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Quantification of puerarin and daidzein and *in vitro* pharmacological evaluation of *Pueraria tuberosa* (Willd.) DC.: An ethnopharmacological approach

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Abstract

Pueraria tuberosa (Willd.) DC. commonly known as Vidarikand, is an indigenous medicinal plant commonly found in Southeast Asia and mainly found in Uttarakhand, Odisha, Assam and Meghalaya states of India. The tuber of the plant is used in ethnomedicine as well as in traditional Ayurvedic systems of medicine for treating various ailments. Puerarin and daidzein, the important isoflavons are the constituents besides several other phytochemicals in the tuber. Simultaneous quantification of two isoflavones, puerarin and daidzein in *P. tuberosa* was achieved through high-performance thin layer chromatography (HPTLC) using mobile phase chloroform:methanol (8:2). Puerarin and daidzein were separated at the R_f value of 0.132 (± 0.002) and 0.605 (± 0.002), respectively and scanned at λ_{max} 340 nm. Calibration analysis of puerarin and daidzein were performed within the range of 2-12 µg/band. The limit of detection and limit of quantification for puerarin were 0.001237 and 0.003747 and that of daidzein were 0.0012 and 0.0037, respectively. Quantity of puerarin and daidzein were found to be 0.701% ± 0.003 (7.01 mg/g) and 0.482% ± 0.004 (4.82 mg/ g), respectively. Significant amount of phenolics (0.016 % ± 0.00), total flavonoids (0.034 % ± 0.00), total tannins (0.4% ± 0.001), total starch (0.33% ± 0.006) and total sugar content (0.22 % ± 0.01) were observed. The *in vitro* antidiabetic, anti-inflammatory and antioxidant analysis were also performed to predict/evaluate the therapeutic potential of the species. The IC_{50} values were determined and found to be 158.256 µg/ml ± 0.223 for antidiabetic activity, 99.58 µg/ml ± 0.582 for anti-inflammatory activity and 11.561 ± 0.436 for antioxidant activity. The significance of the findings have been discussed in light of the therapeutic properties of the compounds.

1. Introduction

Pueraria tuberosa (Willd.) DC., commonly known as 'Vidarikanda' (Bhukushmandi in Sanskrit) is a therapeutically important perennial herb, distributed throughout Southeast Asia. In India, it is found mainly in Uttarakhand, Assam, Odisha, and Meghalaya states (Barooha and Ahmed, 2014). This species is also known as 'Indian Kudzu' due to its resemblance with the common 'Kudzu' and both belongs to Fabaceae family.

The species is widely used in ethnomedicine as well as in traditional Ayurvedic systems of medicine. It was used in the preparation of various ayurvedic formulation, restorative tonic, antiageing, spermatogenic, immune booster and in the treatment of cardiovascular diseases, hepatosplenomegaly, fertility disorders, menopausal syndrome, sexual debility and spermatorrhoea **(**Maji *et al*., 2014**)** *P. tuberosa* is used as main ingrediant in *Vajikarana rasayan* (An Ayurvedic formulation) used in treatment of sexual deficiencies/disorders. In this formulation, several plant parts are used to improve sexual functions (Chauhan *et al.*, 2013). Traditionally, *P. tuberosa* is used for fertility regulation by nomadic tribes in Jammu-Kashmir and Assam states of India (Barooha and Ahmed, 2014). The major bioactive metabolites includes puerarin, genstein, daidzein and tuberosin. Besides these, this species also contains puerarone, tuberosol, isotuberosin, *etc*. (Amal *et al.*, 2014). Individually or collectively phytochemicals may be responsible for the therapeutic potential against a wide range of ailments and about their activity as immune booster, aphrodisiac agent, anti-inflammatory, cardiotonic and brain tonic. Previous *in vivo* and *in vitro* studies provided the support in favour of the traditional use of the tuber as spermatogenic, immune booster, aphrodisiac, anti-inflammatory, cardiotonic and brain tonic (Chopra *et al.*, 1995; Maji *et al.*, 2014). Isoflavones are used in different medicinal compositions. In this article, special emphasis was given on quantification of two isopflavones, *viz.*, puerarin and diadzein in *P. tuberosa*. Puerarin is reported to be used widely in the treatment of cardiac diseases. Daidzein is also reported in different medicinal formulations used for the treatment of cancer, ageing and as antioxidant. Daidzein also plays role in the cosmetic industry due to its ability to prevent skin ageing and photo-damaging.

In this article, results of pharmacognostical and pharmacological evaluation of *P. tuberosa* were reported along with quantification of the two important isoflavones, puerarin and diadzein, which would be helpful in future exploration of the plant species through identification and extraction of the therapeutically important compounds and utilisation in useful drugs.

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2. Materials and Methods

2.1 Chemicals

Puerarin (99.3%) and daidzein (99.5%) were purchased from Chroma Dex, Inc. USA. Other organic solvents, namely; chloroform, acetone, and diethyl amine of analytical and high-pressure liquid chromatography (HPLC) grade, were obtained from Merck, Mumbai (India). Solvents were filtered (0.45 mm filter, Millipore, Bedford, MA, USA) and sonicated for 15 min before use. HPTLC plates (20 cm × 20 cm, precoated silica gel aluminium plates 60 F254, 0.25 mm) were purchased from E. Merck (Darmstadt, Germany).

2.2 Sample collection

The *P. tuberosa* (tubers) were collected during September from Jeolikote, Uttarakhand, India. Specimen was authenticated by Dr. Sharad Srivastava, Senior Principal Scientist, Pharmacognosy Division, CSIR-NBRI, Lucknow, India and deposited in repository of CSIR-NBRI with individual voucher number.

2.3 Anatomical analysis

The anatomical studies (transverse section and powder microscopy) were performed as per standard method (Kokate, 2010; Shukla *et al.*, 2018). The fresh plant material was preserved in 70 % ethanolic solution for anatomical studies.

2.4 Pharmacognostical studies

The pharmacognostical parameters, *viz.*, moisture content, total ash, acid insoluble ash, and extractive values (hexane, petroleum ether, alcohol, water, ethyl acetate, chloroform and benzene soluble extractives) were evaluated as per standard protocol of Ayurvedic Pharmacopoea of India-2005 (Anonymous, 2005a).

