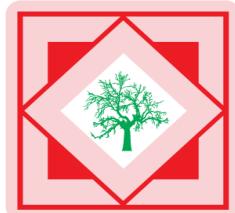




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Quantitative determination of L-dopa in seeds of *Mucuna pruriens* Linn. collected from different geographical sources as well as marketed formulations by high performance thin layer chromatography

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ABSTRACT

Mucuna pruriens Linn. (Leguminosae), commonly known as the cowhage or velvet bean or atmagupta in India, is a climbing legume endemic in India and in other parts of the tropics including Central and South America. It is used for the management of male infertility, nervous disorders and also as an aphrodisiac. L-Dopa is commonly used as a marker compound. Literature survey reveals that there are very few validated HPTLC methods for the estimation of, L-Dopa in seeds collected from different geographical sources as well as their quantification in selected over the counter (OTC) market formulations. Hence an attempt has been made in the present study to develop, validate and apply HPTLC method for evaluation of these marker compound in seeds collected from different geographical sources along with their pharmacognostical evaluation and marketed single and polyherbal formulations.

Key words: L-Dopa, HPTLC, validation, Mucuna seeds, microscopy, formulations

INTRODUCTION

In Ayurvedic system of medicine, *Mucuna pruriens* is used for the management of male infertility, nervous disorders and also as an aphrodisiac [1]. Its different preparations from the seeds are also used for the management of ageing, rheumatoid arthritis, diabetes, male infertility and nervous disorders [2]. Other parts of the plants are also used for various ailments, e.g., trichomes of pods are used for anthelmintics and decoction of root in delirium. Leaves are useful in ulcers, inflammation, cephalgia and general debility. Seed powder contains high amount of L-Dopa, which is a neurotransmitter precursor and effective remedy for the relief in Parkinson's disease [3-4]. In addition to levodopa, seeds contain tryptamine, 5-hydroxytryptamine (5-HT), mucunine, Velvet beandine, prurienine and prurieninine. It is also rich in fatty content [5-6]. L-Dopa is commonly used as a marker compound.

Hence an attempt has been made in the present study to develop and validate HPTLC methods for evaluation of these marker compounds in seeds collected from different geographical sources and various herbal formulations.

MATERIALS AND METHODS

Seeds of *Mucuna pruriens* (Velvet bean), family Leguminosae, were purchased from different suppliers. Gujarat and Maharashtra variety were obtained from Zandu Foundation, Vapi, Gujarat, Madhya Pradesh and Andhra

Pradesh variety from Datt Enterprise, Navi-Mumbai. L-Dopa was procured from Sun Pharmaceuticals Limited, Silvassa (U.T). All the solvents were procured from E Merck and Rankem Labs.

Silica Gel 60 F254 Aluminium plates, 10x10cm with 200 μ m thickness, Merck were used as stationary phase. Butanol: Water: Glacial acetic acid in the ratio of 4:1:1 was used as mobile phase. The method development was done by using computerized Camag HPTLC system (Camag, Muttenz, Switzerland) consisting of a Camag Linomat IV semiautomatic spotting device connected to a nitrogen tank and Win CATS software a Camag TLC scanner III densitometer equipped with mercury, tungsten and deuterium lamp driven by the same Win CATS software, a Camag 100 μ l HPTLC sample syringe (Hamilton, Bonaduz, Switzerland) and a Camag glass twin-trough development chamber.

Marketed formulations a) Zandopa churna (Label claim: Each 7.5 g contains: *Mucuna pruriens* 6.525 g), b) Addyzoa capsule (Label claim: *Mucuna pruriens* extract 150 mg), c) Vigorex SF capsule (Label claim: *Mucuna pruriens* 100 mg), d) AP/HCD capsule (Label claim: *Mucuna pruriens* : 125 mg), e) Shilajeet Gold Capsules (Label claim: *Mucuna pruriens* extract 90mg), f) Vita-Ex Gold capsule (Label claim: *Mucuna pruriens* : 100mg) were obtained from local pharmacy stores.

Seeds were collected and authenticated by Archana Khemani, Head, Department of Botany, Zandu Pharmaceuticals Pvt Ltd (now Emami), Vapi, Gujarat by studying morphological features. The herbarium of the specimen has been deposited at Botany Department of Zandu Pharmaceuticals Pvt Ltd (now Emami), Vapi, Gujarat. After authentication seeds were dried at room temperature until they become free from moisture, stored in airtight containers powdered and used for further evaluation.

Pharmacognostical evaluation

All samples were subjected to morphological and microscopical examination to study the variations among them [7].

Morphology: All samples were subjected to macroscopical identification based on color, odor, taste, form, size and fracture of the drug. Morphological features were compared with standard description.

Microscopy: All samples were subjected to microscopy. Photomicrographs were obtained by observing free hand sections as well as powder of drug under Digital Binocular Motic microscope (DMB series PAL System) with built in analogue camera. Computer images were captured using Motic Image Software (VQCI038). Images of microscopy are shown in **Figures 1-2**.

HPTLC Analysis of L-Dopa

Preparation of standard and sample solution.

Standard

10mg of L-Dopa was dissolved in 10ml 1.0M methanolic hydrochloric acid to give a concentration of 1 μ g/ μ l.

***Mucuna* Seed Samples**

100 gm seed powder of each *Mucuna pruriens* samples collected from different geographical source were cold macerated with 1.0M methanolic HCl and 1M aqueous HCl solutions for 24 hrs. Extracts obtained were stored in air-dried containers, labelled and used for HPTLC analysis. 100mg of 1.0M aqueous hydrochloric acid extracts and 1.0M methanolic hydrochloric acid extracts of all *Mucuna pruriens* samples were dissolved in 10ml of 1.0M methanolic hydrochloric acid and filtered through Whatmann filter paper.

