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Simultaneous estimation of genistein and daidzein in *Pueraria tuberosa* (Willd.) DC by validated high-performance thin-layer chromatography (HPTLC) densitometry method

Swaha Satpathy^a, Arjun Patra^{a,b}, M. Delwar Hussain^b, and Bharti Ahirwar^a

^aInstitute of Pharmacy, Guru Ghasidas University, Bilaspur, India; ^bDepartment of Pharmaceutical and Biomedical Sciences, College of Pharmacy, California Health Sciences University, Clovis, California, USA

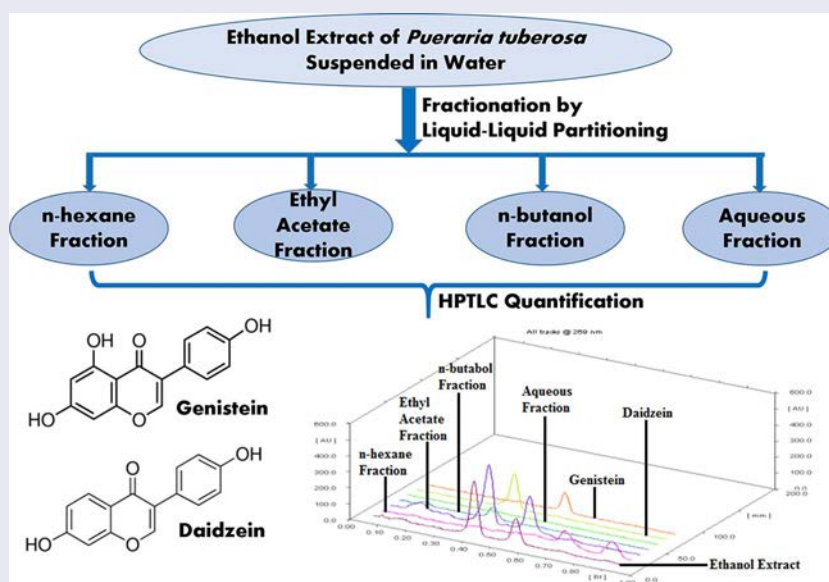
ABSTRACT

The present study was performed to estimate the concentration of genistein and daidzein in ethanol extract of tubers of *Pueraria tuberosa* (Indian kudzu or Vidarikanda) and its various fractions (*n*-hexane, ethyl acetate, *n*-butanol, and aqueous) by high-performance thin-layer chromatography (HPTLC). The separation of bioactive compounds was performed using mobile phase, toluene:ethyl acetate:acetone:formic acid (20.0:4.0:2.0:1.0) and detected at wavelength 269 nm. The method was validated for linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), etc. by International Conference on Harmonization guidelines. The calibration range was found to be 100–600 ng/band for both the bioactive compounds. Daidzein was separated with an R_f value of 0.39 ± 0.02 and genistein with an R_f value of 0.54 ± 0.02 . Average recovery was 99.96 and 99.90% for genistein and daidzein, respectively. The LOD and LOQ were 14.786 and 44.805 ng, respectively, for genistein, and 9.607 and 29.114 ng, respectively, for daidzein. Both the phytoconstituents were found in ethanol extract and its ethyl acetate fraction only. The developed HPTLC method was simple, precise, robust, specific, rapid, and cost effective and could be used for quality control analysis and quantification of genistein and daidzein in different herbal formulations containing the plant species.

KEYWORDS

Daidzein; genistein; HPTLC; ICH guidelines; method validation; *Pueraria tuberosa*

GRAPHICAL ABSTRACT



Introduction

Pueraria tuberosa (Willd.) DC, commonly known as Indian kudzu or Vidarikanda, is an important medicinal plant in Indian system of Ayurvedic medicine. The tubers of the plant in Ayurveda is described as emollient, laxative, aphrodisiac, galactagogue, diuretic, emetic, cardiogenic, rejuvenate, and

expectorant. The tuber is used in various formulations as a restorative tonic, antiaging, vital energy booster, galactagogue, spermatogenic, immune booster, lactagogue, cholagogue, and nutritive. Certain Indian tribes consume the tuber as a complementary food and for the treatment of diabetes. It is an important ingredient of different Ayurvedic formulations

