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Research Article

Chromatographic Evaluation of a Phytoestrogen Genistein from *Flemingia vestita* Benth: an Endemic Plant of Northeast India

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ABSTRACT: Background: *Flemingia vestita* Benth (Fabaceae) is a much-branched trailing herb with tuberous roots, found throughout the Himalayas and Khasi Hills up to an elevation of 8,000 ft. Formononetin, pseudobaptigenin, diadzein and genistein are major isoflavones present in the tuberous root of *F. vestita*. **Materials and Methods:** In this research work, tubers of *F. vestita* were collected from different regions of Northeast India and analyzed for their genistein content using two chromatographic techniques (HPTLC and HPLC). Both the chromatographic methods have been optimized and validated as per ICH guidelines. **Results:**Comparative evaluation of genistein content in different samples of *F. vestita* revealed that the marker content varies significantly. Tubers collected from Lyngiong were found to be rich in genistein. **Conclusion:**Thus, chromatographic characterization of *F.vestita* using HPTLC and HPLC would enable its rational utilization and these methods can be applied for the quality evaluation of *F. vestita*.

KEYWORDS: *Flemingia vestita* Benth, genistein, HPLC, HPTLC.

INTRODUCTION

Flemingia vestita Benth and Hooker (Ver. *Soh-phlang*, Fabaceae) is an indigenous medicinal plant found in Meghalaya, Northeast India.[1–3] Its fleshy tuberous roots are consumed by local tribal people of Meghalaya to cure intestinal helminth infections and female reproductive disorders.[3,4] Tubers of *F. vestita* are reported to possess major isoflavones such as formononetin, pseudobaptigenin, diadzein and genistein.[5–8]

Plant and plant products are subject to wide variation in their phytochemical profiles due to their variety climatic conditions, maturity, post harvest processing, storage, stability etc. Therefore it is extremely important to standardize these drugs based on their marker compounds using suitable chromatographic technique in order to identify morphological and geographical variations.[9–12]

F. vestita; being an endemic plant of North-East India, its distribution is confined to a restricted area due to its specific ecological niches and soil related requirements. Therefore, in order to maintain the quality of drug when the plant is sourced from some different areas of Northeast India, its phytochemical evaluation seems imperative. Based upon the reported therapeutic activities of genistein like estrogenic, vermifugal, anticancer, antibacterial and neuroprotective etc,[4,13] it was selected as a marker to evaluate the quality of *F. vestita* tubers.

In the present research work, genistein content in *F. vestita* tubers collected from various geographical provinces of North-East India was determined and compared using two validated chromatographic techniques (HPTLC and HPLC). As an application of the method other morphological parts of *F. vestita* (leaves, stem, flowers, peel of tuber, tubers without peel, whole tubers) have also been characterized for their genistein content.

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MATERIALS AND METHODS

Different parts of *F. vestita* were collected from Shillong whereas tubers were collected from different regions of Northeast India namely, Shillong, Nongspung, Lyngiong, Jakrem and Sohra rim. The representative samples of *F. vestita* were authenticated from Dr. PB Gurung, Dept. of Botany, North East Hill University, Shillong. The samples were thoroughly washed, cleaned and shade dried for a week. The materials were then packed in absorbent paper, oven dried at 45°C for three days, powdered using a mixer-grinder and sieved through BSS mesh number 85.

Reference standard and reagents

Genistein (98% purity, Figure 1) was procured from Sigma Aldrich chemical company (Steinheim, Germany). All the chemicals used were of analytical grade and were procured from Merck Specialities Pvt. Ltd.

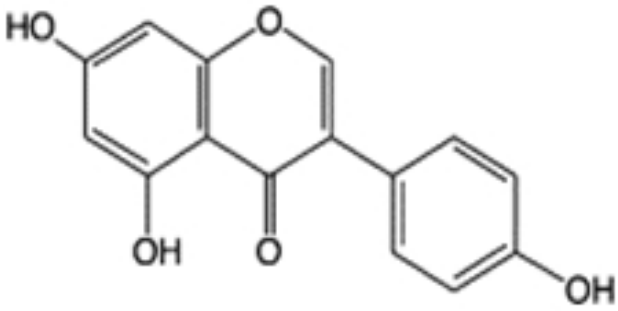

Figure 1. Structure of genistein.