Standard and Sample application: 2,4,6,8 and 10 μ l of the standard solution and 15 μ l of all sample solutions were applied as bands on the plate.

Marketed formulations

Preparation of sample solution:

Procedure for Churna

0.5 g churna weighed and macerated with 10ml 1.0M methanolic hydrochloric acid for 24 hrs. This was then filtered through Whatmann filter paper.

Procedure for Capsules

The capsule was emptied and the powder within was weighed. This was dissolved in 10ml 1.0M methanolic hydrochloric acid and filtered through Whatmann filter paper.

Chromatographic conditions

Chromatographic estimation was carried out using following conditions.

Silica Gel 60 F254 Aluminium plates, 10x10cm with 200 μ m thickness, Merck were used as stationary phase and the mobile phase used constituted of Butanol: Water: Glacial acetic acid in the ratio of 4:1:1. Chamber saturation time employed was 20 minutes and the developing distance was 85mm. Densitometric scanning was performed on TLC scanner III in the absorbance mode at 280 nm at slit width 8 mm \times 0.40 mm, scanning speed 20 mm/s and data resolution 100 μ m/step. Peak area and R_f value of sample and standard were compared.

Derivatization: The plates spotted with sample and standard were sprayed with 0.2 %w/v solution of ninhydrin in acetone and dried in oven at 105°C for 5 minutes.

Method Validation:-

The method was validated in compliance with ICH guidelines. The following parameters were validated.

Linearity

The linearity of responses for L-Dopa was assessed in the range of 2-10 μ g/spot. Five different concentrations of the standards solutions were applied five times to study the linearity.

Accuracy and recovery studies

Both accuracy and recovery were studied. Accuracy of the method was tested by taking three concentrations and three determinations of each analytical concentration. The recovery study was carried out by addition of known amounts of standards to the product. Standards added were 80%, 100% and 120% of 1 μ g of L-Dopa. Three determinations were done to study the recovery. The % recovery of L-Dopa was compared with the actual amounts. Areas of sample, standard and spiked samples were recorded and % recovery was calculated using the following formula.

$$\% \text{ Recovery} = \frac{A+B}{C} \times 100$$

Area of sample = A

Area of standard = B

Area of sample + Area of standard (spiked) = C

Precision**a) System precision**

The system precision study basically focuses on the exactness of the instrument.

Repeatability of sample application and measurement of the peak area were studied. Six determinations at a concentration of 2 μ g/spot, for L-Dopa were applied. The repeatability of sample application and the repeatability of measurement of the peak area were evaluated by comparing their %RSD values which are obtained from the peak area measurements.

b) Method precision

To study the precision of the method, both intra-day and inter-day precision were applied. Intra-day precision was studied by applying three different concentrations 2, 4 and 6 μ g/spot of L-Dopa three times in triplicate to see variation in their peak areas within a day. For inter-day precision the same concentrations were applied but their peak area variation was studied for three different days.

Limit of detection and quantification

Detection Limits (DL) and quantification limits (QL) were calculated from the calibration equations obtained from the experiment. The determinations of the detection and quantification limits were based on the standard deviation of the response and the slope.

The limit of detection and limit of quantification of the proposed method was calculated according to $3.3 \times S.D/S$ and $10 \times S.D/S$ criterions, respectively, where S is the slope of the corresponding calibration curve and S.D is the standard deviation of the y-intercept (n=5) of the calibration curve.

Robustness

In order to study the robustness of the method, slight but deliberate changes were made in some parameters. Parameters such as the mobile phase composition, total mobile phase amount, time from application to development and time from development to scanning were used to study the robustness. Concentrations of 2 μ g/spot of L-Dopa were applied for the analysis.

Summary of validation parameters is tabulated in **Table 1**.

Estimation of L-Dopa using validated HPTLC method.

For quantification of L-Dopa 15 μ l of all sample solutions were spotted along with different concentrations of standard solution.

The chromatograms were developed and scanned at 280nm. The amount of L-Dopa present in each extract was calculated by comparing the peak area of standard and respective samples.

The following formula was used to quantify the active constituent,

$$\% \text{ L-Dopa} = \frac{\text{Area of sample} \times \text{conc. of standard} \times 100}{\text{Area of standard} \times \text{conc. of sample}}$$

HPTLC pattern of extracts along with standard are given in **Figures 3-4**. Results of quantification of acidic aqueous and methanolic extracts of *Mucuna pruriens* samples are reported in **Table 2** and **Figure 5**.

The HPTLC pattern and HPTLC chromatogram of market formulations with standard is given in **Figures 6-9**. Results of quantification are shown in **Table 3** and **Figure 10**.

RESULTS AND DISCUSSION

Macroscopy

All samples were subjected to macroscopical identification based on colour, odour, taste, form, size and fracture of the drug. Morphological features were compared with standard description.

Morphologically no much variation was observed in Velvet bean samples except some small black spots on the seeds of Velvet bean. However, other features were found to be similar to those mentioned in standard monographs.

Seed ovoid, slightly laterally compressed, with a persistent oblong, funicular hilum, dark brown with spots; usually 1.2-1.8 cm long, 0.8-1.2 cm wide, hard, smooth to touch, not easily breakable, white in colour.

Odour : Not distinct

Taste : Sweetish-bitter

Microscopy:

All samples were subjected to powder microscopy.