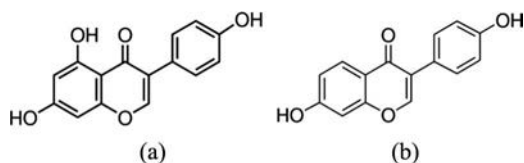


Figure 1. Molecular structure of genistein (a) and daidzein (b).

like shatavaryadi ghrita, mahavisagarbha taila, marma gutika, nityananda rasa, saraswatarishta, ashwagandharishta, chyavanaprasam,^[1] goknura vidari ghrita, and macatmagupta sarpi.^[2] The plant contains various bioactive compounds, namely, puerarin, daidzein, genistein, daidzin, genistin, puerarone, tuberosin, 4-methoxypuerarin, hydroxytuberosone, quercetin, biochanin A, biochanin B, irisolidon, tectoridin, robinin, tuberostan, puerarostan, puetuberosanol, etc.^[3] Genistein has been studied for antidiabetic,^[4] hypolipidemic,^[5] antioxidant,^[6] hepatoprotective,^[7] fibrinolytic,^[8] anti-inflammatory,^[9] immunomodulatory,^[10] nootropic,^[11] anxiolytic,^[12] antihypertensive,^[13] neuroprotective,^[14] cardiovascular,^[15] antifertility,^[16] wound healing,^[17] and nephroprotective^[18] properties. Daidzein has been studied for antidiabetic, hypolipidemic,^[4] antioxidant,^[19] hepatoprotective,^[20] anti-inflammatory,^[21] immunomodulatory,^[22] nootropic,^[23] anxiolytic,^[24] neuroprotective,^[25] and wound healing^[26] activities. These two bioactive compounds have been quantified by high-performance thin-layer chromatography (HPTLC) method in two different Chinese herbal medicines (*Pueraria lobate* and *Pueraria thomsonii*), which were officially included in Chinese Pharmacopoeia till 2000 edition under the same name “Gegen” (*Radix Puerariae*, RP).^[27,28] These phytoconstituents have also been analyzed in four different formulations of *Glycine max*: Menoplant Soy-a 40+, soya meno, Menostop and Soyfem using HPTLC-densitometric method^[29] but are not estimated in *P. tuberosa*. Considering the wide pharmacological potentials of genistein and daidzein (Figure 1), the present study was performed to estimate the concentration of these two compounds in ethanol extract of tubers of *P. tuberosa* and its various fractions (*n*-hexane, ethyl acetate, *n*-butanol and aqueous) by HPTLC method.

Experimental

Chemicals and reagents

All the solvents were of analytical grade. Toluene (Fisher Scientific, India, 99.0% purity), ethyl acetate and acetone (Sd Fine Chem Ltd, India, 99.5% purity), formic acid (Merck, India, 99.0% purity); the HPTLC reference standard, genistein and daidzein were purchased from Sigma-Aldrich, India.

Plant material and preparation of extract

The tubers of *P. tuberosa* were collected from Bilaspur, Chhattisgarh, India through the traditional practitioners of Chhattisgarh and authenticated through ICAR-National Bureau of Plant Genetic Resources, Regional Station, Phagli, Shimla, India. A voucher specimen has been preserved in Institute of

Pharmacy, Guru Ghasidas University, Bilaspur, India for future references. The tubers were washed, cut into small pieces, dried under shade, and then coarsely powdered. The coarse powder material was extracted with 95% ethanol using Soxhlet apparatus. The extract was dried under reduced pressure with the help of rotary vacuum evaporator (Heidolph) and preserved in airtight container until further use. Portion of the extract was suspended in distilled water and successively fractionated with *n*-hexane, ethyl acetate, and *n*-butanol by liquid-liquid partitioning. Finally, the remaining aqueous fraction was also prepared.

