

Seasonal dynamics of Shatavarin-IV, a potential biomarker of *Asparagus racemosus* by HPTLC: Possible validation of the ancient Ayurvedic text.

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The medicinal property of *Asparagus racemosus* is primarily attributed to its constituent steroidal saponins, particularly the major component, shatavarin-IV. Thus, it can serve as a biomarker and its level can decide of the utility of the plant cultivar as a drug. Hence, a sensitive, reliable and quantitative High Performance Thin Layer Chromatography (HPTLC) method has been established for quantification of shatavarin-IV in the methanolic extracts of the roots collected in both summer and rainy seasons. The extracts of the powders of dried roots were applied to silica gel 60 F₂₅₄ aluminum-supported precoated TLC plates and developed with n-hexane: ethyl acetate: methanol, 80:10:10 (v/v), as the mobile phase. Shatavarin-IV was detected and quantified by densitometry at $\lambda = 336$ nm. The accuracy of the method was checked by conducting recovery studies at three different levels of shatavarin-IV. The average recovery was found to be 101% and 107% for summer and rainy seasons respectively. The shatavarin-IV contents, as estimated by the proposed method were $12.5 \mu\text{g gm}^{-1}$ and $10.9 \mu\text{g gm}^{-1}$ in summer and rainy roots respectively. The entire method was performed six times (n=6) to check the repeatability. The proposed HPTLC method for quantitative monitoring of shatavarin-IV in *A. racemosus* roots collected in different seasons strictly adhered to the validation issues laid down by the ICH guidelines. The method is reliable reproducible and highly precise and selective.

Keywords: HPTLC, Quantification, Seasonal, Shatavarin-IV

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The perennial climbing shrub, *Asparagus racemosus* Willd. (family: Asparagaceae) grows in low jungle areas throughout the Indian subcontinent¹ and is also found in tropical Asia, Africa, and Australia. It is known as shatavari (meaning curer (vari) of hundred (shatum) diseases) in Ayurvedic text, and the plant roots are frequently mentioned in traditional and Ayurvedic scriptures². The plant is a constituent of more than 64 traditional and few Ayurvedic formulations³. The ancient classical Ayurvedic literature has recommended as a galactagogue and for treatment of reproductive disorders⁴ and threatened abortion⁵. In addition, *A. racemosus* root is also used against mental, neurological and hepatic disorders; as an antiulcer, anti-inflammatory, antidiabetic, anti-ageing and anti-tumor agent^{6,7}. Its immunomodulatory and immunoadjuvant effects may reduce the toxic side effects of chemotherapeutic drugs without compromising their anti-cancer activity^{8,9}. Some of the ancient claims have also been mechanistically rationalized in animal models¹⁰.

It is well-established that the phytoconstituents contribute to the medicinal benefits of the herbs/plants. Several in vitro, pre-clinical and clinical trials have confirmed that the steroidal saponins, shatavarin I-IV are responsible for the physiological actions of *A. racemosus* roots, and shatavarins I and IV are the major components of the shrub roots¹¹. Based on several laboratory results, the steroidal saponin, shatavarin IV (chemical structure shown in Fig. 1) is reported to be one of the active principles of *A. racemosus* roots. It inhibited the core Golgi enzymes transferase in cell free assays and exhibited immunomodulation activity against specific T-dependent antigens in immuno compromised animals¹². A recent study has shown that shatavarin-IV elicits lifespan extension and alleviates Parkinsonism¹³. Hence, it is used as a marker for quality control and standardization of *A. racemosus* roots.