Microscopically, there were no variations among the samples. All other characteristics were in concordance to those described in standard literature.

Microscopy of *Mucuna pruriens*:

Transverse section of seed shows testa with palisade like cells with thickened anticlinical walls in epidermis. Hypodermis comprises of I shaped bearer cells, which is followed by parenchyma with tangentially elongated cells. Cotyledons comprise of epidermis, and parenchymatous cells containing oil globules and oval starch grains.

Powder is greyish white in colour. Fragments of epidermis, I shaped bearer cells and parenchymatous cells with oil globules and starch grains are the characteristic features [8]. Images of transverse section and powder microscopy are shown in **Figures 1-2**.

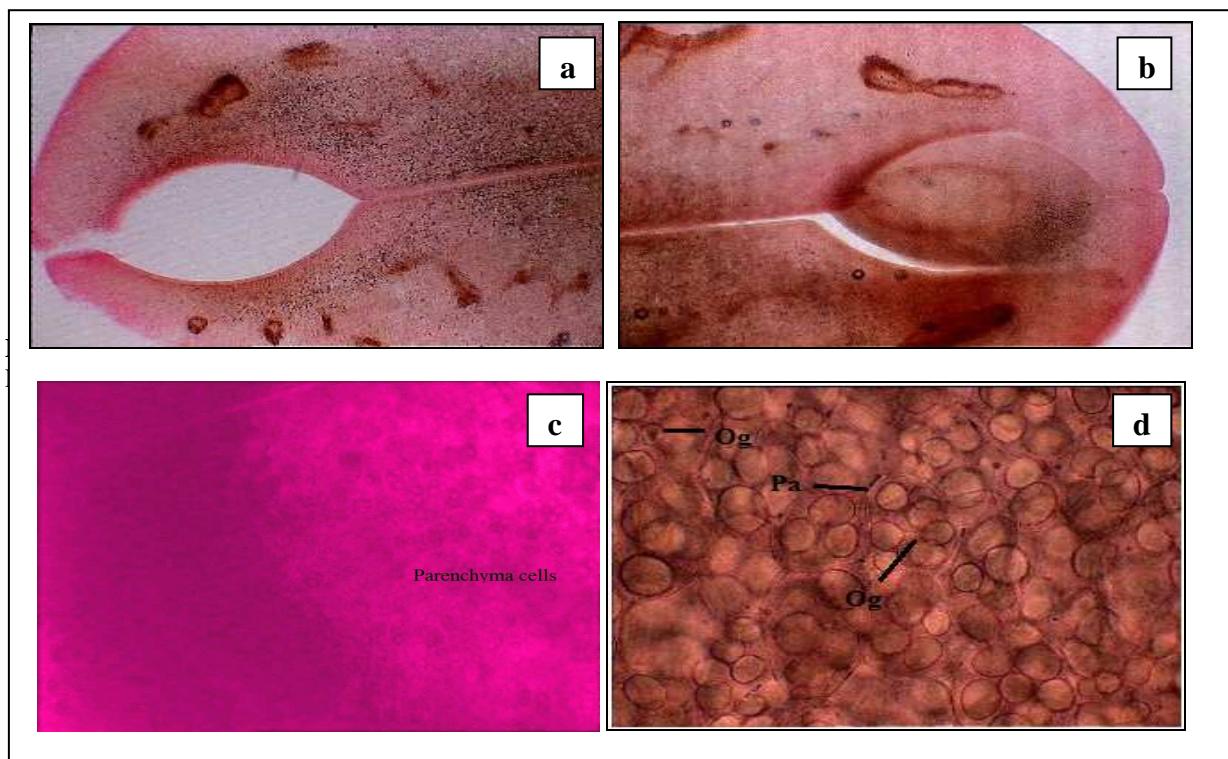


Figure 1: (a-d) Transverse section of *Mucuna pruriens* seed
Pa- Parenchyma, Og- Oil globules, Sg- Starch grains