Extraction conditions

To the accurately weighed powdered drug (1.0 g), 10.0 mL of methanol was added, vortexed for 5 min and kept standing overnight. Next day, it was filtered through Whatman filter paper No. 1 and the filtrate was diluted with methanol (1:10, v/v). Similar extraction procedure was employed for HPLC analysis (the samples were filtered through nylon micro filter (0.45 µm) prior to injecting in HPLC system).

Optimized instrumental and chromatographic conditions

High Performance Thin Layer Chromatography (HPTLC)

Chromatographic analysis was performed on TLC plates pre-coated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with aluminum sheet support. Samples were spotted using CAMAG Linomat 5 (Switzerland) equipped with syringe (Hamilton, 100.0 µL). Plates were developed in a glass twin trough chamber (CAMAG) pre-saturated with mobile phase toluene: ethyl acetate: acetone: formic acid (10:2:1:0.5, v/v/v/v), dried and scanned with CAMAG TLC Scanner 4 conjugated with win CATS software at 261 nm. The temperature was maintained at 22 ± 2°C. Photo documentation of the plate was done at 254 nm using CAMAG Reprostar 3 system.

High Performance Liquid Chromatography (HPLC)

Chromatographic analysis was performed at room temperature using Jasco's HPLC system comprising of two PU-1580 pumps (HG-1580-31) and rheodyne injector and photo diode array detector (MD-1510). Chromatograms were recorded by means of Jasco-Borwin chromatography software version 1.50. Separation was achieved on Cosmosil C_{18}-column (150 mm x 4.6 mm, 5.0 µm) using mobile phase 0.025 M phosphate buffer (in water): Acetonitrile (68: 32, v/v, pH 2.4) delivered at a flow rate of 1 mL/min. The samples were injected into HPLC system and the peaks were recorded at 261 nm using PDA detector (MD - 1510).

Preparation of standard solutions

A stock solution of genistein (1000 µg/mL) was prepared in methanol. Eight calibrant samples (2.00 µg/mL to 100.00 µg/mL for HPTLC and 40.0 ng/mL to 60000.0 ng/mL for HPLC) and three quality control samples namely Low (LQC), mid (MQC) and high (HQC) (3.0, 15.0, 80.0 µg/mL respectively for HPTLC while 50.0, 1500.0, 50000.0 ng/mL respectively for HPLC) of genistein were prepared in methanol using the stock solution.

Method validation

The HPTLC and HPLC method for determination of genistein content was validated as per ICH guidelines.[10,11] The validation parameters addressed were linearity, sensitivity, precision, accuracy, ruggedness, recovery and stability.

Estimation of genistein in *Flemingia vestita*

Different samples of *F. vestita* were analyzed using HPTLC and HPLC for their genistein content using optimized chromatographic conditions. During HPTLC analysis, samples (10 µL each) were spotted on HPTLC plate along with genistein standard (10 µg/mL, 10 µL) and analyzed under optimized chromatographic conditions. During HPLC analysis, samples (20 µL each) were injected into the system.

Statistical analysis

Microsoft Excel-2007 was used to determine mean, standard deviation (SD), coefficient of variation (CV) and mean difference during the analysis.

RESULTS AND DISCUSSION

F. vestita is an important medicinal plant endemic to Northeast India.Amongst the major isoflavones present in the plant, genistein is reported to possess antihelminthic, antibacterial, neuroprotective, estrogenic properties.[3–6] So far, there is no report on the evaluation of the genistein content from *F. vestita* using chromatographic techniques. However, chromatographic characterization of plants like *Glycine max, Mangifera indica, Trifolium pratense*, etc. in terms of genistein content using HPTLC and HPLC has been carried out.[14–16] Thus, in this research work, a comparative account on the genistein content in *F. vestita* samples have been addressed using two validated chromatographic techniques.

