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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].

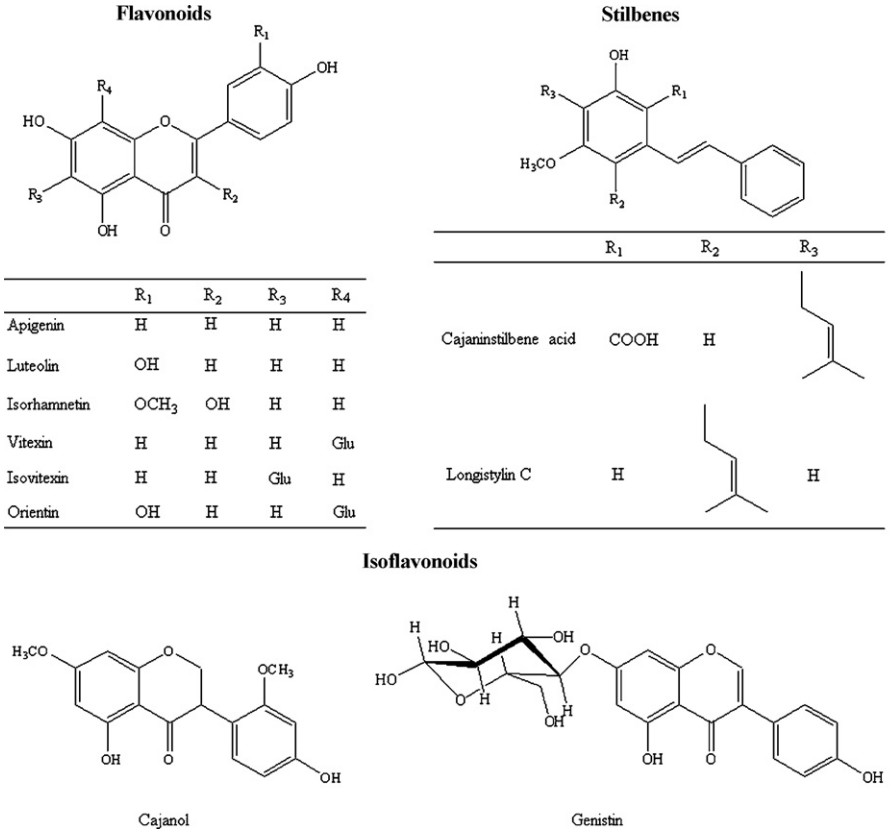
The aim of the present study is to establish a highly sensitive and accurate method using LC–MS/MS for simultaneous qualitative and quantitative analysis of ten active phenolic compounds including six flavonoids (apigenin, luteolin, isorhamnetin, vitexin, isovitexin and orientin), two isoflavonoids (cajanol 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.