The concentrations of the phytoconstituents vary depending on the season, maturity and locality of the plants during collection. This was realized centuries ago and finds mention in the ancient

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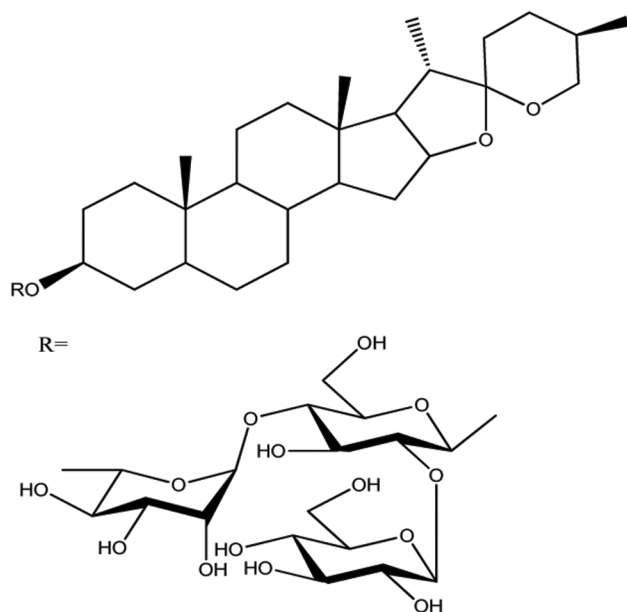


Fig. 1 — Chemical structure of shatavarin-IV

Ayurvedic scriptures (Sushruta, Chapter 36, Verse 5).

‘भेषजद्रव्यमपि किञ्चित् सौम्यं किञ्चिदाग्नेयम्। तेन यत् सौम्यं तत् सोमगुणातिरिक्तेषु सौम्येषु ऋतुषु सम्पूर्णवीर्यतया ग्राह्यम्, एवमाग्नेयमान्नेयेषु, सर्वथा मूलाद्यङ्गे ग्राह्यम्। यत्तु ग्रीष्मादिषु मूलादिग्रहणं तदनुपपत्तिकत्वादसाधु।

सु. सू. 36:5.

‘bhesajacravyamapi kincit saumyam kincidagneyam/ tena yat saumyam tat somagunatiriktesu saumyesu rtusu sampurnaviryataya grahyam, evamagneyamagneyesu sarvatha muladyange grahyam/ yattu grismadisu muladigrahanam tadanupapattikatvadasadhu’

Sushruta, Chapter 36, Verse 5.

The ancient ayurvedic medicinal system claims that *A. racemosus* roots must be collected in summer, but not in the rainy season for higher potency¹⁴. This may be due to seasonal variation in the concentrations of the major active compound of the plant. Earlier, shatavarin-IV concentration in *A. racemosus* roots has been quantified using gas chromatography¹⁵, HPTLC¹⁶ and HPLC¹⁷ techniques. However, there is no report on the quantification of shatavarin-IV in the plant roots, collected in different seasons. It was felt that such a study is essential to validate the ancient Ayurvedic claim, made in Sushruta thousands of years ago. Hence, the objective of the present study was to develop and validate a rapid, simple, sensitive, robust and reproducible densitometric quantitation method for estimation of shatavarin-IV in

A. racemosus roots collected in summer and rainy season. For this, we used HPTLC technique that has emerged as an efficient tool for phytochemical evaluation of herbal drugs, because of its simplicity and the need of minimum sample cleanup.

Methodology

Reagents and standards

GR grade solvents *n*-hexane, ethyl acetate and methanol were obtained from E. Merck Ltd, Mumbai, India. Shatavarin-IV standard was procured from Natural Remedies Ltd., Bangalore, India (Lot No. T11E117).

Plant materials

Roots of *A. racemosus* were collected from the cultivated Medicinal and Botanical Plant Garden (GPS position latitude 30°18'55" N, longitude 76°23'28" E) of NIAPR, Patiala, Punjab, India, once in summer (end of April), designated as SS and in rainy days (mid of July), designated as RS. Roots were authenticated by BSI (Botanical Survey of India), Howrah and the respective voucher specimen numbers 41246 & 49358 are preserved there and in the place of research, i.e., Central Ayurvedic Research Institute of Drug Development, Kolkata, Ministry of AYUSH, Government of India, for future reference.

