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Determination and quantification of active phenolic compounds in pigeon pea leaves and its medicinal product using liquid chromatography–tandem mass spectrometry

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ABSTRACT

A novel method using liquid chromatography coupled to electrospray ionization mass spectrometry (LC–ESI-MS) has been optimized and established for the qualitative and quantitative analysis of ten active phenolic compounds originating from the pigeon pea leaves and a medicinal product thereof (Tongluo Shenggu capsules). In the present study, the chromatographic separation was achieved by means of a HiQ Sil C18 V reversed-phase column with a mobile phase consisting of methanol and 0.1% formic acid aqueous solution. Low-energy collision-induced dissociation tandem mass spectrometry (CID-MS/MS) using the selected reaction monitoring (SRM) analysis was employed for the detection of ten analytes which included six flavonoids, two isoflavonoids and two stilbenes. All calibration curves showed excellent coefficients of determination ($r^2 \geq 0.9937$) within the range of tested concentrations. The intra- and inter-day variations were below 5.36% in terms of relative standard deviation (RSD). The recoveries were 95.08–104.98% with RSDs of 2.06–4.26% for spiked samples of pigeon pea leaves. The method developed was a rapid, efficient and accurate LC–MS/MS method for the detection of phenolic compounds, which can be applied for quality control of pigeon pea leaves and related medicinal products.

1. Introduction

Pigeon pea [*Cajanus cajan* (L.) Millsp.] is a major nontoxic edible herb grain legume present in crops of the semi-tropical and tropical world [1]. Other common names used for pigeon pea are red gram, no-eye pea, Angola pea, Congo pea, Gungo pea, etc. Various medicinal applications have been recorded for this plant for treatment of diabetes in India [2], as febrifuge medication to stabilize the menstrual period, for dysentery in South America [3], and for the treatment of hepatitis and measles in Africa [4]. In China, the young leaves of pigeon pea are chewed for treating aphtha, and the decoction of leaves are used to treat traumatism, burnt infection, bedsore, cough and diarrhoea [5]. Pigeon pea leaves have shown extraordinary therapeutic effects on ischemic necrosis of the femoral head and other bone-related diseases.

Chemical investigations, pharmacological and clinical studies on pigeon pea leaves have demonstrated that phenolic compounds are the major compounds responsible for their beneficial bioactivities [6–9]. Phenolics are compounds containing one or more aromatic rings with one or more hydroxyl groups. They are a widespread family of phytochemical with diverse biological functions in plants. Apart from their beneficial properties, which have conferred to them a relevant role in the pharmaceutical and nutraceutical industry, the phenolic compounds can be excellent chemical markers for the quality control of medicinal plants and their corresponding products [10]. Flavonoids, isoflavonoids and stilbenes represent the main phenolic compounds found in pigeon pea leaves [11–15].

Previous studies on pigeon pea mainly focused on the structural identification, content analysis and activity assay of flavonoids including apigenin, luteolin, isorhamnetin, vitexin, isovitexin, orientin, pinostrobin and quercetin. Recently, the isoflavonoids and stilbenes in pigeon pea leaves have drawn more and more attention and become a research hot spot. Cajanol is an isoflavanone of exceptionally rare occurrence, which has only been obtained in *Cajanus cajan* (L.) Millsp., *Stizolobium deeringianum*, *Campylotropis hirtella* (Franch.) Schindl. and several other species. Cajanol has antifungal and antioxidant activities; it also exhibits inhibitory activity on prostate specific antigen secretion in LNCaP cells [16]. Genistin (a well-known isoflavone glucoside) is an inhibitor of protein tyrosine kinase and DNA topoisomerase, which has some role as a chemopreventive agent against cancer in humans [17]. The two

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stilbenes cajaninstilbene acid and longistylin C have been reported to possess estrogenic, hypoglycemic, hypotriglyceridemic, hypocholesterolemic activities and potential effect in the treatment of postmenopausal osteoporosis [7–9].

Quality control analysis of the active components is an important issue for the safe and effective use of herbal medicines and their preparations. However, this goal remains as an analytical challenge because of the diversity of the chemical compounds present in these complex herbal matrices. Different analytical techniques have been employed for the analysis of constituents in pigeon pea leaves. These include thin layer chromatography (TLC), gas chromatography (GC) [5], high-performance liquid chromatography-ultraviolet detector (HPLC-UV) [18–21] and liquid chromatography–mass spectrometry (LC–MS) [22,23]. However, these methods are insufficient, since they are used for the analysis of only few classes of essential oil, flavonoids and stilbenes. LC separation with detection by collision-induced dissociation tandem mass spectrometry (CID-MS/MS) using a triple quadrupole (TQ) instrument in the multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) mode presents excellent sensitivity and selectivity for the quantification of target compounds in plants or biological matrices [24].

