IJPSR (2011), Vol. 2, Issue 11 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 10 July, 2011; received in revised form 21 August, 2011; accepted 25 October, 2011

COMPARISON OF HPLC AND HPTLC TECHNIQUES FOR DETERMINATION OF UMBELLIFERONE FROM DRIED TUBER POWDER OF *IPOMOEA MAURITIANA* JACQ.

Vidya Dighe and Shreeda Adhyapak*

Department of Chemistry, Ramnarain Ruia College, Matunga, Mumbai- 400080, Maharashtra, India

Keywords:

Umbelliferone,
Ipomoea mauritiana Jacq.,
HPTLC,
HPLC,
Method validation

Correspondence to Author:

Shreeda Shreesh Adhyapak

Ph. D Research Scholar, Department of Chemistry, Ramnarain Ruia College, Matunga, Mumbai – 400 019

ABSTRACT

Umbelliferone is a widely occurring phenolic compound of plant origin, for which many biological activities against chronic diseases have been reported. A comparative study of HPLC and HPTLC method has been developed for the quantitation of umbelliferone from dry tuber powder of *Ipomoea mauritiana* Jacq. Both the methods were validated for linearity, precision, limit of detection (LOD), limit of quantitation (LOQ), system suitability and accuracy in accordance with ICH guidelines. The accuracy of the HPLC and HPTLC method was checked by a recovery study conducted at three different levels and values of the average percentage recoveries for these two methods were found to be 97.90 and 98.90 respectively for umbelliferone. The study showed that both the methods are of similar efficiency, sensitivity and can be used for determination umbelliferone from dried tuber powder of *Ipomoea mauritiana* Jacq.

INTRODUCTION: *Ipomoea mauritiana* Jacq. (Family- Convolvulaceae; syn: *Ipomoea digitata* Linn.) is commonly known as Milk yam in English or Kshirvidaari and Vidaarikanda in local language in India. It is an extensive perennial climber and is found all over India commonly in Eastern Bihar, West Bengal and the Tarai areas of Uttar Pradesh and generally grows in moist areas, monsoon forests and in coastal areas [1].

Ipomoea mauritiana Jacq. is used in various mixtures; which are considered to be useful as nutritive, diuretic, expectorants. It finds use in treatment of fevers and bronchitis; and is also used as vitalizer, lactagouge and aphrodisiac [2]. The tubers of this plant are used as tonic, alterative, aphrodiasiac, galactogogue, demulcent, lactagogue, purgative, cholagogue and have antioxidant and immunomodulatory activities [3-6].

An ether soluble fraction from the tubers of *Ipomoea mauritiana* Jacq. has been reported to posses hypotensive and muscle relaxant activities [7].

Some of the chemical constituents present in tubers of *Ipomoea mauritiana* Jacq. are β- sitosterol [8], β-sitosterol-3-O-β-D-glucoside, Umbelliferone, Taraxerol acetate, scopoletin and scoparnone [9] etc.

Umbelliferone is known to prevent the complications of Type 2 diabetes [10], and is also known to be used in Cancer Prevention Therapy [11].

A HPLC method for quantitaion of Umbelliferone from immature Marsh grapefruit (*Citrus paradise*) [12] has been reported in literature.

A TLC method for detection of Umbelliferone, a coumarin from Rutae herba (Ruta graveolens L.) has been reported [13].

HPTLC method for simultaneous quantification of umbelliferone, psoralen, and eugenol from the dried fruit pulp of *Aegle marmelos* and in the fruit of *Trachyspermum ammi* and *Foeniculam vulgare* has been reported [14].

However, a comparative study of HPLC and HPTLC method for quantitation of Umbelliferone from tubers *Ipomoea mauritiana* Jacq. has not been reported in the literature. In the present research work, simple, sensitive and accurate High Performance Layer Chromatography (HPLC) and High Performance Thin Layer Chromatography (HPTLC) methods have been developed and validated for the quantitation of Umbelliferone from tubers *Ipomoea mauritiana* Jacq. and their results are compared.

MATERIALS AND METHODS:

Standard, Chemicals and Plant material: Standard Umbelliferone was procured from Sigma-Aldrich Chemie GmbH (Aldrich Division; Steinheim, Federal Republic of Germany) with 99.00% purity.