The separation of genistein was achieved from the *F. vestita* samples on HPTLC plates using toluene: ethyl acetate: acetone: formic acid (10:2:1:0.5, v/v/v/v) as a mobile phase (Figure 2 and 3). A characteristic band of genistein was obtained at R_f = 0.25 in the matrix of *F. vestita* and its identity was confirmed by overlaying the densitometric chromatograms and comparing the spectra of samples with that of genistein. The representative HPTLC chromatogram of genistein and *F. vestita* is represented in Figure 4.

Separation of genistein on HPLC was achieved using 0.025 M Phosphate buffer: Acetonitrile (68: 32, v/v)as a mobile phase. A well resolved peak of genistein was eluted at R_t of 9.44 minute from the complex matrix of *F. vestita* under the optimized HPLC conditions. The presence of genistein in the plant matrix was putatively confirmed by comparing its UV absorption spectra at 261 nm with that of the standard genistein. The representative HPLC chromatogram of genistein and *F. vestita* is represented in Figure 5.

Eight calibrant samples of genistein were analyzed using the optimized chromatographic conditions and the response values for each concentration were obtained (area under curve). A straight-line fit made through the data points by least square regression analysis did not show a constant proportionality. Hence, prior to the regression analysis, logarithmic transformation of the data set was carried out.These log values (response and respective concentration of genistein) were subjected

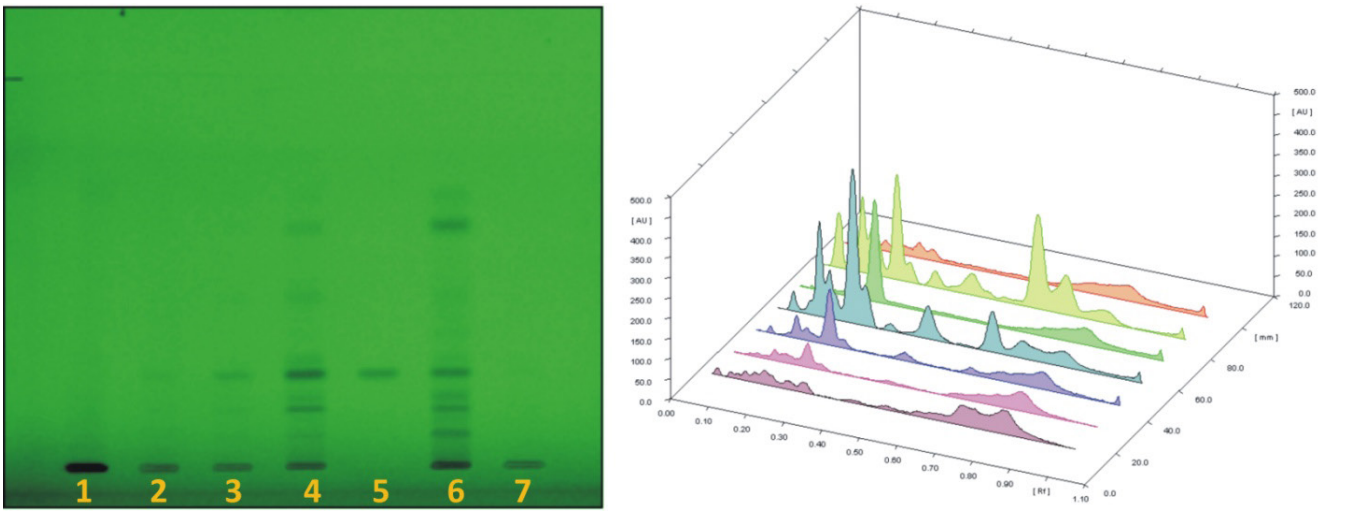

Figure 2. HPTLC plate photo and overlay showing the presence of genistein in different parts of *F. vestita* at 261 nm. Track details; 1) leaves, 2) stem, 3) tubers without peels, 4) whole tubers, 5) genistein 10 µg/mL, 6) peel of tubers and 7) flowers.