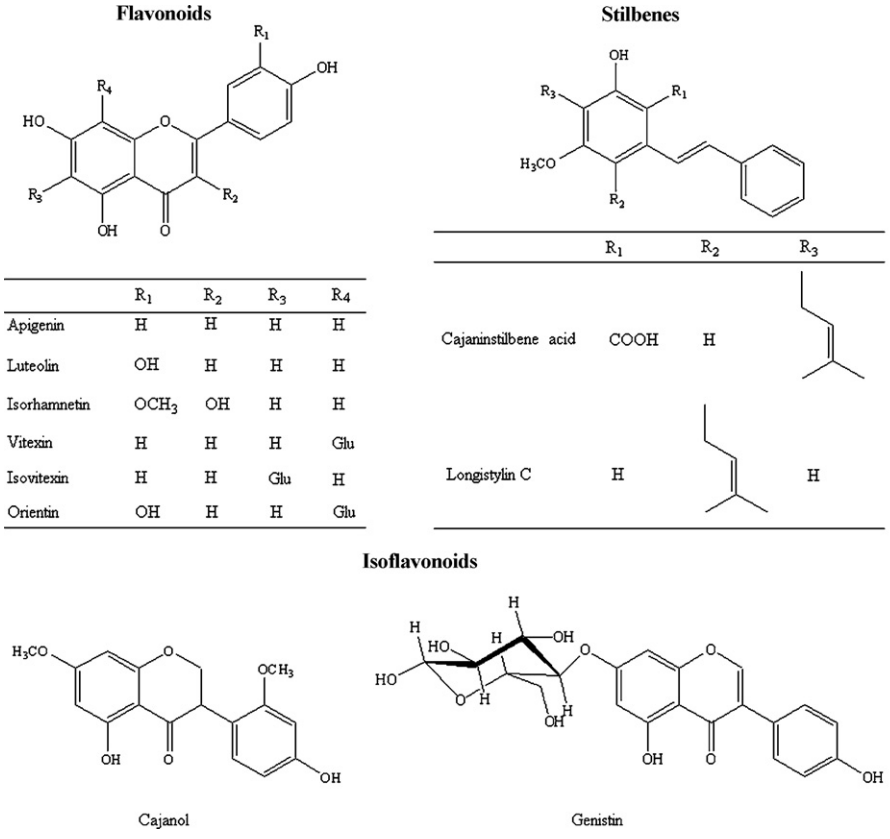
The aim of the present study is to establish a highly sensitive and accurate method using LC–MS/MS for simultaneous qualitative and quantitative analysis of ten active phenolic compounds including six flavonoids (apigenin, luteolin, isorhamnetin, vitexin, isovitexin and orientin), two isoflavonoids (cajanol and genistin) and two stilbenes (cajaninstilbene acid and longistylin C), as well as for quality control of pigeon pea leaves and its medicinal products. Among the analytes, vitexin, isovitexin and genistin represent three isobaric compounds; the isomeric isorhamnetin and cajanol have the same molecular weight and extremely similar structures making their separation a difficult task. As far as we know, there has been no study which provides more comprehensive information on the determination and quantification of flavonoids, isoflavonoids and stilbenes in pigeon pea leaves and its medicinal products.

2. Experimental

2.1. Chemicals and reagents

Apigenin (4′,5,7-trihydroxyflavone, ≥95%), luteolin (3′,4′,5,7-tetrahydroxyflavone, ≥98%), isorhamnetin (3′-methoxy-3,4′,5,7-tetrahydroxyflavone, ≥95%) and genistin (4′,5,7-trihydroxyisoflavone-7-glucoside, ≥95%) were bought from Sigma–Aldrich (Steinheim, Germany). Vitexin (4′,5,7-trihydroxyflavone-8-glucoside, ≥96%) was purchased from Fluka (Buchs, Switzerland). Isovitexin (4′,5,7-trihydroxyflavone-6-glucoside, ≥98%) was obtained from Extrasynthese (Lyon, France). Orientin (3′,4′,5,7-tetrahydroxyflavone-8-glucoside, ≥98%), cajanol (2′,7-dimethoxy-4′,5-dihydroxyisoflavanone, ≥95%), cajaninstilbene acid (3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid, ≥98%) and longistylin C (3-hydroxy-6-prenyl-5-methoxystilbene, ≥95%) were separated and purified in our laboratory. Their structures were elucidated by spectroscopic methods (UV, IR, MS, ^{1}H NMR and ^{13}C NMR) [11–14,25,26] and the structural formulas were shown in Fig. 1.

Methanol of HPLC grade was purchased from J&K Chemical Ltd. (Beijing, China). Formic acid of HPLC grade was purchased from Dima Technology Inc. (Muskegon, MI, USA). Other solvents were analytical grade from Tianjin Chemical Reagents Co. (Tianjin, China). Deionized water was purified by a Milli-Q Water Purification system (Millipore, MA, USA). All solutions and samples prepared for LC–MS were filtered through 0.45 μm nylon membranes (Millipore, MA, USA) prior to use.



	R_1	R_2	R_3	R_4
Apigenin	H	H	H	H
Luteolin	OH	H	H	H
Isorhamnetin	OCH_3	OH	H	H
Vitexin	H	H	H	Glu
Isovitexin	H	H	Glu	H
Orientin	OH	H	H	Glu

	R_1	R_2	R_3
Cajaninstilbene acid	COOH	H	
Longistylin C	H		H

Fig. 1. Chemical structures of the studied phenolic compounds. Glu: glucose.

Table 1
Mass spectrometric parameters for ten phenolic compounds.

Analyte	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)	MRM (amu)
Apigenin	−150	−47	−5	269.0 → 117.0
Luteolin	−20	−50	−6	284.8 → 133.0
Isorhamnetin	−50	−45	−13	315.1 → 151.0
Vitexin	−30	−49	−6	431.0 → 283.0
Isovitexin	−30	−49	−6	431.0 → 283.0
Orientin	−60	−28	−8	447.3 → 327.0
Cajanol	−46	−21	−9	315.1 → 178.9
Genistin	−105	−39	−6	431.3 → 268.0
Cajaninstilbene acid	−60	−25	−7	337.4 → 293.0
Longistylin C	−90	−32	−5	293.3 → 235.0

2.2. Preparation of standard solutions

Standard stock solutions of 10 accurately weighed reference compounds were directly prepared in methanol. The concentrations of apigenin, luteolin, isorhamnetin, vitexin, isovitexin, orientin, cajanol, genistin, cajaninstilbene acid and longistylin C in the standard stock solutions were 20, 20, 20, 200, 50, 500, 20, 20, 500 and 250 μg/mL, respectively. Working standard solutions containing each of the 10 phenolic compounds were prepared by diluting the stock solutions with methanol to a series of proper concentrations. The standard stock solution and working standard solutions were all stored at 4 °C.