All solvents used were of HPLC grade. Toluene (purity 99%), isopropanol (purity 99.00%), methanol (purity 99.00%), ammonia solution (95.0%) were obtained from Spectrochem Pvt. Ltd., Mumbai, India and Acetonitrile (purity 99.8%) was procured from Merck, India. Distilled water used was purified with Sartorius (Arium 61315, made in USA) water purification unit.

Ipomoea mauritiana Jacq. tubers were collected as wild plant, from Ghodbunder Road, Chenna Pt, Mumbai, India. A herbarium of *Ipomoea mauritiana* Jacq. was prepared in duplicate and was authenticated from Botanical Survey of India, Pune. The plant material was thoroughly washed to remove dust particles. Tubers of the plant were separated and then air dried. Immediately after drying, tubers were powdered using an electric mixer-grinder and sieved through a BSS mesh no. 85 sieve. The sieved powder was used for the present research work.

Preparation of Sample Solution: About 1.00g of Ipomoea *mauritiana* Jacq. tuber powder was accurately weighed and transferred to a stoppered test tube (capacity 20.0 mL) and 10.0 mL of methanol was then added to it.

It was then shaken at 30 rpm, on a rotary shaker overnight at room temperature ($28^{o}C \pm 2^{o}C$). The contents of tube were then filtered through Whatmann filter paper No. 41 (E. Merck, Mumbai, India) and the filtrate was further used as the sample solution for the assay experiment.

Preparation of Mobile Phase: The mobile phase used in quantitation of Umbelliferone from tubers *Ipomoea mauritiana* Jacq. by HPLC method, was prepared by mixing distilled water and acetonitrile in volume ratio of 77:23 and then sonicated for 10 minutes.

The mobile phase used in quantitation of Umbelliferone from tubers *Ipomoea mauritiana* Jacq. by HPTLC method, was prepared by mixing toluene, isopropanol and ammonia in volume ratio of 8:2:0.1 and then sonicated for 10 minutes.

Preparation of stock solution of Umbelliferone (1000µg/mL): The stock solution of umbelliferone was prepared by dissolving 10.0 mg of umbelliferone standard in 5.0mL of methanol in a 10.0 mL standard volumetric flask, followed by shaking. The contents of the flask were then diluted up to the mark with methanol.

Preparation of working standard solutions of Umbelliferone: For HPLC, 1.0 mL of the stock solution of umbelliferone (1000.00 µg/mL), was diluted to 10.0 mL, with mobile phase comprising of a mixture of distilled water and acetonitrile, in the volume ratio of 77:23. Thus standard solution of umbelliferone of 100 µg/mL concentration was prepared.

The aliquots (0.01 mL to 2.00 mL) of 100.00 µg/mL solution of umbelliferone were transferred to separate 10.0 mL volumetric flasks and the volume of each flask was made upto 10.0 mL, with the mobile phase, to obtain the working standard solutions of umbelliferone, in the concentration range of 0.10µg/mL to 20.00µg/mL.

For HPTLC, the aliquots (0.01 mL to 0.30 mL) of stock solution of umbelliferone (1000.00 µg/mL) were transferred to separate 10.0 mL volumetric flasks and the volume of each flask was made upto 10.0 mL, with methanol, to obtain the working standard solutions of umbelliferone, in the concentration range of 1.00µg/mL to 30.00µg/mL.

Chromatographic conditions: HPLC analysis was performed with a Chemito LC 6600 High Performance Liquid Chromatography system, equipped with Isocratic HPLC pump and Rheodyne injector, having 20µL loop. The detector used was UV- Visible detector. The UV spectrum of Umbelliferone was recorded using a UV/VIS Spectrophotometer (Shimadzu UV-1650 PC)

A Kromasil C_{18} reversed phase column (250 mm x 4.6 mm i.d.,5µm) was used for the analysis. The system was run at a flow rate of 1.0 mL/min, 20 µL of sample was injected in the chromatographic system and the detection was done at 325nm. The chromatograms and data were recorded by means of Iris 32 Chromatographic software.