2.5 Phytochemical analysis

The phytochemical parameters, *viz.*, total phenolics, flavanoids, tannins, sugar and starch were quantified by spectrophotometric method. Total flavonoid and phenolic content was estimated and expressed in terms of mg/g of QE (quercetin equivalent) and mg/g GAE (gallic acid equivalent) based on calibration curve of quercetin and gallic acid as standard (Ordonez *et al*, 2006). Tannic acid was used as the reference for the quantification of total tannins and is expressed as TAE (tannic acid equivalent), whereas sugar and starch was quantified as per API (2005) and is expressed in percentage.

2.6. Quantification of marker compounds through HPTLC

2.6.1 Apparatus

A CAMAG automated thin layer chromatography (ATS-4) sampler was used to dispense the aliquots of the standard stock solution and the prepared samples. The plates were developed in CAMAG automatic developing chamber (ADC-2, 20 cm × 20 cm). The slit dimensions were 5 mm × 0.30 mm and scanning speed was 100 mm/s. Scanning of bands was performed using Camag TLC Scanner 4 in ultraviolet (UV) absorbance mode by Vision Cats software using deuterium lamp source.

2.6.2 Chromatographic conditions

Chemical profiling and method optimization for simultaneous quantification of puerarin and daidzein were carried out on TLC aluminium pre-coated plate (20 cm × 10 cm) with 0.2 nm layer thickness of silica gel 60 F_{254} (TLC silica gel 60$F_{250,}$ EMD Millipore Corporation). Tracks (standard and sample) were applied as 6 mm bandwidth using CAMAG automated TLC sampler (ATS-4) (Camag, Switzerland) under a flow of N_2 gas. The linear ascending development was carried out with chloroform: methanol (8:2 v/v) as a mobile phase in a CAMAG automatic developing chamber (ADC-2, 20 cm × 20 cm). The saturation time of chamber was conditioned and optimized to 20 min at room temperature (25°C ± 2) and relative humidity for better resolution with mobile phase vapors. The plate was allowed to develop up to a height of approximately 85 mm from the point of application (total length run by mobile phase), and the total run time was standardized at room temperature (25°C ± 2) and relative humidity of 55% ± 2. After development, the plates were air-dried for 30 min, and scanning was performed using Camag TLC Scanner at λ_{max} of 250 nm for both puerarin and daidzein in UV absorbance reflectance mode operated by Vision Cats software. Quantification was performed using peak area versus content of standard marker using regression analysis in the range of 200-1200 ng /band. Images of TLC plate were taken at two wavelengths, *viz.*, 254 nm and 366 nm.

2.6.3 Sample and standard preparation

The stock solution of puerarin and daidzein (1 mg/ml) was freshly prepared in analytical grade methanol. A stock solution of 1000 µg/ml of puerarin and daidzein were diluted with same solvent to obtain a six working solutions of concentration ranging from 200, 400 600, 800,1000 and 1200 ng/band for further analysis. The solutions were filtered through a 0.45 µm millipore membrane filter (Pall, USA) before application. 10 mg/ml of the plant extract was used for HPTLC studies.

2.6.4 Validation of method

In the employed experimental condition, the HPTLC method for simultaneous quantification of puerarin and daidzein included evaluation of the performance parameters, *viz.*, specificity, linearity, sensitivity, accuracy, recovery, precision, and robustness as per ICH guidelines (Anonymous, 2005b).

2.7 *In vitro* activity analysis

The antidiabetic, anti-inflamatory and antioxidant activities were performed *in vitro* as per the methods detailed below:

2.7.1 *In vitro* antidiabetic assay: Two methods, *viz.*, Dinitrosalicylic acid method (DNS) and starch-iodine method were followed for *in vitro* antidiabetic activity studies:

2.7.1.1 Dinitrosalicylic acid method (DNS)

The inhibition assay was performed using DNS method (Miller, 1959). The results were expressed as percent inhibition calculated using the following formula:

Inhibition activity (%) = Abs (Control) – Abs (extract) × 100 /Abs (Control)

2.7.1.2 Starch-iodine method

The inhibition assay was performed using starch-iodine method following Xiao *et al.* (2006). The results were expressed as % inhibition calculated using the following formula:

Inhibition activity (%) = Abs (Control) – Abs (extract) × 100 /Abs (Control)

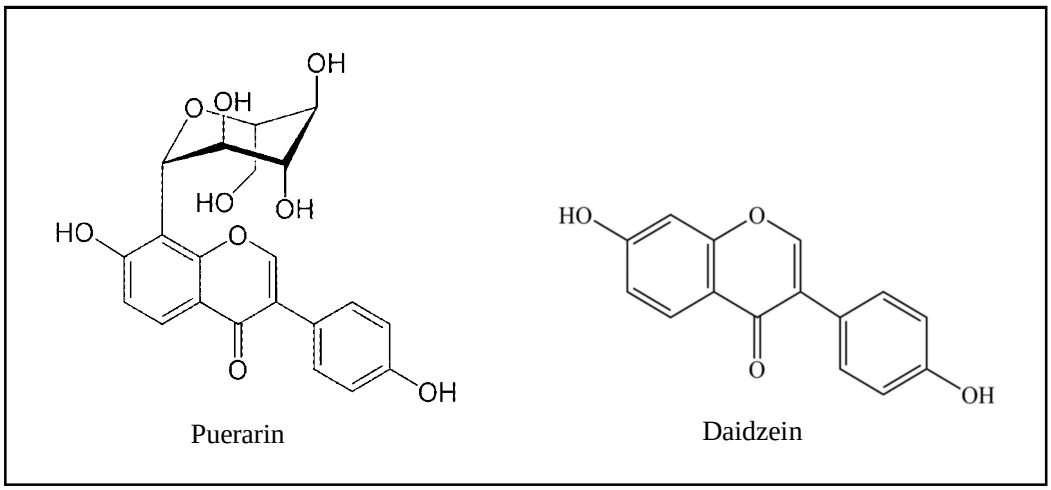

Figure 1: Chemical structure of marker compounds puerarin and daidzein.