Preparation of sample solution

The each seasonally collected roots of *A. racemosus* were separately dried in the shade¹⁸, powdered, and the powder was passed through an 80 mesh sieve and stored in an airtight container at 25°C. The dried powder of each root (1 g each) was accurately weighed and placed in two different stoppered containers. Methanol (10 mL) was added and the samples were sonicated for 45 min and left to stand overnight at room temperature (28±2°C). The contents were filtered through Whatman No. 41 paper (E. Merck, Mumbai, India). The clear supernatants were collected in dry 10-mL volumetric flask diluted up to the mark with methanol. The extracts of roots of *A. racemosus* were marked as RS and SS respectively for roots collected in rainy days and roots collected in summer days and these solutions were used for the assay experiment.

Preparation of shatavarin-IV standard solution

A stock solution of shatavarin-IV (0.1 mg mL⁻¹) was prepared by dissolving 10 mg accurately weighed

shatavarin-IV in methanol and diluting it to 100 mL with methanol. An aliquot (1.0 mL) of the stock solution was transferred to a 100 mL volumetric flask and the volume was adjusted to 100 mL with methanol to obtain the working standard solution containing 1 ng mL^{-1} .

Calibration plot for shatavarin-IV

Chromatography was performed on $10\text{ cm} \times 10\text{ cm}$ TLC plates precoated with 0.2 mm layers of silica gel 60 F₂₅₄ (E. Merck). Before use, the plates were washed with methanol. Working standard of shatavarin-IV solution of different volume were applied to the plates, as 8 mm bands at 15 mm above from bottom edge of the plate, by means of a Camag ATS-4 automatic TLC sample applicator. The plate was developed with *n*-hexane: ethyl acetate: methanol, 8: 1: 1 (v/v) as the mobile phase in a twin trough chamber (maker: CAMAG, Switzerland) at $23 \pm 2^\circ\text{C}$ and relative humidity as $\sim 48\%$. The development distance was 70 mm . The plate was then dried in air and derivatised by dipping it in 20% aqueous sulphuric acid solution and further heating at 105°C for 10 min . Derivatised plate was then scanned at $\lambda = 336\text{ nm}$ by Camag TLC Scanner 4 and WINCATS software (version 1.4.6). Peak areas were recorded for shatavarin-IV and a calibration plot was obtained

by plotting peak area against shatavarin-IV concentration. The plot was linear in the range $6\text{--}14\text{ ng}$ on plate with a correlation coefficient of 0.999 . The limit of quantification was found to be 6 ng and the limit of detection was 2 ng .

Estimation of shatavarin-IV in samples of RS and SS

The sample solutions of RS and SS were applied $4\text{ }\mu\text{L}$ and $2\text{ }\mu\text{L}$ respectively, in triplicate, to the precoated silica gel 60 F₂₅₄ plates, with the Camag ATS-4 automatic TLC sample applicator. The plate was developed, derivatised and scanned in similar manner. The peak areas and absorption spectra were recorded. The amount of shatavarin-IV in the RS and SS samples were calculated for each solution by use of the calibration plot, a quantification plot is represented in Fig. 2.

Method validation

Method validation^{19,20} was performed to check the reliability of the method developed. Linearity ranges, limit of detection and quantification, precision (both intraday and interday precision) were studied to check the reproducibility. Accuracy of the method developed was confirmed by the recovery study through known dilution of the standard spiking in the sample. The robustness or ruggedness of the method was studied by varying a few parameters.