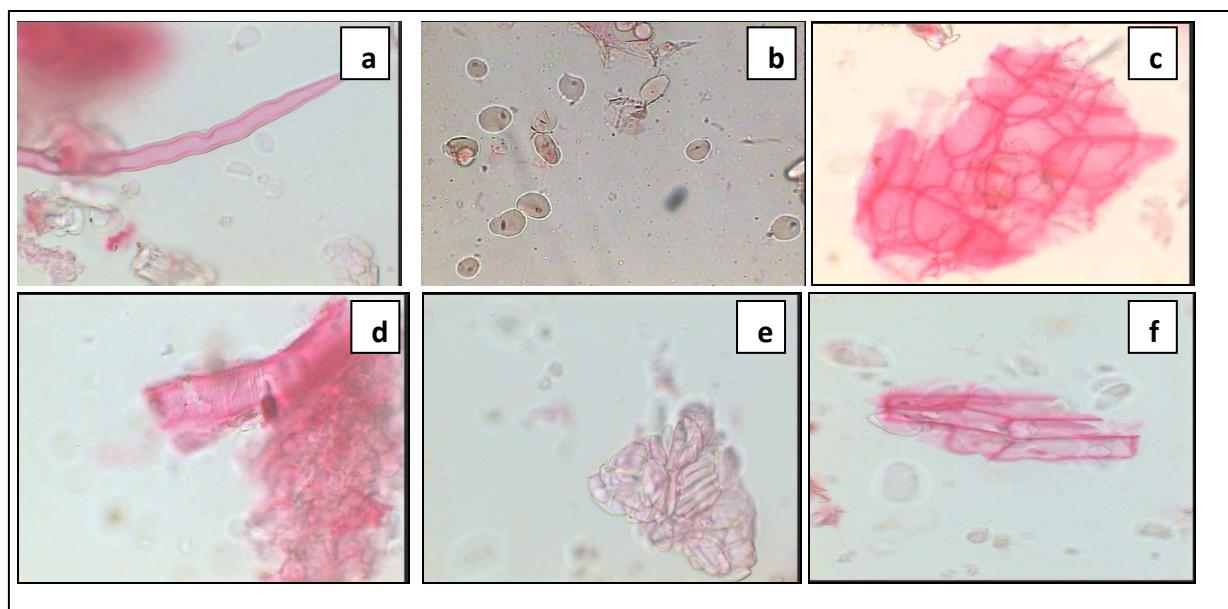


Figure 2: Powder microscopy of *Mucuna pruriens* seeds

a- Lignified trichomes, b- Parenchyma cells with oil globules and starch grains, c- Group of parenchyma cells, d- Epidermal cells, e- Epidermal cells with I shaped bearer cells, f- Elongated parenchyma cells

HPTLC Results of L-Dopa

The mobile phase butanol- water-glacial acetic acid (4:1:1 v/v/v) gave good resolution. Well-defined spots (compact dense spots) were obtained when the chamber was saturated with the mobile phase for 20 min at room temperature. The R_f value of L-Dopa was found to be 0.49 ± 0.02 respectively. The developed method was found to have all parameters within limits of ICH guidelines

Table1: Summary of validation parameters for estimation of L-Dopa by HPTLC method

| Parameter | L-Dopa |
|---|--------------------------|
| λ max (nm) | 280 |
| Linearity range ($\mu\text{g}/\text{spot}$) | 2-10 |
| Correlation Coefficient | 0.9988 \pm 2.65857E-05 |
| Regression equation | $y = 2790.25x + 1121.21$ |
| Limit of detection ($\mu\text{g}/\text{spot}$) | 0.016 |
| Limit of Quantification ($\mu\text{g}/\text{spot}$) | 0.049 |
| Recovery (Mean \pm S.D.) | 99.93 \pm 0.316 |
| Precision (% RSD) | |
| Repeatability of application (n=6) | 0.15 |
| Repeatability of measurement (n=6) | 0.14 |
| Intra-day* | 0.094 |
| Inter-day** | 0.106 |
| Robustness | Robust |

* Mean of three concentrations in triplicates in the same day.

** Mean of three concentrations in triplicates in three different days.

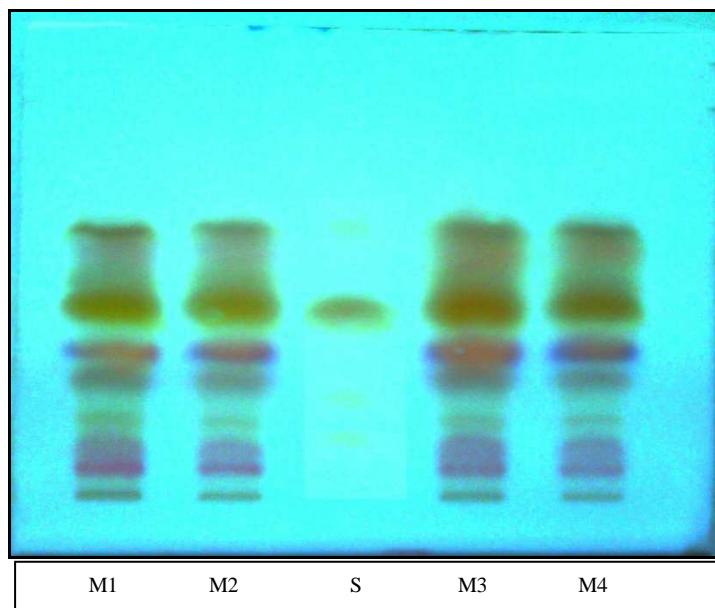


Figure 3: HPTLC pattern of 1 M methanolic hydrochloric acid extracts of *Mucuna pruriens* along with standard L-Dopa sprayed with ninhydrin reagent

M1 – 1 M methanolic hydrochloric acid extract of Gujarat variety

M2 – 1 M methanolic hydrochloric acid extract of Madhya Pradesh variety

S – L-Dopa standard in 1N methanolic hydrochloric acid

M3 – 1 M methanolic hydrochloric acid extract of Andhra Pradesh variety

M4 – 1 M methanolic hydrochloric acid extract of Maharashtra variety

Table 2: Amount of L-Dopa in *Mucuna pruriens* extracts by HPTLC

| Sample | Methanolic Extract | Aqueous Extract |
|-------------------------|--------------------------------|--------------------------------|
| | %L-Dopa ^a \pm S.D | %L-Dopa ^a \pm S.D |
| Sample-1 Gujarat | 12.41 \pm 0.037 | 11.39 \pm 0.078 |
| Sample-2 Madhya Pradesh | 10.73 \pm 0.037 | 11.43 \pm 0.145 |
| Sample-3 Andhra Pradesh | 12.61 \pm 0.085 | 11.45 \pm 0.154 |
| Sample-4 Maharashtra | 13.34 \pm 0.056 | 10.76 \pm 0.376 |