Instrument and chromatographic conditions

High-performance thin-layer chromatography plates consisted of $20 \times 10 \text{ cm}^2$, precoated with silica gel G₆₀ F₂₅₄ (E. Merck, India) (0.2 mm thickness) with aluminum sheet support. The plates were saturated with methanol followed by dry heat activation at 110°C for 10 min to avoid interference of moisture. The spotting device was a CAMAG Linomat V automatic sample spotter (Camag Muttenz, Switzerland); the syringe, 100 µL (Hamilton, Bonaduz, Switzerland); UV cabinet with dual-wavelength UV lamp; the developing chamber was a CAMAG glass twin-trough chamber ($20 \times 10 \text{ cm}^2$) (Muttenz, Switzerland); the densitometer consisted of a CAMAG TLC Scanner 3 linked to winCATS software version 1.4.4.6337; the experimental temperature was $25 \pm 2^\circ\text{C}$ and relative humidity was 40%. Ten microliters of sample and standard solutions were applied to plates at a constant rate of 15 nL/s using nitrogen aspirator, 6 mm band length at 10 mm from the bottom edge and 10 mm distance between bands. The CAMAG twin-trough glass chamber was saturated with the mobile phase for 20 min and then linear ascending development chromatography was performed. The length of chromatogram run was 9.0 cm from the base. Developed plates were dried with the help of hair drier and then scanned in the reflectance-absorbance mode at 269 nm. The slit dimension was $5.0 \times 0.45 \text{ mm}^2$ and scanning speed was 100 nm/s.

Preparation of sample solution

Total alcoholic extract and its various fractions (*n*-hexane, ethyl acetate, *n*-butanol, and aqueous) were suitably dissolved in methanol by sonication to get a concentration of 5.0 mg/mL. All the solutions were filtered through 0.45 µ membrane filter before analysis.

Preparation of stock solution of genistein and daidzein

Stock solution of 600 µg/mL of genistein was prepared by dissolving 3.0 mg of accurately weighed genistein in methanol and the volume was made up to 5.0 mL with methanol in volumetric flask. The solution was sonicated for 5 min. Stock solution of daidzein was also prepared similarly.

Preparation of standard solution of genistein and daidzein for calibration curve

The above stock solution of genistein was diluted appropriately by methanol to get final concentrations of 60.0, 50.0,

40.0, 30.0, 20.0, and 10.0 µg/mL. For plotting the standard curve, 10 µL of standard solution of genistein was applied as bands on HPTLC plates to obtain concentrations in the range of 100–600 ng/band. Similarly, daidzein stock solution was diluted appropriately as above and 10 µL of daidzein standard solution was applied to obtain concentrations in the range of 100–600 ng/band.

Preparation of mobile phase

The mobile phase was prepared by mixing toluene, ethyl acetate, acetone, and formic acid in the ratio of 20.0:4.0:2.0:1.0 (v/v/v/v). The mixture was sonicated for 5 min.

Method validation

The HPTLC method developed was validated as per International Conference on Harmonization (ICH) guidelines by determining the following parameters.^[30,31]

Linearity and range

Calibration curve for genistein and daidzein was plotted over a range of 100–600 ng/band.

Accuracy

The accuracy of the method was determined by performing recovery study by spiking three different levels (80, 100, and 120% addition of genistein and daidzein) to the analyzed sample. The samples were reanalyzed to calculate percent recoveries and the average percent recoveries. Recovery analysis was repeated three times for each level.

Limit of detection and limit of quantification

To evaluate the limit of detection (LOD) and limit of quantification (LOQ), different concentrations of the standard solutions of genistein and daidzein were applied, and LOD and LOQ were calculated using equations as per ICH guidelines.

$$\text{LOD} = 3.3 \sigma/S$$

$$\text{LOQ} = 10 \sigma/S$$

where σ is the standard deviation of the response and S is the slope of the corresponding calibration curve.

System suitability (robustness)

Robustness is a measure of the method which remains unaffected by small variations in the method conditions and is an indication of the method reliability. For system suitability, different parameters like mobile phase composition, developing TLC distance, chamber dimensions, and chamber saturation time were studied and the relative standard deviation (%RSD) after changes was measured.