In HPTLC analysis, the samples were spotted in the form of bands of width 6mm and 14mm apart with a Camag microlitre syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminium Plate 60 F_{254} having 20 cm × 10 cm dimensions (E. Merck, Darmstadt, Germany supplied by Anchrom Technologists, Mumbai.) using a Camag Linomat IV sample applicator (Muttenz, Switzerland).

Linear ascending development was carried out in 20 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature. The length of chromatogram run was 80mm.

Densitometric scanning was performed on Camag TLC scanner III in the reflectance – fluorescence mode at 325 nm and was operated by CATS software (V 3. Camag).

Method Validation:

Linearity: Linearity for both the methods was evaluated by injecting different concentrations in the range of 1.0µg/mL to 12.00µg/mL for HPLC and 5.0µg/mL to 17.00µg/mL for HPTLC, of working standard solutions of umbelliferone. Each solution was analysed three times and the values of peak areas of umbelliferone and mean peak area of umbelliferone for each concentration were recorded. The calibration curve was obtained by plotting a graph of mean peak areas vs. corresponding concentrations of umbelliferone. The results indicated in Table 1.0, show that within the concentration range indicated, there was a good correlation between mean peak area and concentration of umbelliferone.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The limit of detection was determined at a signal to noise ratio of 3:1. The limit of quantitation was determined at a signal to noise ratio of 10:1. The LOD and LOQ values obtained for both the methods are listed in **Table 1**.

Precision: The method was validated in terms of instrumental precision, repeatability, and intermediate precision.

The instrumental precision for HPLC method was studied by analyzing standard solution of umbelliferone of concentration 2.0µg/mL, in ten replicates, and for HPTLC method was studied by analyzing standard solution of umbelliferone of concentration 7.0µg/mL, in ten replicates, in the chromatographic system under the specified conditions.

Repeatability for HPLC and HPTLC was evaluated by analyzing three different concentrations of sample solution i.e., methanolic extract of dried tuber powder of *Ipomoea mauritiana* Jacq. in triplicates on the same day in the same laboratory. The Intermediate precision of the method was evaluated by analyzing the sample solution in triplicate on three different days, in the chromatographic system, under the specified conditions.

The results were expressed as percentage R.S.D of peak area of umbelliferone. The values of percent R.S.D of peak area for instrumental precision, repeatability and intermediate precision are given in Table 1. The values of percent R.S.D of peak area for instrumental precision, repeatability and intermediate precision were less than 2, indicating that the proposed method is precise and reproducible.

TABLE 1: METHOD VALIDATION PARAMETERS FOR THE ESTIMATION OF UMBELLIFERONE BY THE PROPOSED HPLC AND HPTLC METHOD

Parameter	Results	
	HPLC	HPTLC
Linear range (n = 3) µg/mL	1.00 – 12.00	5.00 – 17.00
Correlation coefficient (r)	0.9990	0.9988
LOD µg/mL	0.40	0.50
LOQ µg/mL	1.00	5.00
Instrumental precision % R.S.D. (n=10)	0.18	0.22
Repeatability % R.S.D. (n=3) (On the same day)	0.40	0.42
Intermediate precision % R.S.D. (n=3) (Mean percent R.S.D. for three successive days)	0.52	0.54

System Suitability: System suitability was carried out to verify that the resolution and reproducibility of the system were acceptable for the analysis.

System suitability test for HPLC method was carried out by injecting 20 µL of umbelliferone solution of concentration 4.0µg/mL in five replicates and for HPTLC method was carried out by applying 10µL of standard solution of umbelliferone solution of concentration 12.0µg/mL on TLC plate in five replicates, under specified conditions.

The values of mean peak areas of umbelliferone for the applied concentration, standard deviation and the percent relative standard deviation were calculated. The parameters used to determine system suitability were repeatability of peak areas and retention time of umbelliferone for HPLC method and retention factor (R_f value) of umbelliferone for HPTLC method for replicate analysis.

The values of mean peak area of umbelliferone, retention time (R_t) of umbelliferone for HPLC method was found to be 316.08 and 8.926 respectively with % R.S.D. value less than 2.

The values of mean peak area of umbelliferone, retention factor (R_f value) of umbelliferone for HPTLC method was found to be 5940.92 and 0.55 respectively with % R.S.D. value less than 2.