^a=Mean, n=3

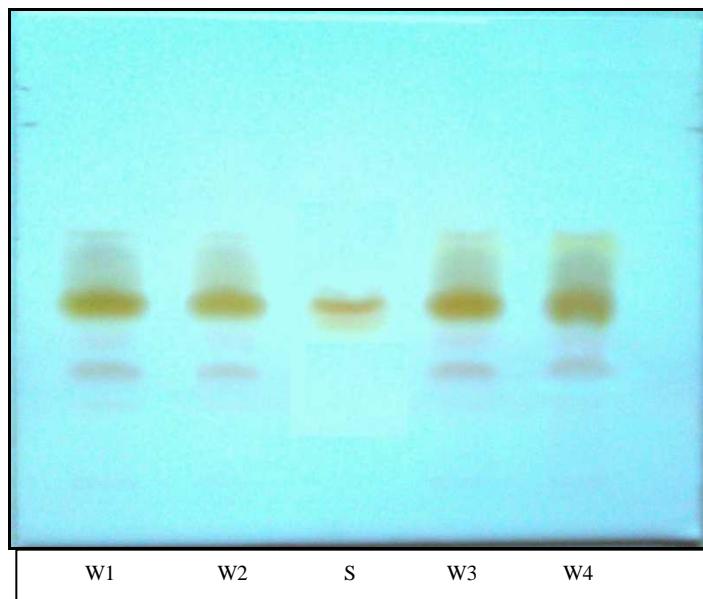


Figure 4: HPTLC pattern of 1 M aqueous hydrochloric acid extracts of *Mucuna pruriens* along with standard L-Dopa sprayed with ninhydrin reagent

W1 – 1 M aqueous hydrochloric acid extract of Gujarat variety

W2 – 1 M aqueous hydrochloric acid extract of Madhya Pradesh variety

S – L-Dopa standard in 1 M aqueous hydrochloric acid

W3 – 1 M aqueous hydrochloric acid extract of Andhra Pradesh variety

W4 – 1 M aqueous hydrochloric acid extract of Maharashtra variety

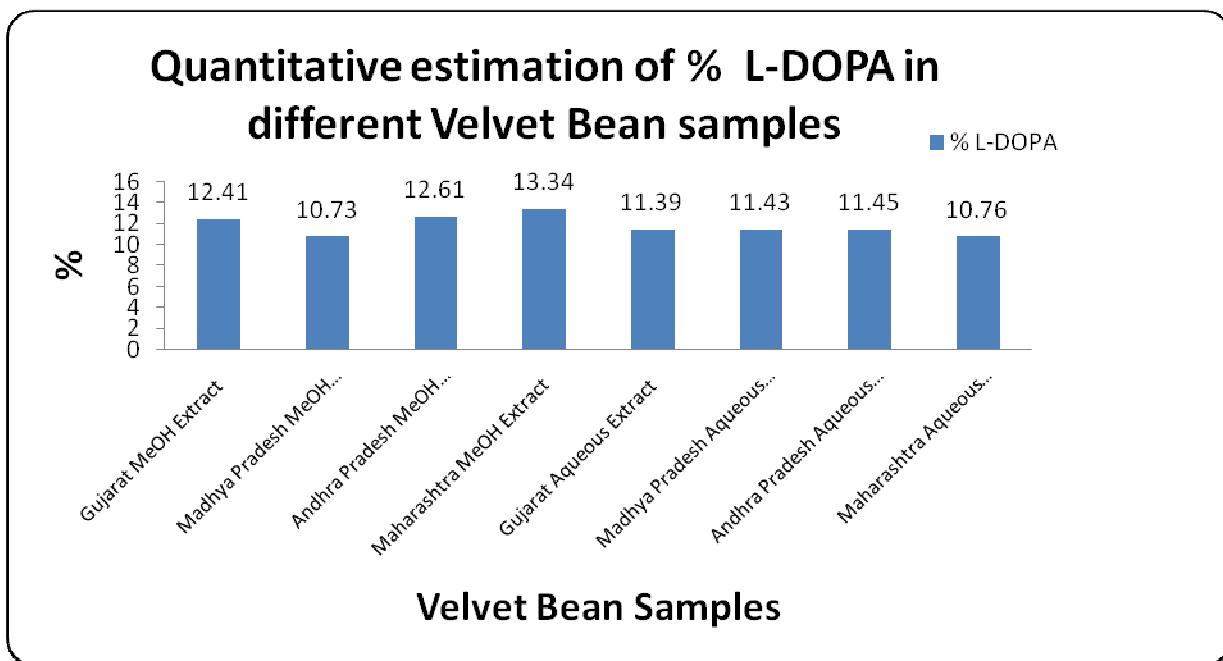


Figure 5: Quantitative estimation of L-Dopa in different *Mucuna pruriens* (Velvet Bean) samples

The amount of L-Dopa in *Mucuna pruriens* samples collected from various geographical regions were spotted developed and calculated by comparing peak area of standard and sample solutions. % of L-Dopa was found to be in the range of 10.73 – 13.34% in 1 M methanolic hydrochloric acid extracts and 10.76-11.45% in 1 M aqueous hydrochloric acid extracts.

The content was found to be within limits given in literature, i.e. not less than 4% [148]. The L-Dopa content was found highest in methanolic extract of Maharashtra sample and lowest in Madhya Pradesh sample. Whereas aqueous extract of Andhra Pradesh samples showed highest L-Dopa content. Variation in L-Dopa content has been observed. The reason may be like climatic conditions, water level, soil and its texture, fertility etc. The present investigation shows that L-Dopa content vary with their respective root source.

