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Rapid reversed-phase high performance liquid chromatography for vitexin analysis and fingerprint of *Passiflora foetida*

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Reversed phase high performance liquid chromatography (RP-HPLC) was developed for quantitation of vitexin and for examining the fingerprint of *Passiflora foetida* leaf extract. The simple isocratic condition consisting of isopropanol : tetrahydrofuran : water (5 : 15 : 80, v/v/v) and 0.3% formic acid with a flow rate of 1.3 ml/min was optimized. Under the optimum condition,

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ten major peaks, including a separated peak of vitexin could be resolved within 18 min. Linearity for vitexin was in the range of 25–500 μg/ml, with a correlation coefficient of 0.99. The vitexin contents in *P. foetida* leaf extract collected from ten different locations varied from 0.04 to 0.13% w/w (%RSDs = 1.1–4.0%). Although the amount of the marker flavonoid (vitexin) was different, HPLC fingerprints of all samples were well conserved. The pattern of the chromatograms and the number of the peaks separated were similar. The data could be valuable for taxonomy, breeding and authenticity testing of products containing *P. foetida*.

Keywords: Fingerprint, high performance liquid chromatography, *Passiflora foetida*, vitexin.

PASSION flower plants belong to the genus *Passiflora*, and are inhabitants of the tropical and semi-tropical zones of North, Central and South America[1]. Leaves, aerial parts and flowers of *Passiflora* have been traditionally used for treatment of stress, anxiety, depression, insomnia, cardiac arrthymia and tension-related asthma[2–4]. Most research work has been focused on *Passiflora incarnata* L. (family Passifloriaceae), the first described passion flower[5], which possesses significant CNS depression activity[6]. Anti-anxiety effect of *P. incarnata* extract in mice was investigated and the results showed that benzoflavone nucleus was the basic moiety essential for the bioactivity of the plant extract[4]. For example, the benzoflavone moiety of the plant could suppress alcohol cessation-oriented hyperanxiety in mice[7]. Moreover, indole alkaloids, maltol and flavonoids as well as lyophilized hydroalcoholic and aqueous extracts from the aerial parts of *P. incarnata* were evaluated for their behavioural effects in mice[2]. Additionally, a combination of *Kava kava* and *P. incarnata* extracts showed a decrease in the amphetamine-induced hypermotility and prolongation of sleeping phase in mice[8]. Several methods have been proposed for the analysis of flavonoids and other constituents in *P. incarnata*. For example, high performance liquid chromatography (HPLC)–mass spectrometry (MS) using collision inducing dissociation (CID)[9] and high performance thin layer chromatography (HPTLC)[10] have been employed for the determination and identification of the C-glycosylflavone isomer pairs orientin/isoorientin and vitexin/isovitexin in *P. incarnata*, *P. alata*, *P. edulis* and *P. caerulea*. Liquid chromatography (LC) has been used for the determination of flavonoids from *P. incarnata* and *P. alata* leaves and fluid extract[11]. High-speed extraction in combination with HPLC has been utilized for the analysis of C-flavonoid glycosides in *P. incarnata*[3]. Additionally, reversed phase HPLC (RP-HPLC) has been employed for qualitative and quantitative analysis of C-glycosylflavones in the plant[12]. The stability of passion flower tinctures has been investigated by LC–diode array detection (LC–DAD) and LC–MS[13]. The possible structures of constituents with sedative properties in *P. incarnata* extract have also been characterized by MS[14,15]. Due to its clinical usefulness and well-established scientific information, *P. incarnata* extract is an official drug from medicinal plants in several pharmacopoeias (e.g. British Herbal Pharmacopoeia 1983, Homeopathic Pharmacopoeia 1981, Pharmacopoeia Helvetica 1987, and the pharmacopoeias of Egypt, France, Germany and Switzerland).

P. foetida belongs to the same family as *P. incarnata* and the plants are indigenous to many countries, including Thailand. Leaves of the plant are also utilized as folk medicine for treatment of anti-anxiety, stress and insomnia. In addition, they are useful in the treatment of hysteria, skin inflammation, cough and fever. Chemical constituents in *P. foetida* include hydrocyanic acid, groups of flavonoids and harman alkaloids. However, the active compounds, which are responsible for its pharmacological activities, are not clearly identified. Due to its clinical usefulness and potential uses in drugs and cosmetics, it is important to obtain supportive information for the standardization of the plant extracts. At present, there is no report on the appropriate marker and HPLC fingerprint pattern of *P. foetida*. The objectives of this study were to develop a HPLC procedure for the analysis of a suitable marker and for the fingerprint of the wild *P. foetida* plant collected from various areas of Thailand. The marker can be used for the standardization of leaf extracts and the fingerprint is valuable for investigation of the authenticity of *P. foetida* leaf extracts.