All the values for standard solution of umbelliferone lie within the acceptable range with % R.S.D values less than 2, indicating suitability of the system.

Robustness: The developed HPLC method was studied by changing the mobile phase composition, flow rate, and detection wavelength and it was found that small deliberate changes made in the conditions had same effect on chromatographic behaviour of the solutes.

Determination of umbelliferone from dried Tuber Powder of *Ipomoea mauritiana* Jacq.: The above validated HPLC and HPTLC methods were used for quantitation of umbelliferone from dried tuber powder of *Ipomoea mauritiana* Jacq. For HPLC assay, 20 µL of the sample solution obtained from the dried tuber powder of *Ipomoea mauritiana* Jacq., was injected in seven replicates, in HPLC system under the specified chromatographic conditions. The identity of umbelliferone peak in the sample solution was determined by comparing the chromatogram of sample solution (**Figure 2**) with that of the standard umbelliferone solution having retention time of umbelliferone as 8.923 min (**Figure 1**).

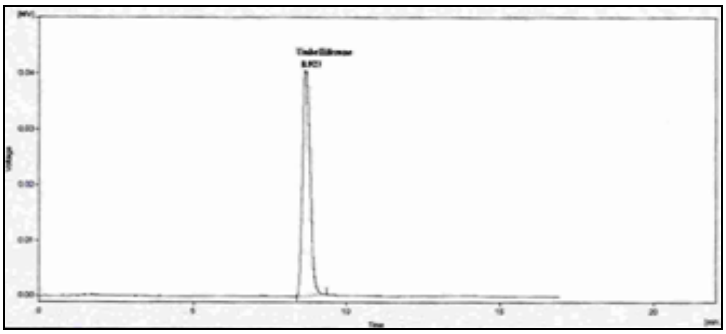

FIG. 1: HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF UMBELLIFERONE
A typical Chromatogram of standard Umbelliferone

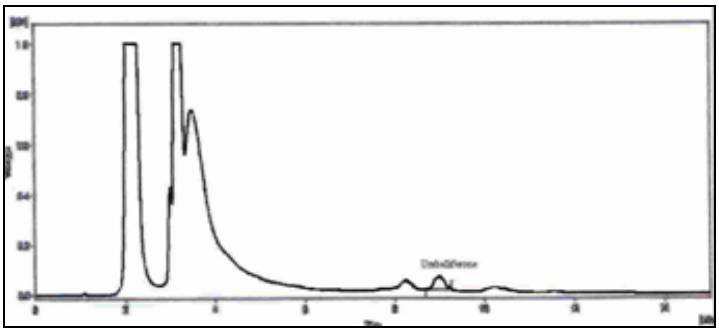

FIG. 2: HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF UMBELLIFERONE
A Typical HPLC Chromatographic pattern for determination of Umbelliferone from methanolic extract of dried tuber powder of *Ipomoea mauritiana* Jacq.

The retention time of umbelliferone in the sample solution was found to be 8.927 min. Amount of umbelliferone present in the sample solution was determined from the calibration curve by using the peak area of umbelliferone in the sample solution. To ascertain the repeatability of the method, the assay experiment was repeated seven times. Mean amount of umbelliferone was found to be 0.0719 mg/g.

For HPTLC assay, 10µL of the sample solution obtained by extraction technique, from the dried tuber powder of *Ipomoea mauritiana* Jacq., was applied as bands in seven replicates, on the TLC plate, with a Camag Linomat IV sample applicator and analysed using the optimized chromatographic conditions.

The identity of peak of umbelliferone in the sample solution was confirmed by comparing the retention factor (R_f) value of the sample with that of the standard solution of umbelliferone having R_f value as 0.55 (**Figure 3**). The identity of the umbelliferone band in the sample solution was also confirmed by overlaying the UV absorption spectra of the sample solution with that of the reference standard solution (**Figure 4**). A typical Chromatogram of plant extract showing the peak of umbelliferone is represented in **Figure 5**.