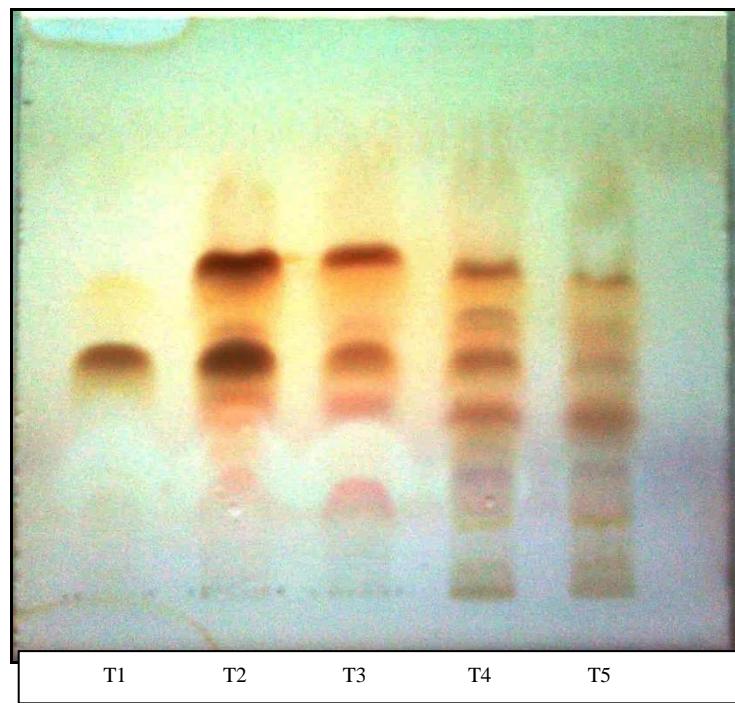


Figure 6: HPTLC pattern of *Mucuna pruriens* formulations sprayed with ninhydrin reagent

T -1 L-Dopa in 1 M Methanolic Hydrochloric acid
T -2 1 M methanolic hydrochloric acid extract of *Mucuna pruriens*
T -3 1 M methanolic hydrochloric acid extract of Zandopa Churna
T -4 1 M Methanolic Hydrochloric acid extract of AP/HCD Capsule
T -5 1 M Methanolic Hydrochloric acid extract of Vigorex SF Capsule

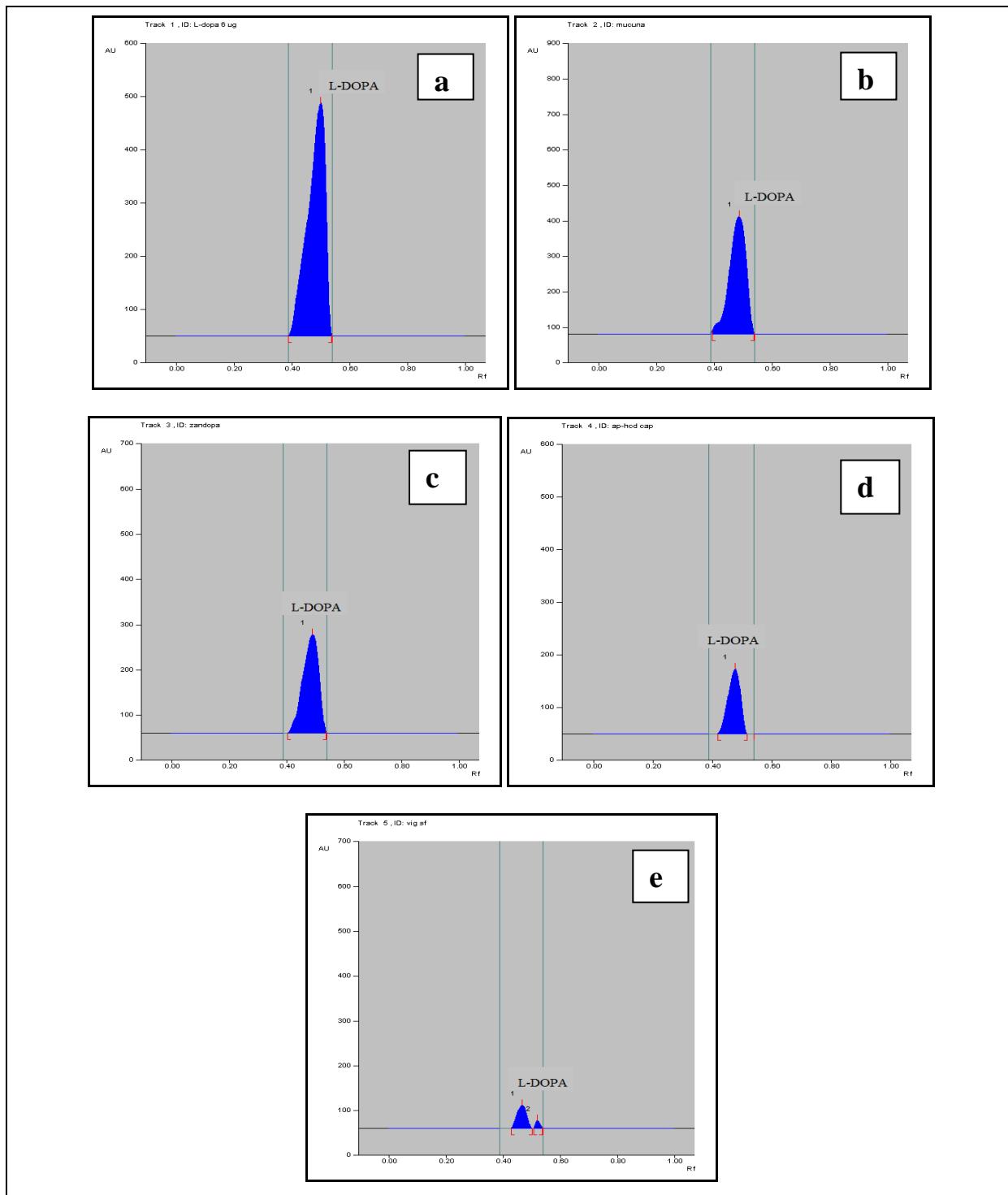


Figure 7: HPTLC chromatogram of 1N methanolic hydrochloric acid extracts of *Mucuna pruriens* marketed formulations along with standard L-Dopa (a-e)