2.3. Materials

Pigeon pea leaves were collected during the autumn season in Hainan Province, China, and authenticated by Prof. Shaoquan Nie from the same laboratory. A voucher specimen (No. 052056001001001) was deposited in the herbarium of this laboratory. The samples were placed in shade and dried to constant weight. The dried leaves were pulverized into a homogeneous size by a disintegrator (HX-200A, Yongkang Hardware and Medical Instrument Plant, China), sieved (40 mesh) and stored in sealed plastic bags.

Tongluo Shenggu capsules (Batch No. 080401) were supplied by Zhejiang Hisun Pharmaceutical Co., Ltd. (Taizhou, China). After removal of the capsules, the contents were powdered and passed through a No. 40 mesh.

2.4. Sample preparation

The dried powder of pigeon pea leaves (5.00 g) was macerated with 100 mL of methanol overnight and then sonicated in an ultrasonic bath (Kunshan Ultrasonic Instrument, China) for 30 min. The mixture was vacuum filtered; the obtained residue was sonicated with 50 mL of methanol again for 30 min.

After extraction, the supernatant extracts were combined and condensed by removing the methanol solvent in a rotary evaporator (RE-52AA, Shanghai Huxi Instrument Co., China) under vacuum at 55 °C. The obtained dry extracts were diluted in methanol (2 mL, HPLC grade). The sample was further diluted and filtered through a 0.45 μm membrane before injecting into LC–MS/MS for analysis.

The powder of Tongluo Shenggu capsules (5.00 g) was accurately weighed and introduced into a flask followed by adding 100 mL of methanol. The sample was extracted and prepared using the method described above for pigeon pea leaves and then analyzed by LC–MS/MS.

2.5. HPLC and MS conditions

Chromatographic analyses were performed by using an Agilent 1100 series HPLC system (Agilent, San Jose, CA, USA), consisting of a G1312A binary pump, a 7725i manual injector and a G1379A degasser. Separation of the analytes was achieved on a HiQ Sil C18 V reversed-phase column (250 mm × 4.6 mm I.D., 5 μm, Kya Tech, Hachioji City, Japan) connected with an Analytical KJ0-4282 C18 guard cartridge system for HPLC (Phenomenex, Torrance, CA, USA). The mobile phase consisted of methanol (A) and 0.1% formic acid aqueous solution (B) using the following gradient elution program for separation: 0–6 min, 47% (A); 6–15 min, 47–9% (A); 15–25 min, 9% (A); 25–30 min, 9–47% (A). The column temperature was maintained at 30 °C, the flow rate was 0.7 mL/min and the injection volume was 10 μL.

Mass spectra were acquired with an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada), equipped with an electrospray ionization (ESI) source operating in the negative-ion mode. The ESI-MS of ten phenolic compounds was recorded using direct infusion of each reference compound. The conditions used for TurboIonSpray interface were nebulizing gas (NEB), curtain gas (CUR) and collision gas (CAD) 12, 10 and 6 a.u. (arbitrary units); dwell time 1.5 s; ion spray voltage −4500 V; the ion source temperature 300 °C; focusing potential (FP) and entrance potential (EP) −75 and −10 V, respectively. The other parameters for CID-MS/MS analyses of the ten phenolic compounds including declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were acquired and summarized in Table 1. Analyst Software (version 1.4) installed on a DELL computer was used for data acquisition.

2.6. Validation study

The linear range, limit of detection (LOD), limit of quantification (LOQ), precision and recovery were studied for the developed method. The linearity of calibration curve was tested by analysis of individual reference compound at eight concentrations. LOD and LOQ for each analyte were evaluated at signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively.

Intra-day and inter-day variations were chosen to determine the precision of the developed method. One gram of the pulverized samples of pigeon pea leaves was accurately weighed and then extracted and analyzed as described in Sections 2.4 and 2.5. For the intra-day variability test, the samples were analyzed in triplicate five times within one day, while for the inter-day variability test, the samples were examined in triplicate on three consecutive days. The RSDs for the retention time and peak area were calculated as measures of precision.

Recovery was determined using the spiked samples with the pigeon pea leaves matrix. A portion of 1.00 g of the pulverized pigeon pea leaves matrix was individually spiked with certain amount of reference compound of apigenin, luteolin, isorhamnetin, vitexin, isovitexin, orientin, cajanol, genistin, cajaninstilbene acid and longistylin C, respectively. Three replicated samples were extracted and analyzed with the same procedures as described in Sections 2.4 and 2.5 for evaluating the accuracy.