Figure 2: Collected tubers of *P. tuberosa*.

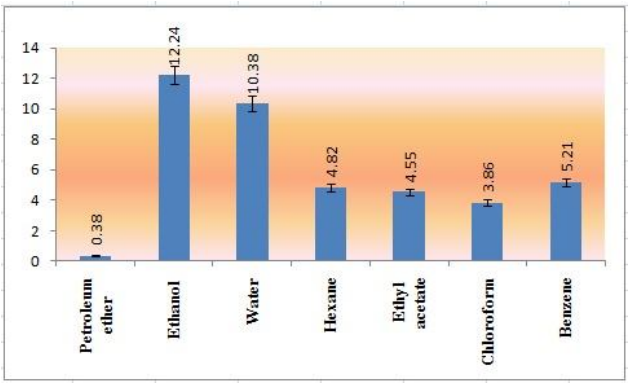

Figure 3: Extractive values in different solvents of *P. tuberosa* (n = 3, values are Mean ± sd).

2.7.2 *In vitro* anti-inflammatory assay

The inhibition assay was performed using BSA method (Williams *et al*, 2008). The results were expressed as % inhibition calculated using the following formula:

$$\text{Inhibition activity (\%)} = \text{Abs (Control)} - \text{Abs (extract)} \times 100 / \text{Abs (Control)}$$

2.7.3 *In vitro* antioxidant activity

Total flavonoid and phenolic content was estimated (Ordon Ez, 2006) and expressed in terms of mg/g of QE (quercetin equivalent) and mg/g GAE (gallic acid equivalent) based on calibration curve of quercetin and gallic acid as standard. The antioxidant potential was analyzed *via* DPPH radical scavenging assay (Shukla *et al*., 2016) and phosphomolybdenum method (Prieto *et al*., 1999).

2.8 Statistical analysis

Observations of each sample were performed in triplicate. The data were recorded as mean ± standard deviations and analysis of variance (ANOVA) was used to calculate the critical F value (F-test) and the statistical significance for the analyzed puerarin and daidzein content by Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA). The significance of the regression coefficients was evaluated by F test. Differences were considered significant at $p<0.05$.

3. Results

3.1 Physicochemical and phytochemical studies

The study revealed that the loss on drying value (LOD) was 10.65 %. The extractive value, *i.e.*, water, ethanol, petroleum ether, hexane, ethyl extractive, chloroform and benzene were analysed. The water soluble extractive value was 10.38 %, while it was 12.24 % for ethanol 0.38 % for petroleum, 4.82 % for hexane, 4.55 % for ethyl acetate, 3.86 % for chloroform and 5.21 % for benzene (Figure 3). Total ash value, water soluble ash and acid insoluble ash were 13.9 %, 13.4 % and 7 %, respectively (Figure 4). Physicochemical results of *P. tuberosa* were found as per the standard limit of Ayurvedic Pharmacopoeia of India -2005 (Anonymous, 2005a).

Phytochemical evaluations showed presence of significant amount of total phenolic content (0.017 % ± 0.002), total flavonoid content (0.033 0% ± 0.001%), total tannin content (0.23 % ± 0.001), total starch content (0.35 0% ± 0.002) and total sugar content (0.40 % ± 0.01) , which are presented in Figure 5.

3.2 Macro and microscopic analysis of *P. tuberosa*

3.2.1 Macroscopic analysis

P. tuberosa is a large perennial climber with very large tuberous roots. Its stems were observed to grow up to 12 cm in diameter. The

tubers were found globose or pot-like in shape, about 25 centimetres (9.8 in) across and the insides being white, starchy and mildly sweet in taste.

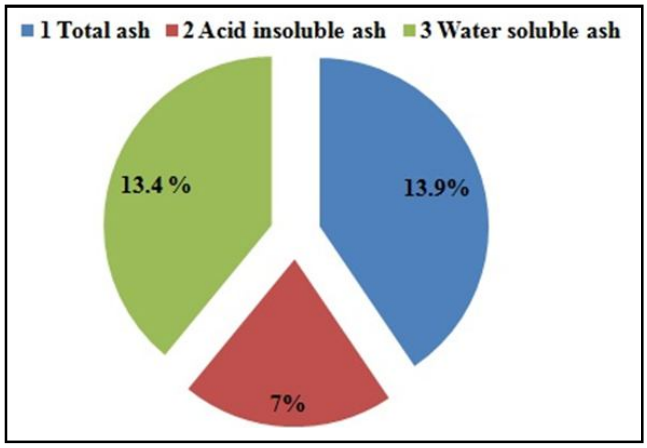

Figure 4: Ash values of *P. tuberosa*.

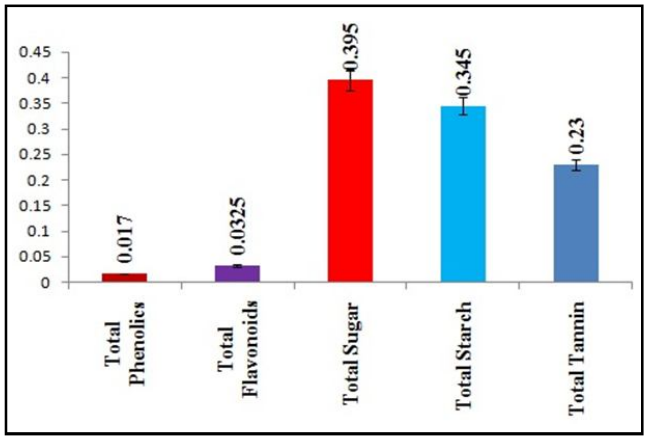

Figure 5: Phytochemical analysis of *P. tuberosa*.