Track -1 L-Dopa in 1 M methanolic hydrochloric acid (a)

Track -2 1 M methanolic hydrochloric acid extract of *Mucuna pruriens* (b)

Track -3 1 M methanolic hydrochloric acid extract of Zandopa churna(c)

Track -4 1 M methanolic hydrochloric acid extract of AP/HCD capsule (d)

Track -5 1 M methanolic hydrochloric acid extract of Vigorex SF capsule (e)

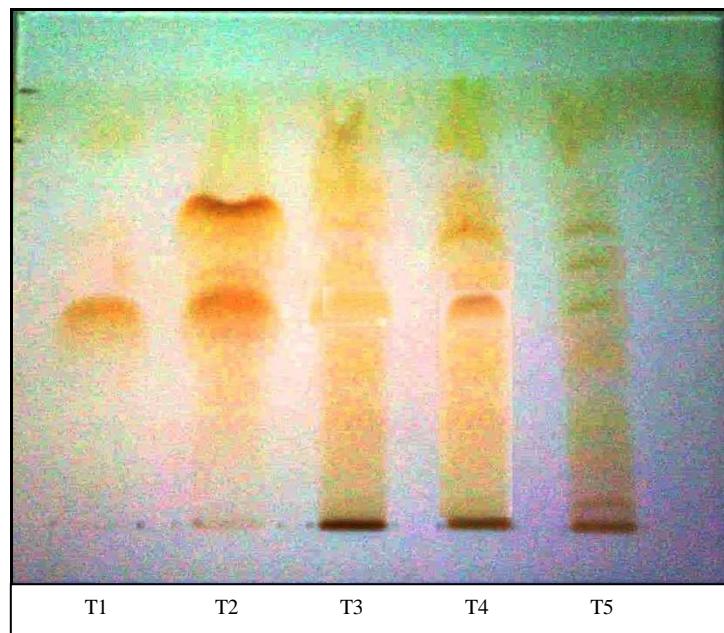


Figure 8: HPTLC pattern of *Mucuna pruriens* formulations sprayed with ninhydrin reagent

T1 - L-Dopa in 1 M methanolic hydrochloric acid extract

T2 - 1 M methanolic hydrochloric acid extract of *Mucuna pruriens*

T3- 1 M methanolic hydrochloric acid extract of Addyzoa Capsule

T4- 1 M methanolic hydrochloric acid extract of Shilajeet Gold Capsule

T5- 1 M methanolic hydrochloric acid extract of Vita X Capsule

Table 3: Percent content of L-Dopa in marketed formulations

| Sample No. | Sample name | % L-Dopa \pm S.D |
|------------|-------------------------|--------------------|
| 1 | Zandopa | 0.56 \pm 0.023 |
| 2 | AP/HCD Capsules | 1.07 \pm 0.025 |
| 3 | Vigorex SF Capsules | 0.46 \pm 0.016 |
| 4 | Addyzoa Capsules | 0.33 \pm 0.055 |
| 5 | Shilajeet Gold Capsules | 1.70 \pm 0.048 |
| 6 | Vita X Capsules | 0.35 \pm 0.020 |