Instrument precision

It was checked by repeated scanning ($n = 7$) of the same spot of genistein and daidzein (400 ng/spot) and expressed as %RSD.

Repeatability

Repeatability of the method was confirmed by analyzing 400 ng/spot of genistein and daidzein ($n = 5$) and expressed as %RSD.

Inter-day and intra-day variations

Variability of the method was performed by analyzing aliquots of standard genistein and daidzein, 300, 400, and 500 ng/spot on the same day for three times (intra-day precision) and on three successive days (inter-day precision). The HPTLC plates were analyzed using optimized chromatographic conditions and the peak area was recorded. The results were expressed as %RSD.

Specificity

Specificity of the method was ascertained by analyzing standard compounds and samples on the same plate. The band for genistein and daidzein from sample solution was confirmed by comparing the R_f values and spectra of the bands with those of the standards. The peak purity of the compound was analyzed by spiking and comparing the spectra at three different levels, i.e., peak start, peak middle, and peak end positions of the bands.

Quantification of genistein and daidzein in ethanol extract and its various fractions

The extract and different fractions were dissolved in methanol by sonication and a final concentration of 5.0 mg/mL was prepared. A total of 10 µL of sample was applied on HPTLC plates and developed using optimized chromatographic conditions to determine the content of genistein and daidzein. The assay experiment was repeated seven times. The quantity of genistein and daidzein present in the extract and the %RSD were calculated. Amount of genistein and daidzein in the sample solution was determined from the calibration curve using peak areas of the compounds recorded.

Stability of standard genistein and daidzein solutions

Stability of standard genistein and daidzein solutions was assessed by comparing the peak areas of standard solution at different time intervals, for a period of minimum 72 hr, at room temperature. Standard genistein and daidzein solutions of 500 ng/band were applied on HPTLC plate at different time intervals and analyzed under standard chromatographic conditions. The results were expressed as %RSD.

Results and discussion

Selection of mobile phase

Composition of mobile phase and ratio of solvents were studied as variables to optimize the chromatographic conditions. The chromatograms were recorded and the R_f value and resolution were calculated. For mobile phase selection, different compositions consisting of different proportions of solvents of varying polarity were tried. For the final estimation, the condition that gave the best resolution/spot symmetry and retention factor was selected. The alcoholic extract of *P. tuberosa* and standard solution of genistein and daidzein were run in different solvent composition (toluene, pyridine, acetone, chloroform, methanol, butanol, formic acid, ethyl acetate, acetic acid, etc.) using different ratios. Solvent system containing toluene, ethyl acetate, acetone, and formic acid produced good results in the alcoholic extract as well as genistein and daidzein without interference of sample components under the laboratory conditions. The proportion of the above solvents (toluene:ethyl acetate:acetone:formic acid 20.0:4.0:2.0:1.0, v/v/v/v) was also optimized for separation, detection, and quantification of genistein and daidzein as this system resolved the compounds with good spot symmetry.

Method validation

Method validation of the analytical method was done by following ICH guidelines. The relationship between concentration of genistein and the peak response was linear in the concentration range of 100–600 ng/spot with coefficient of determination 0.9978 (r^2 value) and for daidzein, the concentration range was also 100–600 ng/spot with coefficient of determination 0.9906 (r^2 value). The LOD and LOQ were 14.786 and 44.805 ng, respectively, for genistein, and 9.607 and 29.114 ng, respectively, for daidzein.

The average percentage recovery at three different levels (80, 100 and 120%) of genistein and daidzein were found to be in the range of 99.24–100.78 and 98.96–101.42%, respectively (Table 1).

The standard and test samples/solutions were applied on HPTLC plate and several slightly different combinations of the four solvents, developing distance, chamber saturation time, and chamber dimensions were used for determining robustness. The modified parameters afforded good resolution with R_f 0.54 ± 0.02 for genistein and 0.39 ± 0.02 for daidzein. The standard deviation of peak areas was measured for each

parameter, and %RSD was found to be less than 2.0 confirming the robustness of the method.