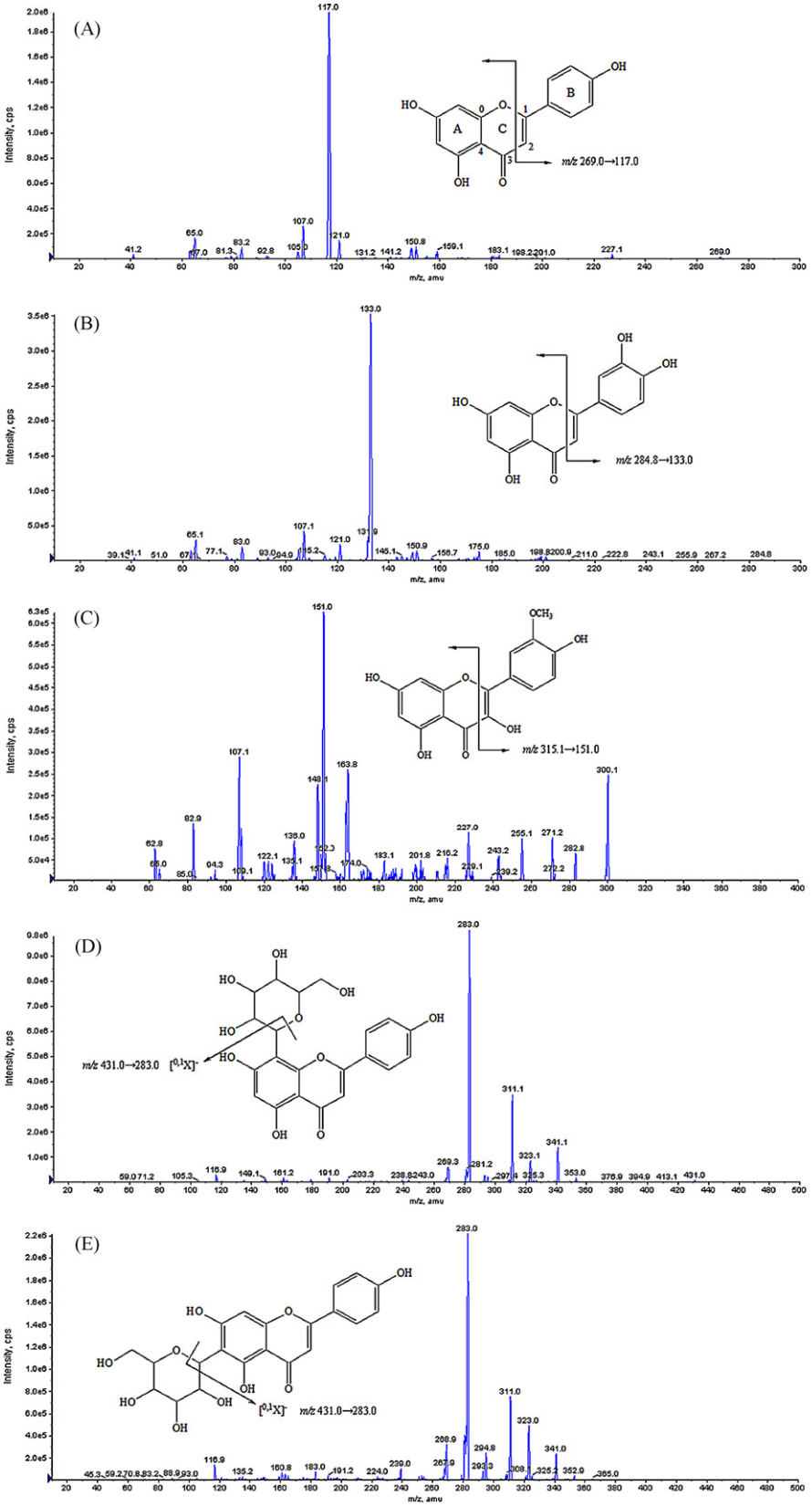


Fig. 2. Product ion mass spectra of $[M-H]^-$ ions of apigenin (A), luteolin (B), isorhamnetin (C), vitexin (D), isovitexin (E), orientin (F), cajanol (G), genistin (H), cajaninstilbene acid (I) and longistylin C (J).