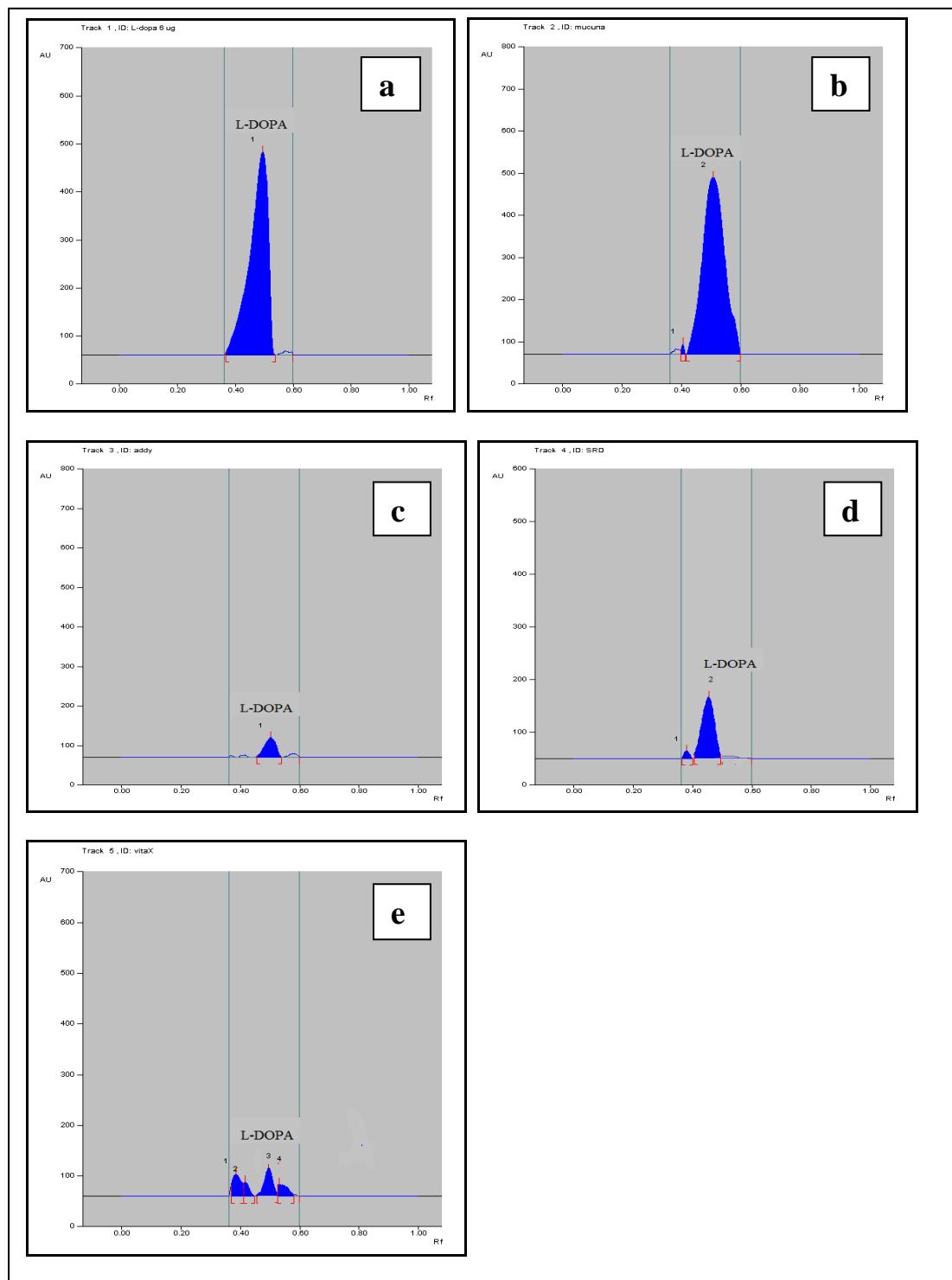


Figure 9: HPTLC chromatogram of 1M methanolic hydrochloric acid extracts of *Mucuna pruriens* marketed formulations along with standard L-Dopa (a-e)

- Track -1 L-Dopa in 1 M methanolic hydrochloric acid extract (a)
- Track -2 1 M methanolic hydrochloric acid extract of *Mucuna pruriens* (b)
- Track -3 1 M methanolic hydrochloric acid extract of Addyzoa Capsule (c)
- Track -4 1 M methanolic hydrochloric acid extract of Shilajeet Gold Capsule (d)
- Track -5 1 M methanolic hydrochloric acid extract of Vita X Capsule (e)

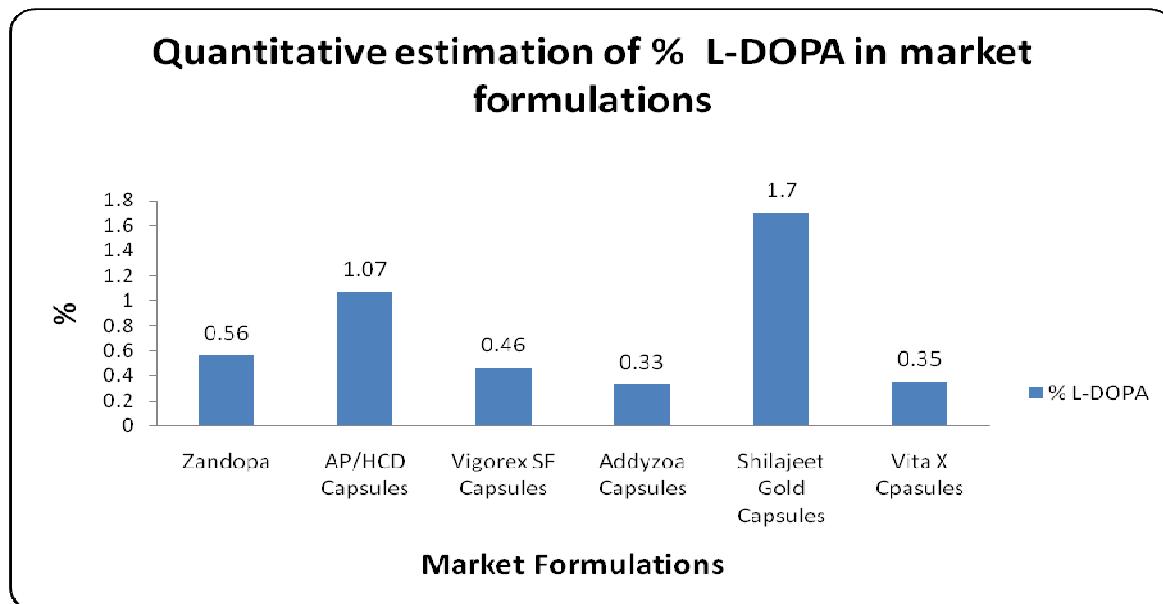


Figure 10: Quantitative Estimation of L-Dopa in different marketed formulations

Various dosage forms comprising of churna and capsules showed presence of L-Dopa in the range 0.33% to 1.70%.

CONCLUSION

A simple, rapid, accurate and convenient method was developed for estimation of L-Dopa by HPTLC. This method was used to standardize different Ayurvedic single and polyherbal formulations containing *Mucuna pruriens*. This developed and validated HPTLC method can be used to determine batch to batch variations and routine analysis by herbal manufacturers of *Mucuna pruriens* formulations.

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