Further, the method was also validated in terms of precision, repeatability, and specificity. The presence of genistein and daidzein in the ethanol extract and its ethyl acetate fraction were confirmed by comparing the R_f value of standard and sample (Figures 2 and 3). In the case of intra-day and inter-day precision, the %RSD varies from 0.11 to 1.61 (Table 2). The summary of results of method validation has been depicted in Table 3.

Stability of standard genistein and daidzein

Stability of standard genistein and daidzein was determined using solvent system toluene:ethyl acetate:acetone:formic acid (20.0:4.0:2.0:1.0, v/v/v/v) at room temperature over 72 hr. The %RSD values of relative retention time and relative peak area of both the compounds were less than 2.0. Therefore, the standard solutions were stable as no significant degradation was observed for a period of 72 hr.

Quantification of genistein and daidzein in the plant

Genistein and daidzein were found to be present in the total alcoholic extract and its ethyl acetate fraction only, and the quantity is more in the ethyl acetate fraction (Table 4).

World Health Organization encourages and promotes the use of herbal medicines for management of various diseases. About 70–80% of world's population relies on traditional medicines for their primary health care.^[32,33] Plant-based medicines are available in the market in different forms like crude drugs, extracts, fractions, single-herbal formulation, and multiherbal formulations. Hence, quality control and standardization of herbal medicines is of paramount importance. These significant challenges could be addressed by reliable quantification of biologically active compounds in the sample or obtaining a chemical fingerprint profile of chemical marker compound(s).^[34] Various analytical methods, namely, TLC, GC, fluorescence, HPLC, HPLC-UV, MS/MS, GC-EI/MS, HPLC-UV-MS, and HPTLC have been used for qualitative and quantitative determination of bioactive compounds in botanicals. HPTLC has been recognized as a favorable techniques because of its several advantages such as: (i) it gives better analytical precision and accuracy as both sample and standard are analyzed simultaneously under similar conditions, (ii) fresh stationary phase is used for each

Table 1. Recovery analysis of genistein and daidzein.

Compound	Amount of compound present in sample (ng/band)	Amount of compound added (ng/band)	Total amount	Amount of compound detected (ng/band)	Percentage mean recovery ($n = 3$)	RSD (%)
Genistein						
80%	278.5	222.8	501.3	500.6	99.86	0.87
100%	278.5	278.5	557.0	552.7	99.24	1.43
120%	278.5	334.2	612.7	617.5	100.78	1.27
Daidzein						
80%	343.5	274.8	618.3	611.87	98.96	0.68
100%	343.5	343.5	687.0	682.33	99.32	1.06
120%	343.5	412.2	755.7	766.43	101.42	0.84

RSD, relative standard deviation.

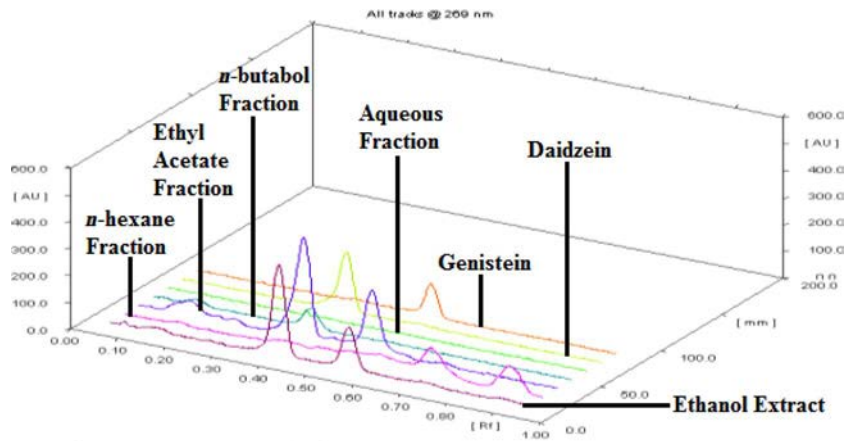


Figure 2. HPTLC chromatogram (3D) of different samples using toluene:ethyl acetate:acetone:formic acid (20.0:4.0:2.0:1.0, v/v/v/v). Note: HPTLC, high-performance thin-layer chromatography.