HPLC analyses were performed using a Thermo Separation instrument (Massachusetts, USA) consisting of an isocratic solvent delivery system (model Contrametric 4100), a variable wavelength spectrophotometric detector (model 3200), and a manual Rheodyne injector. Data analysis was performed on Barspec Data Computer System (Massachusetts, USA). The HPLC column (Waters, Massachusetts, USA) was a Spherisorb ODS 5 μm (250 × 4.6 mm) with a Spherisorb ODS 10 μm (100 × 4 mm) as a guard column. Analysis of vitexin in *P. foetida* was investigated in various mobile phase compositions with different flow rates (Table 1). The injection volume was 20 μl and UV detection was monitored at the wavelength of 340 nm.

Vitexin and isovitexin (Figure 1) standard were purchased from Roth (Karlsruhe, Germany). HPLC grade solvents were from Labscan (Bangkok, Thailand) and AR grade solvents were from J. T. Baker (New Jersey, USA).

Table 1. Chromatographic conditions

Condition	Mobile phase composition (%) (*i*-PrOH : THF : H_2O, v/v/v)	Flow rate (ml min^{-1})	Reference
1	1.6 : 8 : 80 + 0.2% ACOH + 10.4% ACN	1.0	16
2	5 : 15 : 85 + 0.2% HCOOH	1.5	17
3	5 : 15 : 80 + 0.3% HCOOH	1.3	Present work

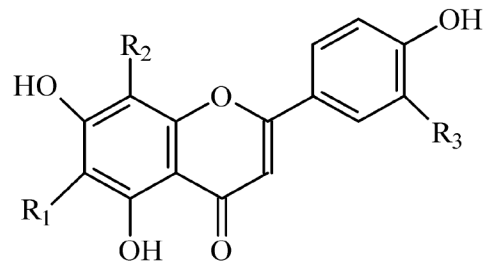
Water was deionized and distilled from Fistream Cyclon system (New Jersey, USA). Other reagents were of analytical grade and used without purification.

Stock standard solution (500 μg ml^{-1}) was prepared by dissolving 50 mg vitexin in 5 ml of warm methanol, adjusted to 100 ml with distilled water and sonicated for 20 min. Standard solutions were prepared by diluting the stock standard solution with 50% methanol to obtain the desired concentrations.

The wild, fully grown *P. foetida* plants were randomly collected from ten different locations in Thailand. Three samples were from central (C1–C3), two from northeastern (NE1, NE2) and eastern (E1, E2), two from southern (S1, S2) and one sample from northern (N1) Thailand. The plants were identified and compared at the Herbarium Section, Forestry Department, Bangkok, Thailand. Leaves of the collected plants were dried at 45°C for 6 h and ground to fine powder. Five grams of the powder was continuously extracted with 40% methanol for 20 h by Soxhlet extraction. The filtered extract was evaporated by a rotary vacuum evaporator. Five hundred milligrams of the extract was diluted to 10 ml with 40% methanol, sonicated at 30°C for 15 min and filtered through a 0.45 μm membrane prior to HPLC analysis.

The initial HPLC condition was modified from the analysis of flavonoids in *P. incarnata* that was reported earlier[16,17]. In the present study, optimization of HPLC condition for analysis of vitexin in *P. foetida* was achieved by varying the mobile phase composition and flow rate, as shown in Table 1. Using condition 1, five major peaks were observed and vitexin was eluted as a broad and asymmetric peak around 14 min. Additionally, the vitexin peak was overlapped with other constituents in the leaf extract. Condition 2 provided a slightly better separation than condition 1. Eight major peaks, including vitexin were eluted. However, most peaks were still broad and the retention time increased to 28 min. Condition 3 was optimized since eight major peaks were eluted and vitexin was well resolved from the other peaks ($R_S > 2$) within 18 min. In addition, the peak shape of vitexin obtained from condition 3 was sharper and more symmetric compared to the other two conditions (Figure 2 *b*). Therefore, this condition was employed for the quantitative analysis of vitexin, which was further used as a marker compound in *P. foetida* leaf extracts. Peak numbering in Figure 2 was done using the numbers of peaks that were observed from the chromatograms and by matching their retention times. Isovitexin (peak no. 9) and vitexin (peak no. 10) were identified by comparing with the authentic standards and by the spiking method. Although isovitexin (peak no. 9) was absent in the leaf extract, the numbering was marked to indicate the position/retention time of the isovitexin peak, in case it is present.