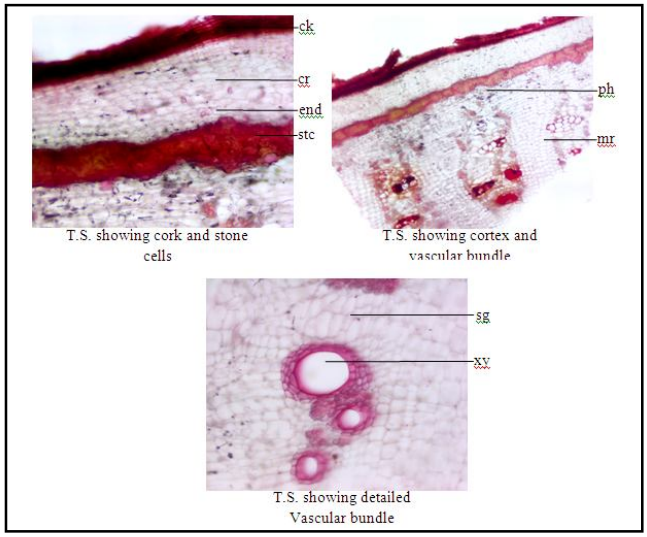

Figure 6: T.S. of *P. tuberosa* tuber abbreviation: ck- cork; cr- cortex; end- endodermis; mr- medullary rays; ph- phloem; sg- starch grains; stc- stone cells; xy- xylem.

3.2.2 Microscopic characters

Transverse section of tuber was observed to be circular in outline, with very narrow range of cork and wider cortex, occupying major area of section, well distinguished endodermal layer and wide stellar system. Detailed structure showed narrow epidermis made of 2 to 4 rows, followed by multi-layered thin-walled cork cambium lie underneath this, epidermis is not discernible; well developed endodermis, fibrous pericycle beneath these multi-layers of stone cells filled with crystals. Phloem was well developed, consisted of sieve tube, companion cells, fiber and parenchyma. Xylem consisted of vessels, tracheids, fibers and parenchyma. Medullary rays were broad and parenchymatous, filled with starch grains which was oval or pentagonal in shape (Figure 6).

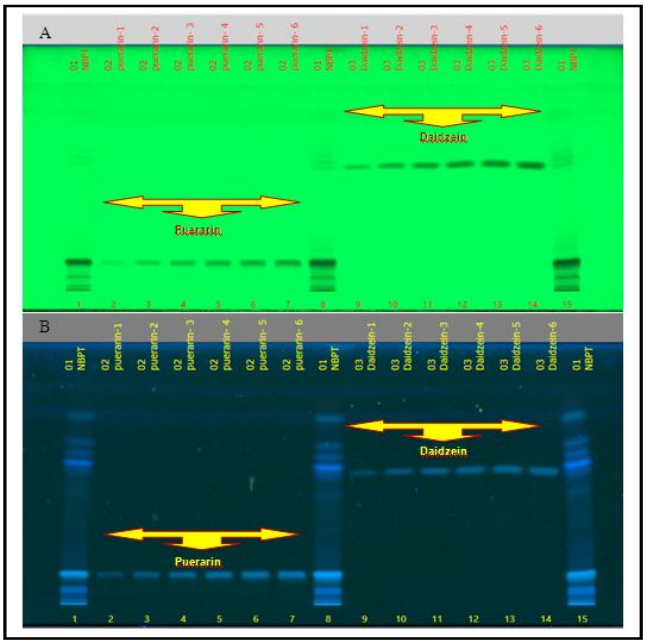

Figure 7: HPTLC fingerprint of *P. tuberosa* at 254 nm (A) and 366 nm (B).

3.3 Method validation

HPTLC method validation included evaluation of linearity, sensitivity, precision, selectivity and robustness parameters according to the guidelines of ICH, 2005 to access the performance of method.

3.4 Calibration parameters

An amount of 2 ml of six calibration standards were achieved with a concentration range of 200, 400, 600, 800, 1000 and 1200 ng/spot of each standard compounds puerarin and daidzein. The TLC plate was developed by using (chloroform:methanol; 8:2 v/v) solvent system to a distance of 85 mm. The plate was dried to obtain the chromatogram and determined the area of peak corresponding to that of puerarin and daidzein. The calibration curve was plotted between area Vs content of puerarin and daidzein. This operation was repeated on three different days in order to select the most appropriate regression model for the response function. Calibration equation of puerarin and daidzein were found ($y = 12.814x + 0.0046$) and ($y = 16.529x + 0.0077$) with regression coefficient 0.9606 and 0.9756, respectively (Table 1).

Figure 8: **HPTLC chromatogram of (A) *P. tuberosa* (B) Puerarin (C) Daidzein.**

3.5 Linearity

Different dilutions were spotted in triplicate on TLC plate at the concentrations of 200, 400, 600, 800, 1000 and 1200 ng/spot of puerarin and daidzein. The data of peak area versus concentration was treated by linear least-square regression equation. The slope (12.81 and 16.52), intercept (0.004 and 0.007) and correlation coefficient (0.9801 and 0.9877) for the calibration curve were determined with six different concentrations of puerarin and daidzein, respectively (Table 1). The results were expressed as percentage of the total area of identified compounds. Based on the calibration curve of puerarin and daidzein content was estimated in the plant sample and expressed on (%) dry weight basis.

Table 1: Calibration parameter of puerarin and daidzein

Calibration parameters	Puerarin	Daidzein
Range (ng/spot)	200-1200	200-1200
Calibration equation	y = 12.814x + 0.0046	y = 16.529x + 0.0077
λ_{max}	250nm	250nm
Regression coefficient (R^2)	0.9606	0.9756
Correlation coefficient (r)	0.9801	0.9877
S.D	0.004892	0.006261
Slope	12.81	16.52
Intercept	0.004	0.007
S.E	0.001085	0.001095
Average	0.01355	0.019283
LOD	0.001237	0.001238
LOQ	0.003747	0.003753

3.6 Sensitivity

Sensitivity of method was determined with respect to limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were calculated from the standard deviation of the response and slope of the calibration curve. The LOD and LOQ of the standards, *i.e.*, puerarin and daidzein were determined as 0.001237, 0.001238 and 0.003747, 0.003753, respectively (Table 1).

3.7 Stability

The reproducibility of method was determined by analyzing marker compound of single concentration (0.1 mg/ml) over three times in same day. The relative standard deviation was used to evaluate the reproducibility of method within the limit of standard. The developed method was also validated for selectivity, specificity and resolution of analyses.