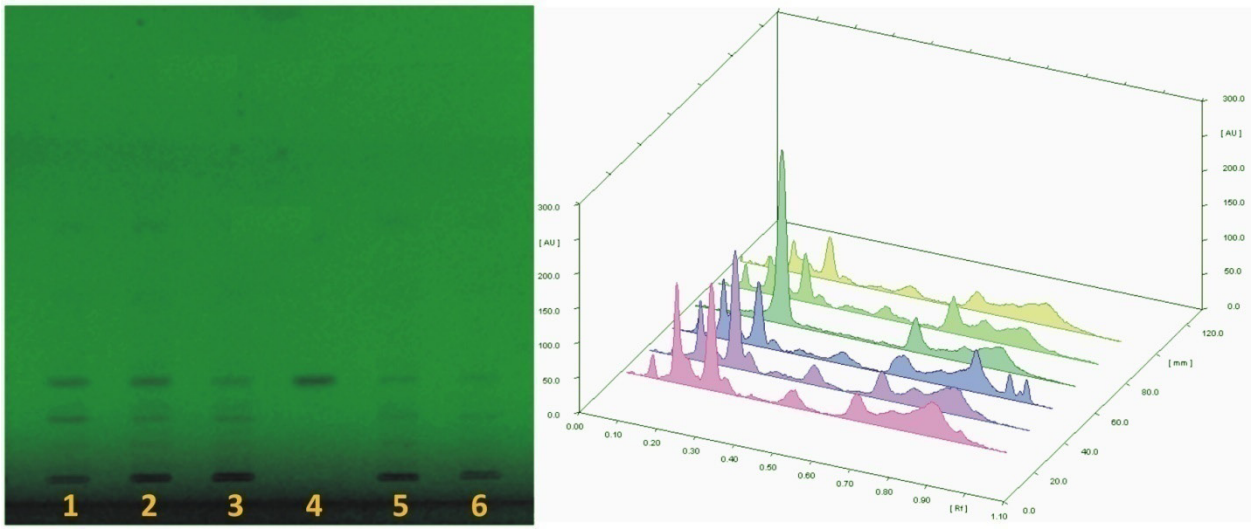

Figure 3. HPTLC plate photo and overlay of *F. vestita* tubers collected from different regions of Northeast India with genistein at 261 nm. Track details; 1) Shillong, 2) Lyngiong, 3) Jakrem, 4) genistein 10 µg/mL, 5) Sohra Rim and 6) Nongspung.