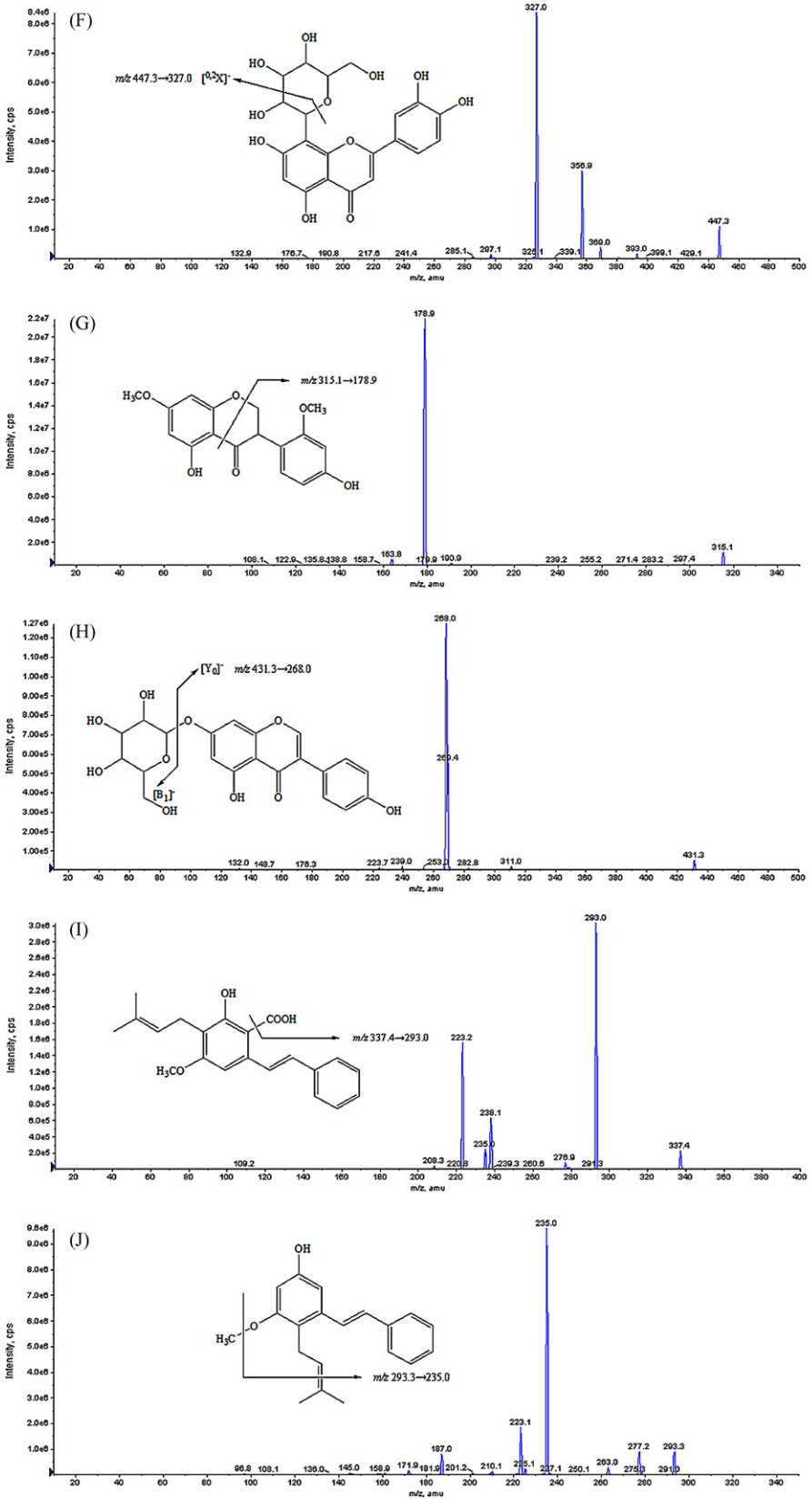


Fig. 2. (*Continued*).

3. Results and discussion

3.1. Optimization of chromatographic separation for phenolic compounds

It is well established that for the separation of phenolic compounds, choice of mobile phase and an appropriate elution program is critical. Because the properties of the mobile phase primarily govern the separation of the analytes and the degree of analyte ionization, optimizing the mobile phase composition is important in LC–ESI-MS analysis.

In our previous study, vitexin and isovitexin were well separated in methanol–water system [22]. It also showed a satisfactory separation for cajaninstilbene acid and longistylin C in other studies [7]. In the separation of flavonoid aglycones such as apigenin, luteolin and isorhamnetin, both methanol–water system [27] and acetonitrile–water system [28] have been successfully applied in the literature. In the present study, methanol was used as the organic phase in the mobile phase considering its better dissolvability to the phenolic compounds and its lower price. The HPLC C18 column using methanol–water system was effective for the separation of samples of pigeon pea leaves and its medicinal product. Formic acid (0.1%) was used to improve the chromatographic behavior and to reduce the peak tailing. Furthermore, comparisons between isocratic elution and gradient elution showed that the latter possessed a distinct advantage for separating these ten phenolic compounds and a high sensitivity for determination by using MS/MS.

Finally, a solvent system consisting of methanol and 0.1% formic acid aqueous solution, which provides greater baseline stability and higher ionization efficiency, was ultimately selected as mobile phase system using gradient elution. Under the optimized LC conditions, retention times of ten phenolic compounds were obtained from multiple injections of intra- and inter-day during the entire method development and sample analysis time period. Orientin, genistin, vitexin, isovitexin, luteolin, apigenin, isorhamnetin, cajanol, cajaninstilbene acid and longistylin C had the retention times of 4.59, 5.79, 6.48, 6.89, 12.96, 13.39, 13.69, 13.86, 17.58 and 19.93 min, respectively. Certain tendencies in the elution order were observed. More polar phenolic compounds elute at the beginning of the chromatogram, showing the following order: flavone (isoflavone) glucosides, flavones, flavonols, isoflavanones and stilbenes. Within the same phenolic class: (a) the retention time decreases as more hydroxyl groups have the phenolic owing to increasing polarity, (b) if the phenolic contains apolar substituents, such as methoxy groups, the retention time increases, and (c) if the chemical structures of phenolic compounds include sugars, the phenolics elute before their corresponding aglycones [10].

3.2. Mass spectra of compounds under study

Recently, the ESI-MS using a triple quadrupole mass spectrometer has shown to be a very powerful instrument for the simultaneous quantification of analytes with high selectivity and sensitivity. Triple quadrupole instruments are used exceptionally well for collision-induced dissociation tandem mass spectrometry (CID-MS/MS) analyses. This low-energy CID-MS/MS analysis is usually accomplished by separating the precursor ion (mass selection) in the first quadrupole (MS1). This is followed by low-energy collision-induced dissociation that promotes the fragmentation of the precursor ion by collision with gas atoms in the radio frequency (RF)-only collision cell. Finally, scanning the product ions in the third quadrupole occurs (MS2). The selected reaction monitoring (SRM) assay is accomplished by selecting a specific precursor → product ion transition. Therefore, the CID-MS/MS analysis provides a very specific and sensitive response for the selected analyte, which can be used to detect and integrate a peak for the target compound in the sample under a simple one-dimensional chromatographic separation. In principle, this MS-based approach can provide very high available specificity for the structural identification of analyte and quantification of analyte concentration [29,30].

In the present study, ESI-MS analyses of ten phenolic compounds were performed in both the positive-ion and negative-ion modes. In order to obtain MS-based information for simultaneous evaluation of ten phenolic compounds, direct flow injection experiments of the reference compounds were initially performed. The full-scan spectra were acquired over an m/z range of 10–1000 amu, dwell time of 1.5 s and step size of 0.1 amu. In virtue of the spectral pattern of ten phenolic compounds and the background noise coming from the real sample matrix, detection signals with negative mode were better than those in positive mode in terms of selectivity and sensitivity. Thus, negative-ion mode was finally selected to detect these ten phenolic compounds.