3.8 Precision

Inter-day and intra-day studies were carried out to test the precision of method and expressed as relative standard deviation % (RSD). Intra-day repeatability was tested by scanning marker compounds three times a day. Intra-day % RSD were determined as 3.89 and 9.79 for puerarin and daidzein, respectively. Similarly, inter-day repeatability was assessed over three consecutive days marker compounds thrice a day and % RSD were measured as 3.730 and 6.059 for puerarin and daidzein, respectively (Table 2).

Table 2: Precision studies for puerarin and daidzein compounds

Markers	Intra-day			Inter-day						
				Day 1		Day 2		Day 3		
		P. tuberosa	%RSD	*P. tuberosa*	% RSD	*P. tuberosa*	% RSD	*P. tuberosa*	% RSD	Mean % RSD
Puerarin		0.02758 0.02965 0.02938	3.89	0.02782 0.02755 0.02812	1.024	0.02801 0.02965 0.02933	2.99	0.02458 0.02665 0.02838	7.169	3.730
	Mean	0.028		0.0278		0.029		0.026		
	SD	0.001		0.00029		0.00087		0.0019		
Daidzein		0.00573 0.00481 0.00573	9.79	0.00473 0.00478 0.00508	3.892	0.00455 0.00484 0.00481	3.369	0.00573 0.00481 0.00473	10.917	6.059
	Mean	0.0054		0.0048		0.004		0.005		
	SD	0.005		0.00019		0.00016		0.0006		

3.9 Accuracy

The accuracy (analyzing by marker addition method) of the method was determined by analyzing the percentage recoveries and mean % RSD of puerarin and daidzein in the plant sample. The samples were spiked with three different concentrations 100, 150 and 200 µg (Table 3). The spiked samples were recovered in triplicate and then analyzed by the developed HPTLC method. The Mean % RSD recovery of puerarin and daidzein were obtained 0.031 and 0.004, respectively (Table 3).

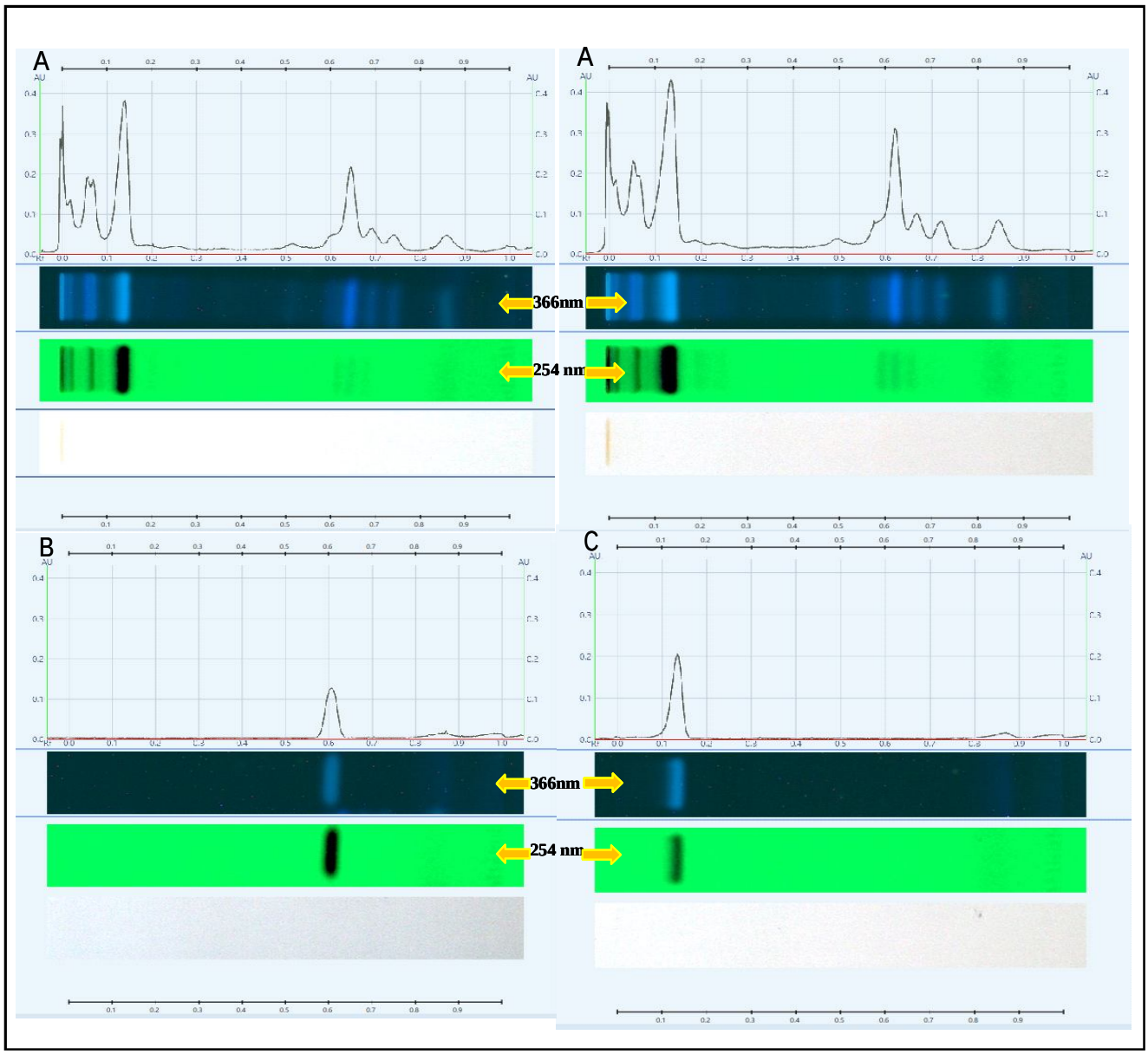

Figure 9: HPTLC chromatogram at 254 nm and 366 nm with spectra of (A) *P. tuberosa* and (B) puerarin and (C) daidzein.