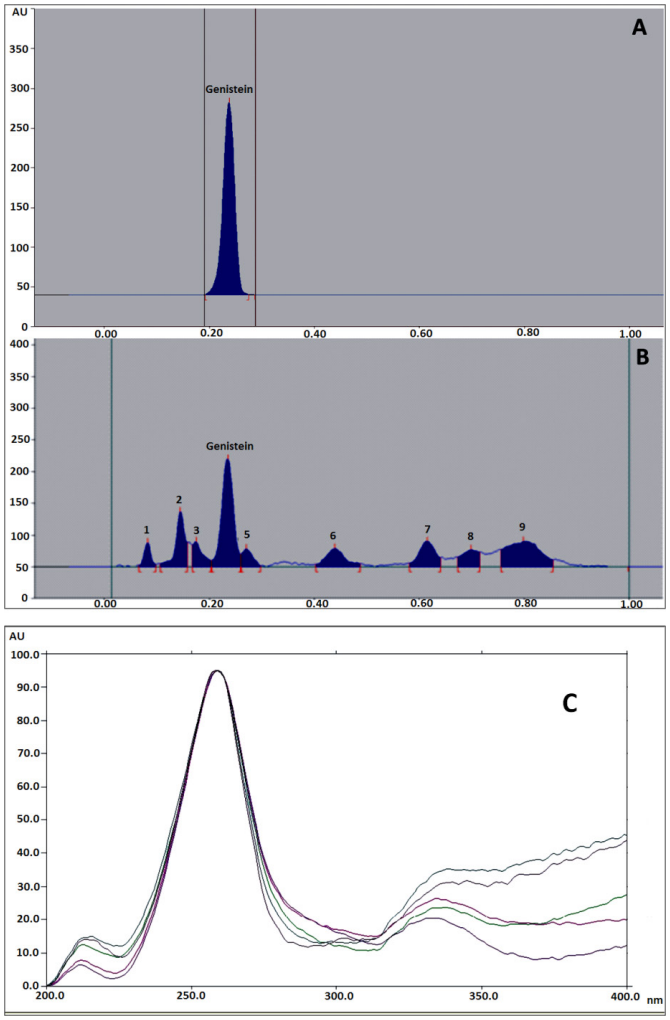

Figure 4. Representative HPTLC chromatograms of A) genistein (10µg/mL), B) *F. vestita* tubers collected from Lyngiong showing presence of genistein and C) spectral confirmation of genistein in *F. vestita* tubers collected from different regions of Northeast India at 261nm.

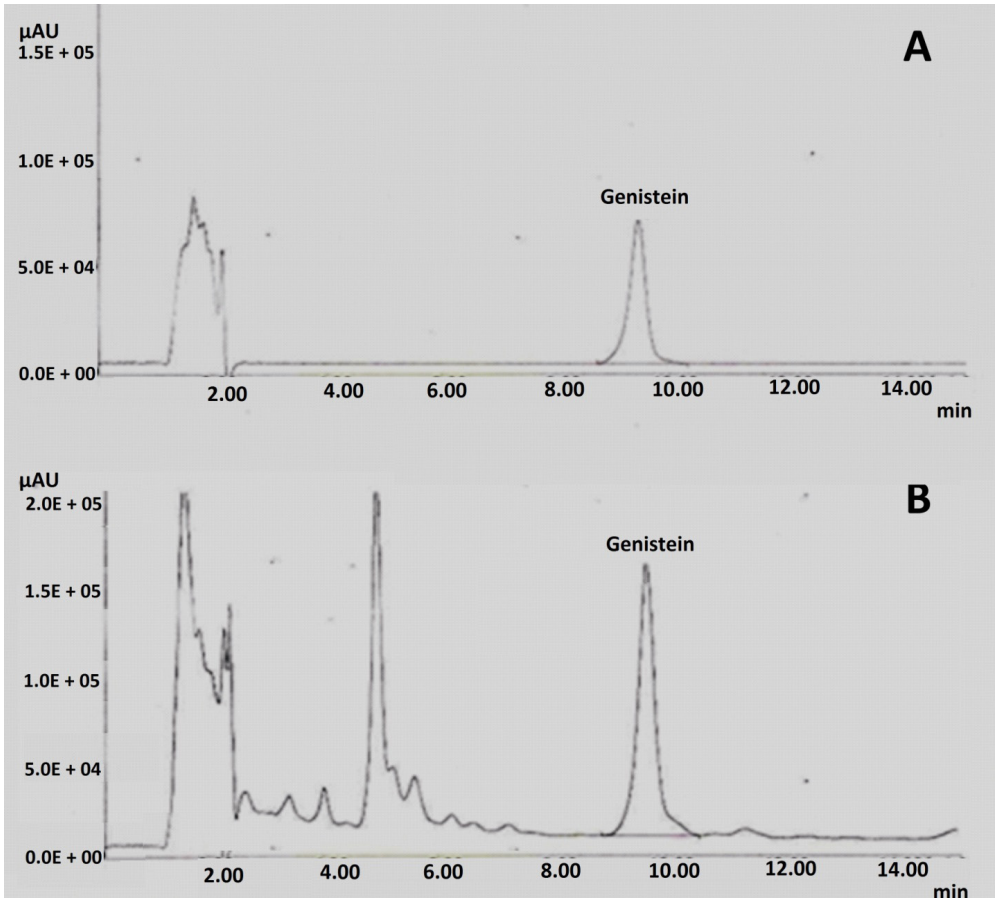

Figure 5. Representative HPLC chromatogram of A) genistein (5000 ng/mL) and B) *F. vestita* tubers collected from Shillong showing presence of genistein at Rt of 9.52 min.

to the regression analysis in order to obtain a regression equation (y = mx + c). The concentration of genistein (x) in the samples analyzed was determined using the regression equation.

