S/N) of orientin peak and vitexin peak of at least 3. LOQ were determined from the signal-to-noise ratios (S/N=10). The LOD for these two flavonoid components were less than 0.02 mg/L and LOQ were less 0.06 mg/L.

The intra-day precision was evaluated by performing five replicates of *Dendrocalamopsis oldham* leaves samples, including extraction procedures. The intra-day precision (relative standard deviation) on the basis of peak area was less than 1.04% for orientin and vitexin. The inter-day precision was also evaluated by performing extraction duplicates on each day within a 5-day period. Inter-day precision (relative standard deviation) on the basis of peak area was less than 1.82% for orientin and vitexin.

CONCLUSIONS

1. An approach of database querying by chemical formula with fragmentations analysis has been previously proven to be very useful for the components identification. This study mainly contributes to a novel strategy for rapid and effectively confirming the structure of flavonoid components in bamboo leaves.
2. Taking *Dendrocalamopsis oldham* leaves as a model material, this strategy significantly enhances the efficiency and sharpness for flavonoid components identifications. A success rate over 76% for identifying the flavonoid components indicates that this strategy is powerful and valuable. The wide applicability of this strategy could be further proven by applying it to another species of bamboo leaves.
3. In terms of the unequivocal identification of flavonoid components, the presently developed strategy and methodology still have some limitations. The inherent limitation of LC/MS based methodology is of that it alone can never suffice for the unequivocal identification of flavonoid components. We cannot exclude the possibility of wrong identifications for some components. However, the presently developed strategy and methodology has been well proven to be useful and valuable in this study by successful application to the identification of flavonoid components from *Dendrocalamopsis oldham* leaves. Such limitations will not prevent its wide application into the identifications of flavonoid components from bamboo leaves.

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