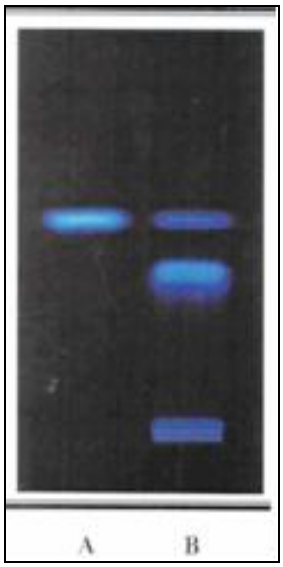

FIG. 3: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY
Separation of standard umbelliferone (A) and methanolic extract of dried tuber powder of *ipomoea mauritiana* jacq. (B)

Amount of umbelliferone present in the sample solution was determined from the calibration curve by using the peak area of umbelliferone in the sample solution. To ascertain the repeatability of the method, the assay experiment was repeated seven times. Mean amount of umbelliferone in dried tuber powder of *Ipomoea mauritiana* Jacq. was found to be 0.0784 mg/g.

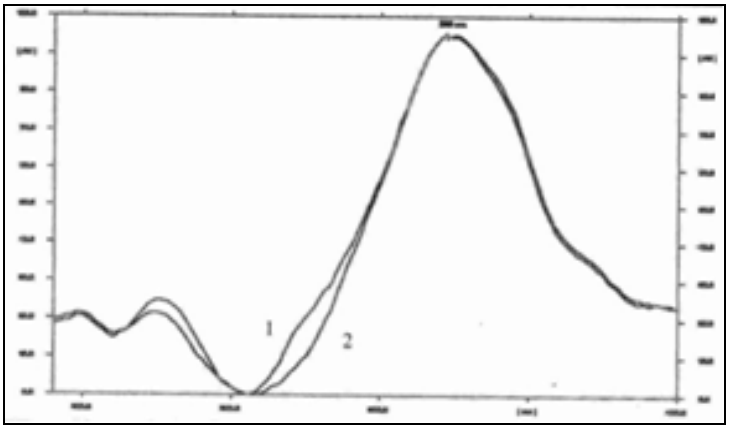

FIG. 4: UV ABSORPTION SPECTRUM
Overlay Spectra of standard umbelliferone (A) and umbelliferone in methanolic extract of dried tuber powder of *Ipomoea mauritiana* jacq. (B)

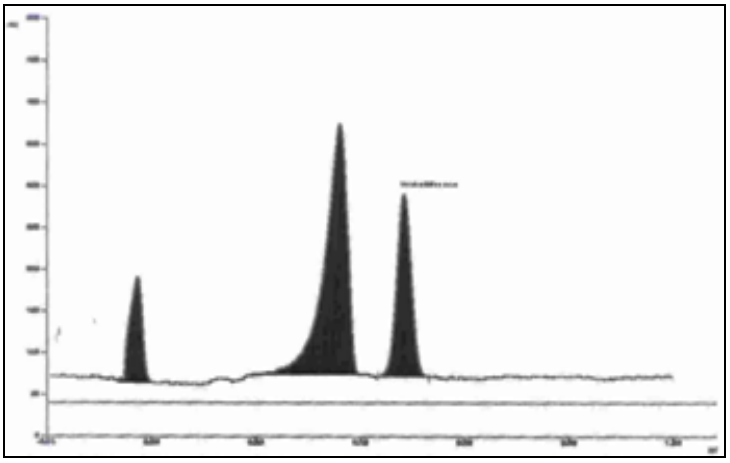

FIG. 5: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY DETERMINATION OF UMBELLIFERONE
A Typical Chromatogram obtained from methanolic extract of dried tuber powder of *Ipomoea mauritiana* Jacq.

Accuracy: Accuracy of the method was tested by performing recovery studies at three different levels for umbelliferone. To accurately weighed about 1.0g of tuber powder of *Ipomoea mauritiana* Jacq., known amounts of umbelliferone (1.0 µg, 2.0µg and 3.0µg), were added, and extracted using methanol and the extracts were estimated by HPLC and HPTLC method, under the specified chromatographic conditions, as described above. The values of percent recovery and average percent recovery for umbelliferone were calculated. The results are recorded in **Table 2**.