Both the methods were validated as per ICH guidelines and were found to be simple, rapid, specific, precise, sensitive and rugged during the validation experiment which on comparison showed that the HPLC method was more sensitive than HPTLC in estimating genistein from plant matrix (Table 1). Both the methods were applied to evaluate the impact of regional variation on genistein content in *F. vestita* tubers collected from different regions of Northeast India. Using the regression equation, the exact content of genistein in the samples was determined.

Variation in the genistein content was observed in different samples analyzed using both the techniques. Different parts of *F. vestita* were evaluated to determine the genistein content and it was found that genistein was maximum in whole tubers whereas it was found absent in the leaves of the plant. Tubers collected from various regions of Northeast India showed the genistein content in the range of 0.56 - 1.346 mg/g (Table 2). Using both the chromatographic methods, it was observed that the tubers from Lyngiong were found to be the rich source of genistein while tubers from Nongspung and Sohra rim showed the minimum content.The possible reasons for such variations may be the significant impact of geographical and environmental factors such as type of soil, exposure to sunlight, temperature, moisture, air, nutrients, etc over the phytoconstituents of medicinal plants.[9–12]

Although genistein is not a plant specific marker, it was chosen for its proven therapeutic efficacy against various ailments for the quality evaluation of *F. vestita* tubers. On the basis of the content of genistein, tubers of *F. vestita* can be selected from a region which has its maximum content and the data may be supported by evaluation of its efficacy.

Table 1: Method validation parameters for estimation of genistein from *F. vestita* samples using HPTLC and HPLC techniques

Parameters	Results	
	HPTLC	HPLC
LOD	0.5 µg/mL	20.0 ng/mL
LOQ	2.0 µg/mL	40.0 ng/mL
Linearity	2.0 - 100.0 µg/mL	40.0 - 60000.0 ng/mL
Regression equation	y = 0.721x + 0.01	y = 1.081x + 5.296
Mean coefficient of determination (r^2)	0.998	0.997
System suitability (% CV, n = 5)		
R_f / R_t	1.50	1.12
Area	1.28	0.55
Precision (% CV, n = 3)		
Within-Batch	0.30 - 1.79	0.12 - 0.67
Between-Batch	0.46 - 1.24	0.16 - 0.52
Recovery (%, n = 7)		
LQC	102.47	95.78
MQC	98.08	93.77
HQC	91.28	94.80
Stability		
Long-term stability		
Standard stock solution stability (For 10 days)	Stable at (4 ± 1ºC)	Stable at (4 ± 1ºC)
Short-term stability		
Bench top stability (For 6.00 hours)	Stable at (25 ± 2ºC)	Stable at (25 ± 2ºC)
Auto sampler stability (For 12.00 hours)	Not applicable	Stable at (4 ± 1ºC)
Ruggedness	Rugged	Rugged

CONCLUSION

Chromatographic methods developed in the current research work can be useful as routine quality control tools for the tubers of *F. vestita*. These methods can also be applied to various plant matrices and polyherbal formulations containing genistein. Using such validated methods, *F. vestita* tubers with precise quality can be encouraged in herbal industries.

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Table 2: Comparative account of genistein content in different samples of *F. vestita* using validated HPTLC and HPLC technique

Samples	Concentration of genistein in mg/g (mean ± SD, n = 7)	
	HPTLC	HPLC
	Morphological variation	
Leaves	---	---
Stem	0.22 ± 0.002	0.28 ± 0.02
Tubers without peel	0.489 ± 0.002	0.521 ± 0.002
Whole tubers	1.462 ± 0.012	1.434 ± 0.02
Peel of tubers	0.912 ± 0.002	0.951 ± 0.002
Flowers	0.148 ± 0.001	0.153 ± 0.005
	Regional variation (tubers)	
Shillong	1.02 ± 0.002	1.434 ± 0.02
Jakrem	0.72 ± 0.001	0.881 ± 0.04
Lyngiong	1.346 ± 0.0015	1.545 ± 0.012
Sohra rim	0.37. ± 0.0014	0.602 ± 0.003
Nongspung	0.56 ± 0.0021	0.546 ± 0.004

CONFLICT OF INTEREST

Nil

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