Weak organic acids, such as formic acid and acetic acid, are often added to the mobile phase when positive-ion ESI is performed. It is commonly accepted that the presence of the acid facilitates protonation of analytes with basic functional groups in positive-ion mode [31]. Therefore, it is reasonable to assume that the addition of a base would facilitate the deprotonation of analytes in negative-ion mode. However, earlier studies of ESI-MS in negative-ion mode showed that volatile bases, such as ammonium hydroxide, resulted in a poor detection limit and less stability in methanolic or aqueous solutions [31]. Whereas weak acids improved the negative-ion ESI responses of the analytes at low concentrations [32]. In the present study, significant increases in negative-ion ESI responses of phenolic compounds were observed using the employed mobile phase containing 0.1% formic acid. Such signal enhancement could be due to the higher gas-phase proton affinity and small molecular volume of formic acid molecules, both factors are known to be beneficial to the ESI process [32]. This finding is consistent with some other studies, which also showed that formic acid at low concentrations were effective in enhancing negative-ion ESI responses of analytes [33,34].

To optimize the SRM assay for each analyte, standard solutions of ten phenolic compounds in methanol were infused into the ESI source with a syringe pump at a flow rate of 0.5 μL/min. It has been demonstrated that optimization of the ESI parameters plays a key role in the achievement of adequate MS signals for any analyte. The effects of ESI parameters including declustering potential voltage, focusing potential voltage and collision energy on signal intensity of analytes were respectively investigated. In general terms, the higher the declustering potential and focusing potential voltages, the greater the energy imparted to the ions entering the analyzing region of the mass spectrometer. The energy helps to decluster the ions and to reduce the chemical noise in the spectrum resulting in an increase in sensitivity. Increasing the voltages beyond optimal conditions can induce fragmentation before the ions enter the mass filters resulting in a decrease in sensitivity. Moreover, the optimal collision energy is selected based on the charge state and mass of the precursor ion. It is manually optimized so as to obtain maximum abundances for the primary fragment ions and give the most informative fragmentation spectrum.

In was found the ESI-MS spectra of the ten phenolic compounds were dominated by the presence of the $[M-H]^-$ deprotonated molecule in the negative mode. The product ion scans of the ten phenolic compounds are shown in Fig. 2. The CID-MS/MS analysis of apigenin produced a product ion $[M-H-C_7H_4O_4]^-$ at m/z 117.0 arised from the cleavage of the *C*-ring linkage in position 1/3 (Fig. 2A). Thus, the SRM transition m/z 269.0 → 117.0 was selected for the identification and quantification of apigenin. Likewise, the SRM mass transitions at m/z 284.8 → 133.0, m/z 315.1 → 151.0, m/z 431.0 → 283.0,

Table 2
Regression data, LODs and LOQs for ten phenolic compounds.

Analyte	Calibration curve[a]	r^2	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Apigenin	$y = 673.5x + 41.4$	0.9974	20–200	1.45	5.87
Luteolin	$y = 764.8x - 124.2$	0.9938	20–200	1.24	4.36
Isorhamnetin	$y = 215.3x + 82.9$	0.9969	20–200	1.69	6.28
Vitexin	$y = 417.5x - 70.8$	0.9955	200–2000	0.89	3.93
Isovitexin	$y = 599.1x + 251.7$	0.9937	50–500	0.86	3.75
Orientin	$y = 378.3x + 93.0$	0.9941	500–5000	1.32	5.91
Cajanol	$y = 489.2x - 56.5$	0.9989	20–200	1.47	5.62
Genistin	$y = 453.6x + 37.9$	0.9973	20–200	0.94	4.59
Cajaninstilbene acid	$y = 533.6x + 157.3$	0.9962	1000–5000	1.38	6.94
Longistylin C	$y = 497.1x - 184.6$	0.9958	500–2500	1.62	7.03

[a] y: peak area of analyte; x: concentration of analyte (ng/mL).

m/z 431.0 → 283.0, *m/z* 447.3 → 327.0, *m/z* 315.1 → 178.9, *m/z* 431.3 → 268.0, *m/z* 337.4 → 293.0 and *m/z* 293.3 → 235.0 were selected for the monitoring of luteolin, isorhamnetin, vitexin, isovitexin, orientin, cajanol, genistin, cajaninstilbene acid and longistylin C (Fig. 2B–J), respectively.

The nomenclature, proposed by Domon and Costello for glycoconjugates [35,36], was adopted to denote the fragment ions for flavone (isoflavone) glycosides (Fig. 2D–F and H). Ions containing the aglycone are labeled ${}^{k,l}X_j$, Y_j and Z_j, where j is the number of the interglycosidic bond broken, counting from the aglycone, and the superscripts k and l indicate the cleavages within the carbohydrate rings. The glycosidic bond linking the glycan part to the aglycone is numbered 0. When the charge is retained on the carbohydrate residue, fragments are designated ${}^{k,l}A_i$ and B_i where i (≥ 1) represents the number of the glycosidic bond cleaved, counting from the non-reducing end [10]. For the *O*-glycoside genistin, we have found that the CID-MS/MS analysis was affected with a lower collision energy. Collision energy of −39 V was enough to produce the main product ion $[Y_0]^-$ at *m/z* 268.0 which involved the cleavage at the glycosidic *O*-linkage with a concomitant H-rearrangement leading to the elimination of the monosaccharide residue $[B_1]^-$ ion (Fig. 2H) [10,37]. It is well known by ESI-MS studies in carbohydrate chemistry that the glycosidic bond is weaker than the bonds within the aglycone molecule. Thus, the CID-fragmentation pathway of *O*-glycolylated isoflavonoids typically is initiated by the cleavage of this bond, leading to elimination of the sugar moiety with the charge being retained on the aglycone fragment. In the case of the *C*-glycosides vitexin, isovitexin and orientin, the sugar unit is linked directly to the C6–C3–C6 aglycone skeleton through a C–C bond, which is stable towards acid hydrolysis. Therefore, the CID-MS/MS of vitexin, isovitexin and orientin need higher collision energies (−49 and −48 V) to fragment than *O*-glycosides. The main fragmentations take place in the sugar, which has the weakest bonds in the molecule. The main product ions in the ESI(−)–CID-MS/MS spectra were due to cleavages of pyranic sugar ring which implies the breakage of the C1"–O bond: $[{}^{0,1}X]^-$ and $[{}^{0,2}X]^-$ (Fig. 2D–F) [10].