TABLE 2: RESULTS FOR RECOVERY EXPERIMENT, FROM DRIED TUBER POWDER OF *IPOMOEA MAURITIANA* JACQ. USING HPLC AND HPTLC METHOD

Level	Weight of sample * (g)	Amount of umbelliferone present in sample (µg/g)		Weight of standard umbelliferone added (µg)	Mean amount of Umbelliferone found (µg)**		Average Percent recovery [%]	
		HPLC	HPTLC		HPLC	HPTLC	HPLC	HPTLC
0	1.002	7.19	7.84	0	7.20	7.81	98.90	97.90
1	1.005	7.11	7.80	1	8.20	8.81		
2	1.004	7.17	7.89	2	9.18	9.72		
3	1.006	7.14	7.82	3	10.17	10.77		

* Sample: Dried Tuber of *Ipomoea mauritiana* Jacq. ** Mean amount of Umbelliferone found (n = 7)

Solution Stability: The stability of standard umbelliferone solution was determined by comparing the peak areas of umbelliferone solution, of concentration 6.0µg/mL in HPLC method and 15.0µg/mL in HPTLC method, at different time intervals, for a period of minimum 48 hrs, at room temperature. The results showed that the peak area of umbelliferone almost remained unchanged (% R.S.D. was less than 2) for both the methods over a period of 48 hrs, and no significant degradation was observed within the given period, indicating the stability of standard solution of umbelliferone, for minimum 48 hrs.

RESULTS AND DISCUSSION: In the present research work, the identity of peak of umbelliferone in sample solution, was confirmed by comparing the retention time (R_t) or retardation factor (R_f) with that of standard umbelliferone. The retention time (R_t) of umbelliferone was found to be 8.923 min (Figure 1) and retention factor (R_f value) was found to be 0.55 (Figure 3).

The values of correlation coefficient of 0.9990 and 0.9988 were found for HPLC and HPTLC method respectively. The values of percent R.S.D of peak area of Umbelliferone for instrumental precision, repeatability and intermediate precision were found to be less than 2. The assay results indicate that amount of umbelliferone estimated by HPLC and HPTLC method was 7.19µg/g and 7.84µg/g respectively and the average percent recoveries of umbelliferone by these two methods at three levels, were 98.90 and 97.90 respectively (Table 2).

The repeatability of peak areas and retention time of umbelliferone for HPLC method and retention factor (R_f value) of umbelliferone for HPTLC method were found to be same for replicate analysis. The values for mean peak areas standard solution of umbelliferone lie within the acceptable range with % R.S.D values less than 2, indicating suitability of the system.

HPLC method was reported in literature [12], for quantitation of Umbelliferone from immature Marsh grapefruit (*Citrus paradise*). The method uses MeOH: H_2O, in the volume ratio of 70:30, as the mobile phase, with UV detection at 254 nm, using C_{18} analytical column, combined with a guard column. The retention time (t_R) of umbelliferone was found to be 20 min. In the present research, work umbelliferone was quantified by HPLC, using a mixture of distilled water and acetonitrile, as the mobile phase, in the volume ratio of 77:23.

A Kromasil C_{18} reversed phase column (250 mm x 4.6 mm i.d., 5µm) was used, and detection was carried out at 325 nm. The retention time of umbelliferone was reduced to 8.923 using the chromatographic method developed in the present research work. Also acetonitrile was required in fewer amounts as compared to the volume of methanol used in reported method.

TLC method reported in literature [13] was used for detection of umbelliferone from *Rutae herba* (*Ruta graveolens* L.) whereas HPTLC method reported in literature [14] was for simultaneous quantification of umbelliferone, psoralen, and eugenol from the dried fruit pulp of *Aegle marmelos* and in the fruit of *Trachyspermum ammi* and *Foeniculam vulgare*. However no HPTLC method was reported for quantification of umbelliferone by comparative HPLC and HPTLC methods from tubers of *Ipomoea mauritiana* Jacq.

Hence, in the present research work, a comparative study of HPLC and HPTLC methods have been carried out for quantification of umbelliferone from tubers of *Ipomoea mauritiana* Jacq.

CONCLUSION: The HPLC and HPTLC methods developed for quantitation of Umbelliferone from tuber of *Ipomoea mauritiana* Jacq. were found to be simple, sensitive and accurate and were validated in terms of linearity, precision, accuracy, system suitability, sample stability. When results of HPLC and HPTLC assay were compared, there was no significant difference in accuracy and precision.

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