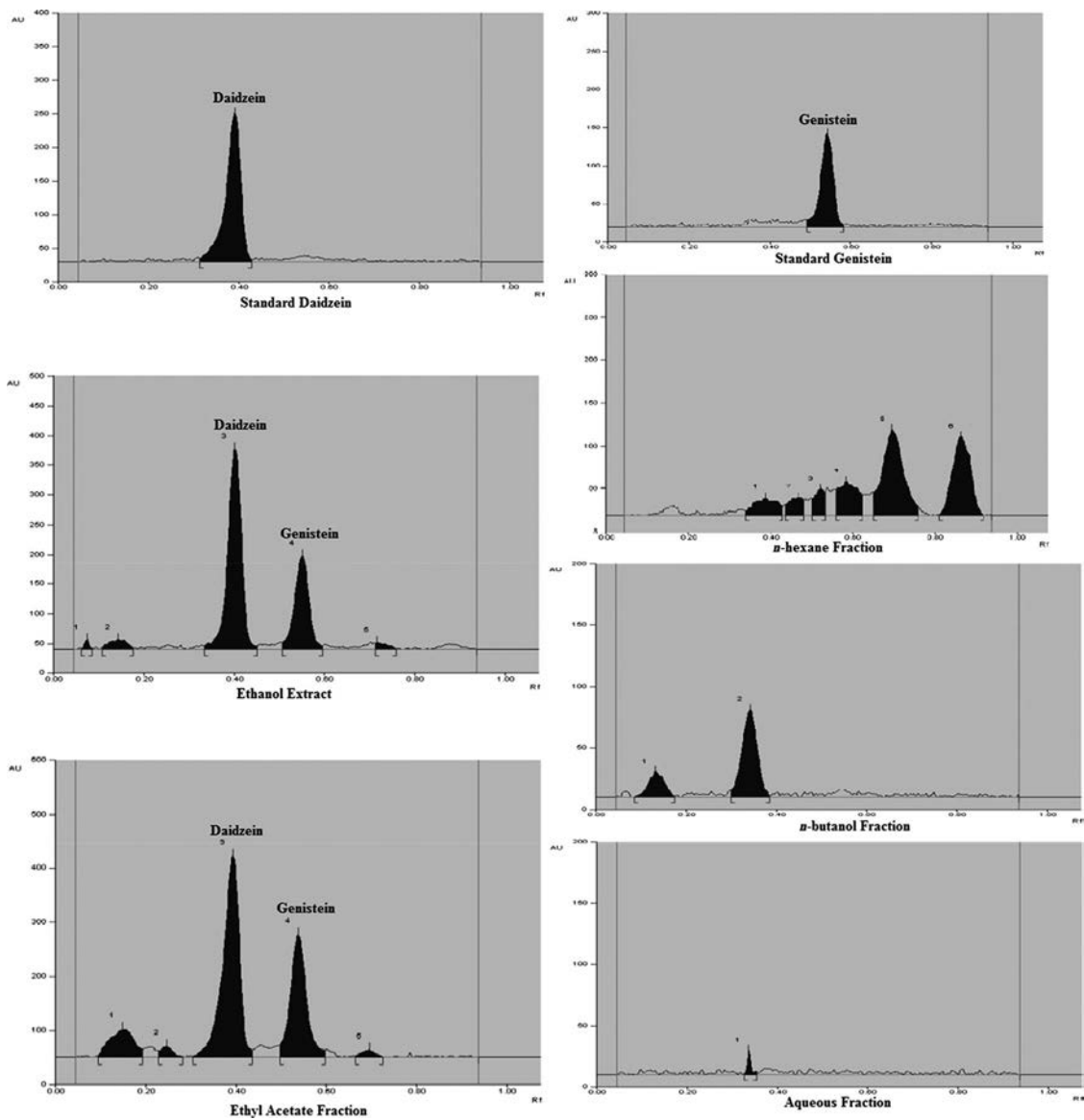


Figure 3. HPTLC chromatogram of different samples using toluene:ethyl acetate:acetone:formic acid (20.0:4.0:2.0:1.0, v/v/v/v). Note: HPTLC, high-performance thin-layer chromatography.