A calibration curve of vitexin in the range of 25–500 μg ml^{-1} was established using the optimized condition described earlier. Calibration data calculated from peak area and height with linear equation and correlation coefficient (r^2) are shown in Table 2. Although peak height provided better correlation coefficient than peak area, the latter was employed for all other calculations since the



	R_1	R_2	R_3
Isovitexin	glc	H	H
Vitexin	H	glc	H

Glc = β-D-glucopyranosyl

Figure 1. Structures of vitexin and isovitexin.

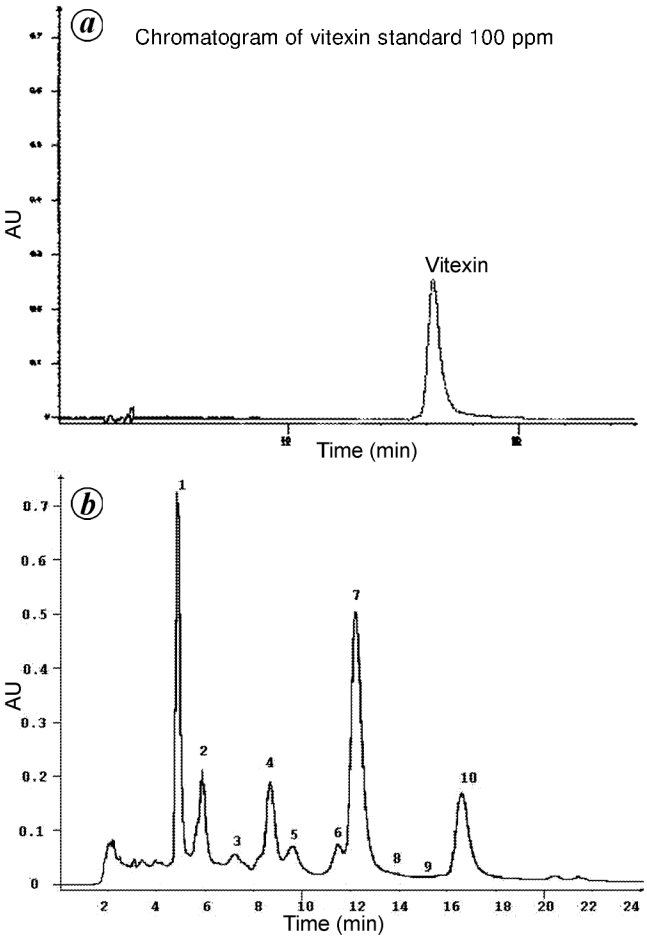


Figure 2. *a*, Chromatogram of standard vitexin. *b*, A typical chromatogram showing separation of vitexin in *Passiflora foetida* leaf extract under the optimum condition. column Spherisorb ODS; mobile phase, Isopropanol : tetrahydrofuran : water (5 : 15 : 80, v/v/v) and 0.3% formic acid; flow rate, 1.3 ml/min; injection volume, 20 μl; detection, 340 nm. Peaks 1–8, Not identified; peak 9, Isovitexin; peak 10, vitexin.

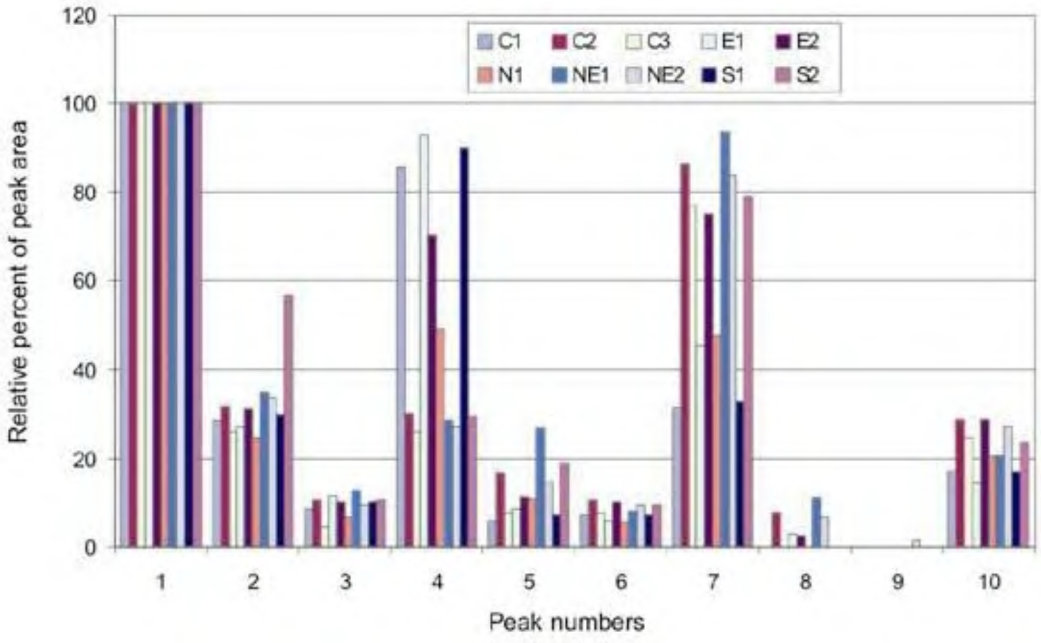


Figure 3. Prevalence of characteristic components in the analysed *P. foetida*.