3.10 Quantification of marker compounds through HPTLC

3.10.1 Chromatographic method optimization

Optimization of HPTLC condition (s), namely; selection of mobile phase, absorption maxima, and slit dimensions were standardized to provide an accurate, precise and reproducible method for simultaneous determination and quantification of puerarin and daidzein. Different combinations of solvent systems were tried based on the chemical nature of targeted isoflavons and finally, chloroform: methanol in the ratio of (8:2 v/v) was selected as the best suited system for efficient separation of these metabolites from other unknown markers. Band length was 8 mm; slit dimension 06 × 0.30 and absorption spectrum of both markers puerarin and daidzein were obtained at λ_{max} 250 nm after scanning the entire UV range, from 200 to 800 nm. Specificity of the developed method reflects the clear and complete separation of marker(s) peak and correspondingly in sample and standard. The relationship between concentration of marker compound and its corresponding peak area in sample band was investigated. The linear relationship was also tested and found suitable for simultaneous quantification of both marker(s). Quantity of puerarin and daidzein were obtained in *P. tuberosa* tuber is 0.701% ± 0.003 and 0.482% ± 0.004, respectively (Table 1).

3.11 *In vitro* activities

Methanolic extract of the sample was screened for *in vitro* study of antidiabetic, anti-inflammatory and antioxidant activities. The percentage inhibition as the IC_{50} value of the samples is tabulated (Table 4).

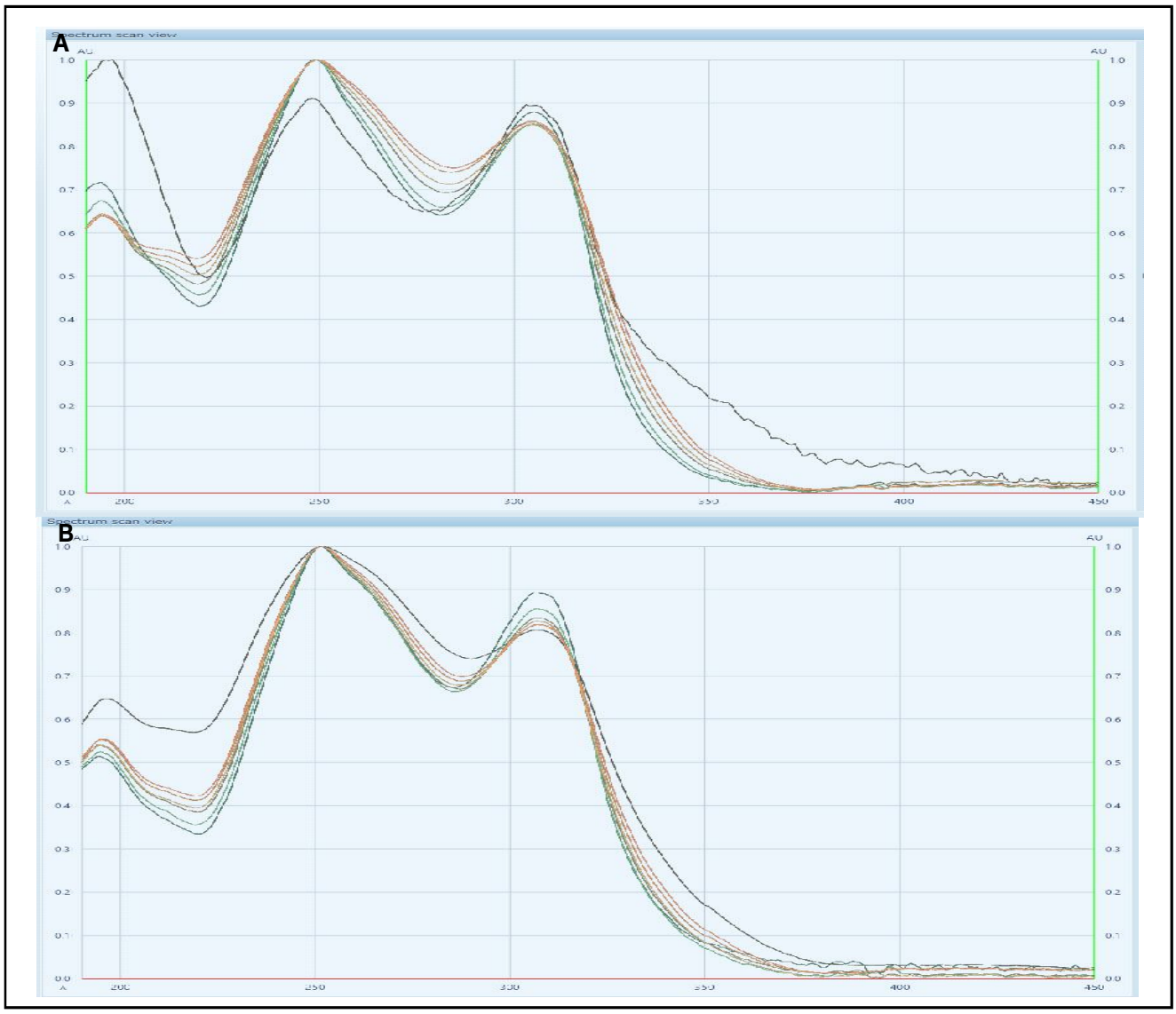

Figure 10: Densitometric purity spectra of puerarin (A) and daidzein (B).

3.11.1. *In vitro* antidiabetic activity

In vitro antidiabetic activity was evaluated by α-amylase inhibition assay based on starch-iodine and DNS method. Analytical data of DNS method revealed that activity increased linearly with concentration, *i.e.*, 0.03-0.15 mg/ml of tested plant extract. The (%) inhibition of *P. tuberosa* species varies from 7.942 % to 48.065 %, IC_{50} value of the sample was found 158.256 µg/ml ± 0.223. Similarly, in the starch-iodine method, with the increase in the concentration, degradation of starch was reduced, and thus indicated the inhibition of enzyme activity. Acarbose exhibited 36.67-95.74 % inhibition at a concentration range of 10-200 µg/ml. IC_{50} of acarbose was observed at 32 µg/ml ± 0.01, while the percentage inhibition of the sample ranges from 3.225 at 50 µg/ml to 48.924 at 250 µg/ml. IC_{50} value was calculated with the regression equation y=0.231x-9.733 and found 258.552 µg ± 0.609. This bioactive potential is expected due to the synergistic action of identified as well as unidentified markers present in the plants.