The different SRM transitions for genistin, vitexin and isovitexin in combination with their different retention times allow the distinction of these three isomers. Likewise, the fragmentation pathways are used to differentiate isorhamnetin (flavonol) and cajanol (isoflavanone) although both analytes have the same molecular mass.

3.3. Method validation

Quantification was performed using an external eight-point calibration curve covering the range from 20 to 5000 ng/mL. The peak area was the average value of three replicate injections. All calibration curves exhibited an excellent coefficient of determination ($r^2 \geq 0.9937$) within the range of tested concentrations. The LODs (S/N = 3) and LOQs (S/N = 10) for the analytes were less than 1.62 and 7.03 ng/mL, respectively (Table 2).

The results of the precision test are summarized in Table 3. The intra-day variations of retention time and peak area were less than 0.56% and 3.14%, and the corresponding inter-day variations were less than 0.63% and 5.36%, respectively. Recovery was examined by using spiked samples of the pigeon pea leaves matrix. The recovery was calculated with the following formula: recovery (%) = (amount found − original amount)/amount spiked × 100. Table 4 shows that the recoveries varied between 95.08% and 104.98% and the RSDs were between 2.06% and 4.26% for ten phenolic compounds. The above data indicated the higher accuracy of the present method for the quantification of phenolic compounds.

3.4. Application to real samples

The developed LC–MS/MS method was subsequently applied for the analysis of ten phenolic compounds in samples of pigeon pea leaves and its medicinal product. Representative SRM chromatograms are shown in Fig. 3. Peak identity was established by both the retention time compared to that of reference compounds and the characteristic transitions (precursor and product ion pair). The calibration curves were used for the quantitative determination

Table 3
Precision of ten phenolic compounds for retention time (RT) and peak area (PA) ($n = 3$).

Analyte	Intra-day variations		Inter-day variations	
	RSD for RT (%)	RSD for PA (%)	RSD for RT (%)	RSD for PA (%)
Apigenin	0.35	2.66	0.28	4.53
Luteolin	0.29	2.38	0.36	3.27
Isorhamnetin	0.38	2.95	0.63	5.22
Vitexin	0.39	2.83	0.45	3.92
Isovitexin	0.26	2.62	0.21	3.86
Orientin	0.42	2.41	0.47	4.84
Cajanol	0.37	2.52	0.35	4.16
Genistin	0.25	2.29	0.33	3.57
Cajaninstilbene acid	0.56	3.07	0.49	5.36
Longistylin C	0.48	3.14	0.55	4.98

Table 4
Recovery of ten phenolic compounds ($n = 3$).

Analyte	Original (mg)	Spiked (mg)	Found (mg)	Recovery (%)	RSD (%)
Apigenin	0.143	0.189	0.329	98.41	2.06
		0.402	0.565	104.98	3.95
Luteolin	0.307	0.356	0.668	101.40	2.83
		0.643	0.967	102.64	2.42
Isorhamnetin	0.104	0.117	0.218	97.44	3.67
		0.264	0.355	95.08	3.58
Vitexin	2.613	1.029	3.611	96.99	2.95
		2.295	4.986	103.40	2.62
Isovitexin	0.425	0.398	0.815	97.99	3.81
		0.823	1.219	96.48	2.14
Orientin	4.439	1.052	5.448	95.91	2.89
		2.961	7.518	103.99	3.54
Cajanol	0.518	0.425	0.934	97.88	3.63
		0.874	1.409	101.95	2.86
Genistin	0.064	0.112	0.171	95.54	4.26
		0.204	0.262	97.06	4.01
Cajaninstilbene acid	10.967	2.559	13.629	104.03	2.76
		5.092	16.191	102.59	2.64
Longistylin C	5.035	2.387	7.476	102.26	3.03
		4.401	9.277	96.39	2.98

of ten phenolic compounds. The results of the contents of ten analytes in samples of pigeon pea leaves and Tongluo Shenggu capsules were presented in Table 5. It was found that the contents of ten phenolic compounds in Tongluo Shenggu capsules were distinct from those in pigeon pea leaves (Table 5). The levels of vitexin, isovitexin, orientin and genistin in Tongluo Shenggu capsules were significantly higher than those in extracts of pigeon pea leaves; while the levels of apigenin, luteolin, isorhamnetin, cajanol, cajaninstilbene acid and longistylin C in Tongluo Shenggu capsules were obviously lower. Apart from the different origins and storage conditions of pigeon pea leaves, etc., the manufacturing procedure of Tongluo Shenggu capsules may account for the significant differences in the contents. In the manufacture of Tongluo Shenggu capsules, pigeon pea leaves were extracted with polar solvents. For this reason, the contents of more polar compounds including flavone (isoflavone) glucosides were higher than the contents of less polar aglycones and stilbenes.