Table 2. Calibration data of vitexin ($n = 3$)

Concentration ($\mu g\ ml^{-1}$)	Mean of peak area (%RSD)	Mean of peak height (%RSD)
25	93,037 (0.3)	0.0228 (3.6)
50	222,121 (5.5)	0.0550 (2.2)
100	432,361 (2.0)	0.1090 (3.7)
150	646,372 (2.8)	0.1505 (1.2)
200	942,078 (2.0)	0.2200 (8.7)
500	1,037,636 (2.6)	0.2551 (3.3)
Linear equation	$y = 4357.9x - 628.5$	$y = 0.001x + 0.007$
Correlation coefficient (r^2)	0.9891	0.9943

Table 3. Assay of vitexin in *P. foetida* leaf extract ($n = 3$)

Source	Vitexin (%w/w)	%RSD
C1	0.04	2.0
C2	0.08	3.0
C3	0.06	2.3
E1	0.05	1.8
E2	0.09	1.1
N1	0.09	4.0
NE1	0.07	1.8
NE2	0.11	2.4
S1	0.06	2.6
S2	0.13	3.6

%RSDs were smaller in most cases. Thus, peak area was used for quantitation of vitexin *P. foetida* leaf extracts.

The method developed was applied for the identification of appropriate markers for *P. foetida* leaves collected from ten provinces in Thailand. Vitexin was selected as a potential marker since it was present in all the extracts and was well resolved from the other peaks. Vitexin peaks were identified in the extracts by comparing the retention time with that obtained from the standard solution, by spiking the standard solution into the sample solution and by a comparison of its UV spectra with that of the standard. The retention time of vitexin peak in the leaf extracts varied from 15.3 to 17.3 min (%RSD = 4.53%), compared to 16.3 min in case of standard vitexin. The shift in retention times was due to the complex matrices in different extracts. Results showed that the selected *P. foetida* leaf extract contained various amount of vitexin (0.04–0.13 %w/w) as one of the constituents (Table 3). The highest amount of vitexin was found in the leaf extract from S2, while the least amount was from C1. Different vitexin contents from each source may be due to the varied climate and nutrient, since the collected samples were wild-grown plants.

The optimized HPLC condition was utilized to reveal the fingerprint of *P. foetida* leaf extract randomly collected from ten different locations in Thailand. The analysed samples were from wild plants and were grouped according to the geographical locations of the collected samples. The chromatogram profiles of the extracts were complex and contained at least ten components. All extracts showed similarities in both pattern and number of compounds, but differed in the concentration of the marker compound, vitexin, which was present in all profiles. In addition to the marker compound, many other peaks could be detected using the optimized HPLC condition. In an attempt to

conclude the fingerprint pattern of *P. foetida* extract and the appropriate marker, a relative percentage of peak area of each component was calculated based on the base peak (peak no. 1). The values were 100, 33, 9, 53, 13, 8, 65, 3, mostly absent and 22% for peaks 1–10 respectively.

Upon examining the relative intensity with the base peak, which was always the most abundant peak in every sample, a certain pattern of *P. foetida* could be seen (Figure 3). The data suggested that this pattern evaluation is suitable for the natural product extract, whose components vary with environmental factors, but roughly of the same number. Although the characteristic components were present at different concentration levels, the fingerprint characteristics were highly conserved. Importantly, vitexin can be isolated and identified from the leaves of Thai *P. foetida* extracts with low %RSDs, suggesting that vitexin is an appropriate marker for the leaf extract of the plant. Therefore, the established HPLC method can be used to validate the quality and authenticity of phytopharmaceuticals containing passion flower leaf extract. Unlike *P. incarnata*, isovitexin was almost absent from all the analysed *P. foetida*. Isovitexin was detected as trace amounts in only one of the collected samples, NE2.

This study describes a rapid and efficient HPLC procedure for the analysis of vitexin in *P. foetida*. The flavonoid was an appropriate marker for the leaf extracts of the plant. Although vitexin has been analysed in several plants[18–20], it has not been determined in *P. foetida*. Additionally, chromatographic analysis can be performed on different samples to generate unique fingerprints that provide a general overview on the patterns and trends of the extracts. The compiled data will be useful for taxonomy, breeding and authenticity testing of phytopharmaceuticals containing *P. foetida* extracts.

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