3.12. *In vitro* anti inflammatory activity

Flavonoids are the naturally occurring bioactive compounds in herb plant that possess anti-inflammatory activity. They promote their interaction with enzymes, cytokines and regulatory transcription factors. Potential of herb/plant not only depends on pharmacodynamics but also pharmacokinetics, *i.e.*, chemical structures, route of drug administration and administrated dose. Anti-inflammatory activity of *P. tuberosa* was screened by the BSA model of protein de-naturation method. In the present investigation, different concentrations of methanolic extract of the sample were examined and percentage inhibitory concentrations were calculated. IC_{50} value was calculated to evaluate the potential (99.58 µg ± 0.582) which showed synergistic potential of the *P. tuberosa* as an anti-inflammatory drug (Table 4).

Table 3: Statistical data for recovery studies for puerarin and daidzein in *P. tuberosa*

Compound analysed	Amount present in sample (µg)	Amount added (µg)	Theoretical-added value (µg)	Amount of compound analyzed (µg)	Recovery (%)	Average recovery	SD	Mean RSD (%)
Puerarin	0.701 0.705 0.712	100 150 200	100.701 150.705 150.712	100.72 150.69 150.70	100.02 99.995 99.997	100.05	0.031	0.031
Daidzein	0.486 0.482 0.479	100 150 200	100.48 150.48 200.47	100.48 150.48 200.47	100.02 99.999 99.997	99.99	0.004	0.004

Table 4: *In vitro* analysis of *P. tuberosa* (Here n = 3, values are Mean ± sd)

S/N	Name of *in vitro* activity	Model	IC_{50} value in *P. tuberosa* (in µg))	IC_{50} value in standards (in µg)
1	Antioxidant activity	DPPH	11.561 ± 0.436	3.861 ± 0.057(µg)
		Phosphomolybdenum model	0.0342 ± 0.003 %	10.38 ± 0.330(µg)
2	Antidiabetic	DNS model	158.256 ± 0.223	32 ± 0.02 (µg)
		Starch-Iodine model	258.552 ± 0.609	25 ± 0.01 (µg)
3	Anti-inflammatory	Protein denaturation method	99.58 ± 0.582	50 ± 0.012(µg)

Table 5: Quantification of puerarin and daidzein in *P. tuberosa*

Standards	*Pueraria tuberosa*
Puerarin	0.701 ± 0.003 %
Daidzein	0.482 ± 0.004 %

3.13 *In vitro* antioxidant activity

Antioxidant activity of *P. tuberosa* (tuber) were calculated by four different models having variable mechanism of action, *viz.* Total phenolic content, total flavanoid content, total antioxidant capacity and DPPH assay. Polyphenolic content, namely; total phenolic and flavonoid contents were estimated. The experimental value of total phenolic and flavonoid contents were (0.016 mg/g GAE ± 0.00) and (0.034 mg/g QE ± 0.00), respectively. The reducing power assay of extract served as a significant indicator of its potentiality as a reducing agent, which in turn signifies its antioxidant activity.

Total antioxidant capacity was calculated by the phospho-molybdenum method. The readings showed that the antioxidant capacity increased along with the increase in the known concentration. The regression equation ($y=4.804+0.136$, $r^2=0.98$) for this curve was calculated and the total antioxidant capacity calculated for *P. tuberosa* is 0.034% .

Free radical scavenging activity of DPPH is the most widely used method for the screening of medicinal plants having antioxidant activity. The scavenging effect of DPPH radical on *P. tuberosa* was concentration dependent and varied among samples as well as standards (ascorbic acid). Ascorbic acid exhibited the maximum inhibition of 77.57% (IC_{50} at 3.86 µg/ ml ± 0.057), where, the IC_{50} of sample was statistically different ($p<0.01$) from standard.

4. Discussion

The present study revealed the importance of *P. tuberosa* with respect to its ethnomedicinal and pharmacological significance. The extractive value was found highest for ethanol, followed by water and benzene. Among the ash contents, water soluble ash content was found higher. Physicochemical results of *P. tuberosa* were found as per the standard limit of Ayurvedic Pharmacopoeia of India -2005 (Anonymous, 2005). Phytochemical evaluations showed presence of significant amount of total phenolic, total flavonoid, total tannin, total starch and total sugar content. These findings supported the utility of the plant species in ethnomedicine as also evident from the traditional therapeutic use of the species. Amal *et al.* (2014) reported major bioactive metabolites includes puerarin, genstein, daidzein, tuberosin, puerarone, tuberosol, isotuberosin, *etc.*, in *P. tuberosa*. Rietjens *et al.* (2017) reported presence of puerarin and genistein, the two very effective estrogens in *P. tuberosa*. Daidzein was reported to have a strong cell cycle arrest activity against breast cancer causing gene in humans (Choi and Kim, 2008). Thus, quantification of these phytochemicals was considered of utmost importance.

Anatomical characterisation of the transverse section of tuber indicated the presence of distinct structural features for storage sinks of starch and other secondary metabolite accumulation. It was also observed that numerous crystals were present in multi-layers of stone cells beneath the fibrous percycle. The results of phytochemical

studies supported the report of traditional use of the tuber in the treatment of various ailments as also reported by Chopra *et al.* (1995), Maji *et al.* (2014) and Chauhan *et al.* (2013). Barooha and Ahmed (2014) reported traditional use of *P. tuberosa* for fertility regulation by nomadic tribes in Jammu-Kashmir and Assam states of India.

A number of studies indicated that the phytochemical screening and chromatographic analysis had crucial role in the pharmacognostical analysis and quality control of herbal drugs (Itankar *et al.*, 2011). In the present investigation, simultaneous quantification of puerarin and daidzein were performed through HPTLC and found their presence in significant amount in *P. tuberosa*. The puerarin and daidzein being flavonoid compounds were reported to have a lot of other potential therapeutic uses (Chopra *et al.* 1995; Maji *et al.*, 2014). Isoflavons present in this plant were reported to have various activities and the quantification will help to confirm the responsible compound(s) in specific activity which may be known by different bioassays.