Table 2. Intra- and inter-day precision study.

Concentration (ng/spot)	Intra-day precision [RSD (%)]		Inter-day precision [RSD (%)]	
	Genistein	Daidzein	Genistein	Daidzein
300	1.01	1.61	0.87	1.37
400	0.46	1.58	0.66	1.01
500	0.11	1.21	0.54	0.87

RSD, relative standard deviation.

Table 3. Summary of results of method validation.

Parameters	Values	
	Genistein	Daidzein
R_f value	0.54	0.39
Linearity and range	100–600 ng/band	100–600 ng/band
Regression equation	$Y = 6.424x + 345.93$	$Y = 10.272x + 695.87$
Coefficient of determination (r^2)	0.9978	0.9906
Instrument precision ($n = 7$)	RSD (%) = 1.17	RSD (%) = 1.00
Repeatability ($n = 5$)	RSD (%) = 1.04	RSD (%) = 1.04
Specificity	Specific	Specific
LOD	14.786 ng/band	9.607 ng/band
LOQ	44.805 ng/band	29.114 ng/band

RSD, relative standard deviation; LOD, limit of detection; LOQ, limit of quantification.

analysis which prevents the interference from previous analysis, (iii) mobile phase consumption per sample analysis is very low, (iv) number of samples can be processed at the same time, (v) instrumentation is inexpensive and user friendly, (vi) quantification of analytes in microgram and nanogram levels, and (vii) time and cost of analysis are less.^[35–38] In the present study, we have reported the presence of genistein and daidzein in various fractions of total alcoholic extract of tubers of *P. tuberosa* by a validated HPTLC method. Liquid–liquid partitioning of alcoholic extract of the tubers revealed the presence of both genistein and daidzein only in the ethyl acetate fraction. These two phytoconstituents have been quantified by HPTLC in four different pharmaceutical products of *G. max*: Menoplant Soy-a 40 + (genistein, 5.56 ± 0.17 mg/g; daidzein, 9.84 ± 0.34 mg/g), soya meno (genistein, 1.70 ± 0.07 mg/g; daidzein, 13.85 ± 0.40 mg/g), Menostop (genistein, 1.10 ± 0.05 mg/g; daidzein, 4.74 ± 0.22 mg/g), and Soyfem (genistein, 17.00 ± 0.43 mg/g; daidzein, 20.32 ± 0.55 mg/g).^[29] Two Chinese herbal medicines, *P. thomsonii* and *P. lobata*, have been studied for the presence of daidzein and genistein by HPLC and capillary electrophoresis (CE). The content of daidzein by HPLC and CE was 1.57 ± 0.50 and 1.65 ± 0.56 mg/g, respectively, and that of genistein was 0.09 ± 0.01 and 0.09 ± 0.01 mg/g, respectively.^[39] The present study confirms that the content of genistein and daidzein in the extracts of *P. tuberosa* is much higher than the above samples and could be a useful source of the two bioactives in different pharmaceutical and nutraceutical preparations.

Table 4. Genistein and daidzein contents in different samples determined by HPTLC method.

Sample	Content of genistein ^a	Content of daidzein ^a
Alcoholic extract	55.7 mg/g (1.08)	68.7 mg/g (0.94)
Ethyl acetate fraction	91.8 mg/g (1.52)	92.1 mg/g (1.24)

HPTLC, high-performance thin-layer chromatography.

Values in parenthesis indicates RSD (%).

^aValues are average of seven readings.

Conclusions

In the present study, the HPTLC method has been developed and validated for quantification of genistein and daidzein from the ethanolic extract and its various fractions of tubers of *P. tuberosa*. Further, the method was found to be accurate, simple, precise, robust, specific, rapid, and cost effective. The developed method could be used for quality control analysis and quantification of genistein and/or daidzein in plant material and herbal formulations containing *P. tuberosa*. Furthermore, the developed method can be used to determine the purity of drugs from various sources and identification of the adulterants/substitutes/controversial plant species.

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