In plants, the flavonoids are *O*-glycosidic compounds in which one or more of the hydroxyl groups of the aglycone are glycosidically linked to a sugar residue. Flavonoids can also occur as *C*-glycosides in which the glycosylation assembly involved a direct linkage of the sugar residue to the basic flavonoid aglycone via an acid-resistant C–C bond. As can be seen from Table 5, we noted that the flavonoid *C*-glycosides vitexin (apigenin 8-*C*-glucoside), isovitexin (apigenin 6-*C*-glucoside) and orientin (luteolin 8-*C*-glucoside) were present in high concentration in pigeon pea leaves.

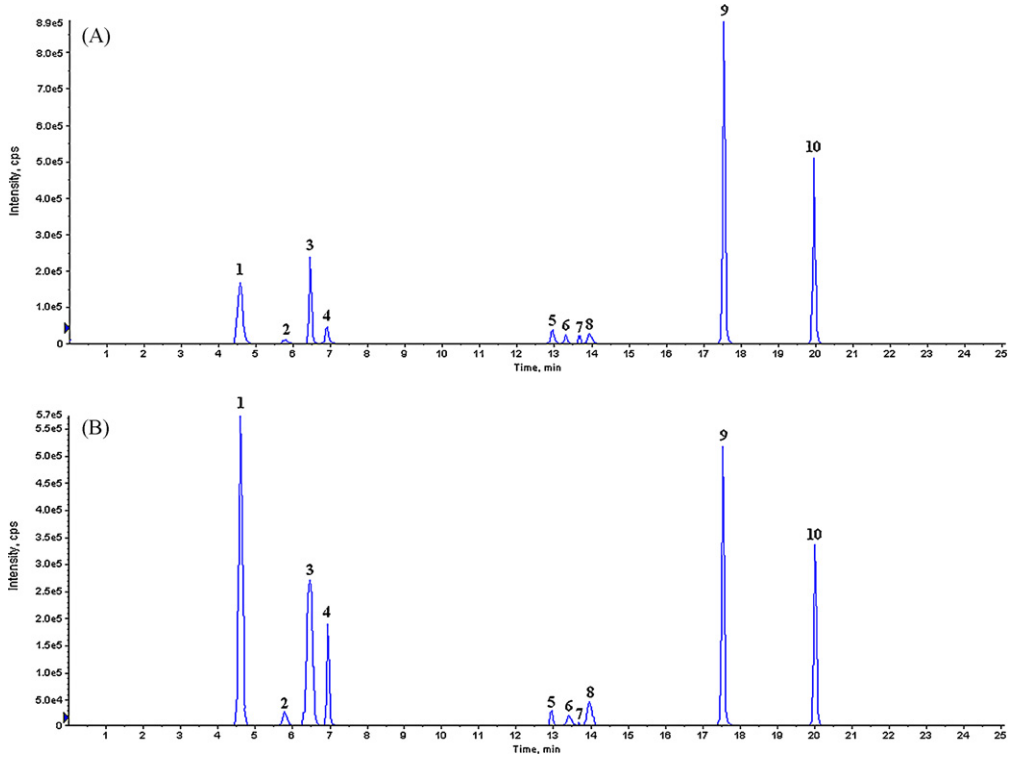


Fig. 3. Representative SRM chromatograms of samples of pigeon pea leaves (A) and Tongluo Shenggu capsules (B). 1: orientin (m/z 447.3 → 327.0); 2: genistin (m/z 431.3 → 268.0); 3: vitexin (m/z 431.0 → 283.0); 4: isovitexin (m/z 431.0 → 283.0); 5: luteolin (m/z 284.8 → 133.0); 6: apigenin (m/z 269.0 → 117.0); 7: isorhamnetin (m/z 315.1 → 151.0); 8: cajanol (m/z 315.1 → 178.9); 9: cajaninstilbene acid (m/z 337.4 → 293.0); 10: longistylin C (m/z 293.3 → 235.0).

Table 5
Contents of ten phenolic compounds in samples of pigeon pea leaves and Tongluo Shenggu capsules ($n = 3$).

Analyte	Pigeon pea leaves		Tongluo Shenggu capsules	
	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)
Apigenin	0.142	2.56	0.074	2.91
Luteolin	0.307	2.82	0.052	2.96
Isothamnetin	0.103	3.93	0.043	3.58
Vitexin	2.607	3.39	4.310	2.61
Isovitexin	0.427	2.95	1.490	3.12
Orientin	4.441	3.07	8.861	2.88
Cajanol	0.519	3.58	0.508	3.14
Genistin	0.064	4.41	0.093	4.02
Cajaninstilbene acid	10.958	2.99	5.061	2.87
Longistylin C	5.038	3.16	3.334	2.95

4. Conclusions

In the present study, an efficient and accurate LC–ESI-MS (negative-ion mode) and CID-MS/MS novel method was developed for the separation and simultaneous quantification of ten active phenolic compounds. The ten analytes were detected with CID-MS/MS analysis using the SRM mode for the efficient and accurate measurements. Therefore, three groups of active components i.e. flavonoids, isoflavonoids and stilbenes were successfully separated and identified in pigeon pea leaves by this LC–MS/MS method for the first time. The method showed a good precision (RSD < 5.36%) and recovery (95.08–104.98%) and was also successfully used for the quantitative determination of active phenolic compounds in samples of pigeon pea leaves and its medicinal product. The developed LC–MS/MS method seems to be a good alternative in terms of both detection levels and structural information and allows quantitative assessment and quality control of pigeon pea leaves and related medicinal products.

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