In this investigation, simultaneous quantification of the two isoflavons were made. Quantity of puerarin and daidzein were found to be 0.701% ± 0.003 and 0.482% ± 0.004, respectively, which are in terms of quantity 7.01 mg/g of puerarin and 4.82 mg/g of daidzein, respectively. These values appeared to be significant as compared to study made earlier in the reference plant soybean and other *Pueraria* species. Prasain *et al.* (2007) quantified puerarin and daidzein in *Pueraria lobata* and found the tuber contained 14.5 mg/g of puerarin and 0.27 mg/g of daidzein. Although, puerarin content was higher, the daidzein content was much lower in this species. On the other hand, *Pueraria radix* was reported to contain 3.279 ± 261 mg/gm of puerarin and 0.238 ± 7.6 mg/gm of daidzein in its tuber (Kumari *et al.*, 2013). In another study, all the parts of *P. tuberosa* tuber were found to contain a significant amount of daidzein. The maximum daidzein (2112.567 ± 0.35 ng/g) content was obtained from a young tuber bark followed by callus (171.903 ± 0.33 ng/g) obtained by culturing tuber (Patel *et al.*, 2020). Soybean has been known as one of the best sources of isoflavones, puerarin and daidzein. For both medicinal and nutritional uses, soybeans are used in an extensive way. The shoot part of the soybean plant was reported to contain puerarin upto 6.627 ± 0.877 mg/gm and daidzein upto 3.908 ± 0.513 mg/gm. The root part was reported to contain puerarin upto 1.156 ± 0.321 mg/gm and daidzein upto 0.714 ± 0.045 mg/gm (Ara *et al.*, 2006). From the above findings, *P. tuberosa* could be considered the best source the two isoflavons, puerarin and daidzein amongst the species investigated so far.

The quantities of puerarin and daidzein observed in the present study could very well support the ethnopharmacological reports available on *P. tuberosa*. Antifertility effect of *P. tuberosa* was reported in rats and it was suggested that phytoestrogens present in the plant was responsible for this (Shukla *et al.*, 1987). Daidzein from *P. tuberosa* acts as signal carriers and responds to the pathogenic attacks and reduces pain during menopause, osteoporosis and also antidiabetic in nature. Puerarin is reported to be used widely in the treatment of cardiac diseases such as angina, cardiac infarction and arrythmia. It prolongs the ventricular action potential duration (APD) in dosage dependent manner in the micromolar range on isolated rat ventricular myocytes (Xu *et al.*, 2016). The effect of daidzein in attenuating breast cancer progression is more effective than tamoxifen (TAM), which is a clinical drug currently used for the treatment of breast cancer (Liu *et al.*, 2012). The phytoestrogen properties of daidzein (extracted from soybean) displayed anti-proliferative properties in three prostate cancer cell lines (LNCaP, DU 145, PC-3) by the induction of cell cycle arrest at G0/G1 phase and the inhibition of angiogenesis *via* altering the expression of cyclin-dependent kinase-related pathway genes. Some of these genes are involved in DNA damage-signaling pathway, and also in the expression of angiogenesis genes; this can lead to the attenuation of growth factor EGF and IGF, thus resulting in tumour growth inhibition (Rabiau *et al.*, 2010). Skin aging is primarily associated with collagen reduction in the dermis, type I and type III collagens are the main component of extracellular matrix (ECM), which is vitally important in maintenance of the dermis structure. Daidzein helps in preventing in skin ageing by maintaining the type I and type III collagens (Sun *et al.*, 2016).

The methanolic extract of the species shows a good activity against alpha amylase diabetic assay. The findings suggested that *P. tuberosa* could be used as a good anti-inflammatory as well as a good free radical scavenging agent. The phenolics and flavonoids are considered to be involved directly in the radical scavenging activity of the natural products (Tapas *et al.*, 2008; Bros *et al.*, 2009; Tungmunnithum *et al.*, 2018; Braca *et al.*, 2002). Puerarin is an isoflavonoid isolated from tubers of the *P. tuberosa* and had been used to improve blood circulation in cardiovascular and cerebrovascular diseases (Yao *et al.*, 2017). The extensive range of pharmacological properties of *P. tuberosa* provides opportunities for further investigation and presents a new approach for the treatment of ailments. Many of them are still unexplored, and there is no supporting data for their activities and exact mechanisms of action. Therefore, further investigations are warranted to unravel the mechanisms of action of individual constituents of this plant (Bharti *et al.*, 2021). *In vivo* and *in vitro* studies have provided the support against traditional demands of the tuber as spermatogenic, immune booster, aphrodisiac, anti-inflammatory, cardiotonic and brain tonic. However, further studies are required to define the active phytochemical compositions and to validate its clinical utilisation in the herbal formulations for human uses (Amal *et al.*, 2014). Epidemiological studies on dietary isoflavones showed that, diets rich in phytoestrogens were beneficial to human health, and hence isoflavones were very much important as a dietary constituent of food as it is known for its medicinal property (Mayo *et al.*, 2019).

The identification and quantification of the two isoflavons, puerarin and daidzein in *P. tuberosa* will be a bench mark guide for further commercial exploitation of the two chemicals from *P. tuberosa* for refinement of useable drugs and their commercial applications.

5. Conclusion

The presence of significant amount of total phenolics, total flavonoids and total tannins indicated the therapeutic potentiality of *P. tuberosa* plant. In the present investigation, quantification of isoflavonoids, *viz*., puerarin and daidzein confirmed the potentiality of the *P. tuberosa* for use as a potent medicine. Further, the observed anti

diabetic, anti inflammatory and antioxidant properties indicated the species to have multiple therapeutic potentiality. Thus, this study will be helpful in future for identification, authentication and quality control of raw material as well as standardization of herbal formulation using this species as an ingredient for treatment of human ailments.

Author contribution

Bikash Sarmah conceptualised the research and performed sample collection, authentication, sample preparation, pharmacognostic and phytochemical evaluation including *in vitro* analysis. Pushpendra Kumar Shukla carried out the quantitative estimation of marker compounds and estimation of puerarin and daidzein. Manoj Kumar Sarma supervised the work, did *in vitro* analysis, drafted and finalised the manuscript. Rolee Sharma supervised the entire research procedures.

All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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