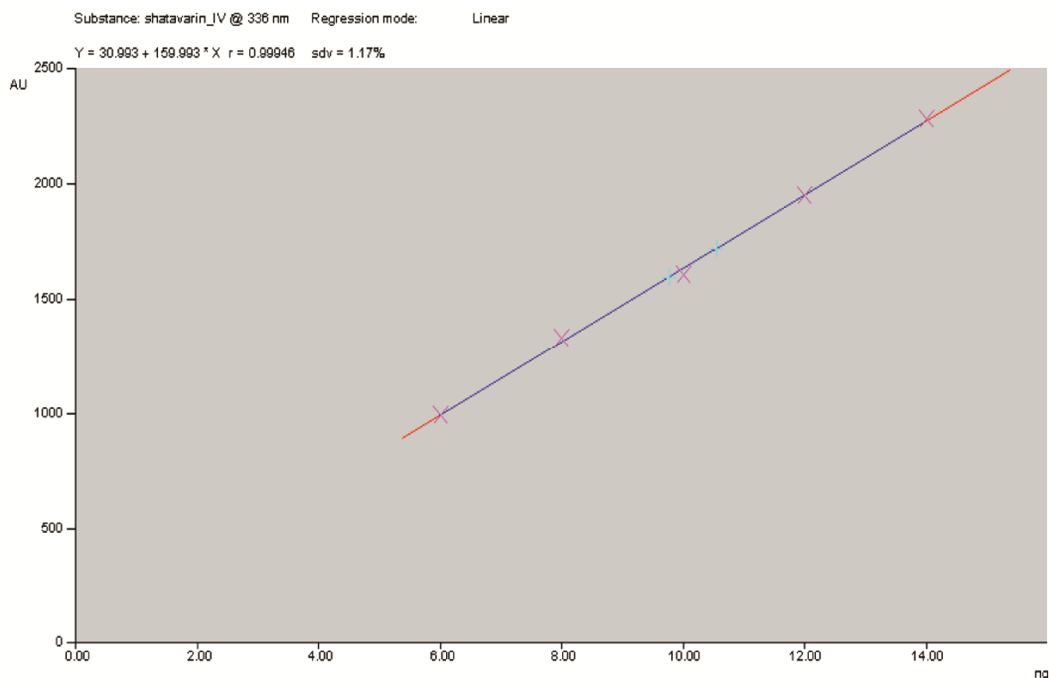


Fig. 2 — Quantification curve of shatavarin-IV

Linearity

Linearity shown by the analyte standard shatavarin-IV has good correlation coefficient in concentration range of 6-14 ng spot⁻¹ ($r = 0.9999$) Linearity was calculated by determining six standard working solutions. The linearity of calibration graph was validated by high value of correlation of coefficient, and the standard deviation (SD) for intercept value was less than 2%. No significant difference was observed in the slopes of standard curves. The data related to linearity study represented in Table. 1 and linearity curve is represented in Fig. 3.

Limit of Detection (LOD), Limit of Quantification (LOQ)

The lowest amount of an analyte in a sample that can be detected but not necessarily quantitated is LOD whether LOQ is the lowest amount of analyte

Table 1 — Linear Regression analysis data for Calibration

Detection Limit (LOD)	2 ng spot ⁻¹
Limit of Quantification (LOQ)	6 ng spot ⁻¹
Linear Range	6-14 ng spot ⁻¹
Number of levels	5
Correlation Co-efficient (by area)	0.999
Linear equation (by area)	$Y=30.864+100.606*X$

that can be detected in a sample with accuracy and precision. In the methods, LOD and LOQ were determined using the following equation:

$$\text{LOD} = 3 * \sigma/S; \text{LOQ} = 3 * \text{LOD};$$

Where “ σ ” is the standard deviation of y-intercept and “S” is the slope of calibration curve.

Accuracy

It was tested by recovery study by standard addition method. The standard analyte was added to pre-analyzed sample solution at 80, 100, and 120% level. The mixed solution of sample and standard were applied for three times for each level. The recovery was calculated by comparing with the expected result. Recovery data are tabulated in Table 2 and curve represented in Fig. 4.

Precision

The method was studied as repeatability, and intra-day and inter-day variation. The precision of the method was expressed as a percentage of relative standard deviation (% RSD); The RSD measured in the study of intra-day and inter-day precision was in the range 1.46-1.71% and 1.78–1.83%, respectively.

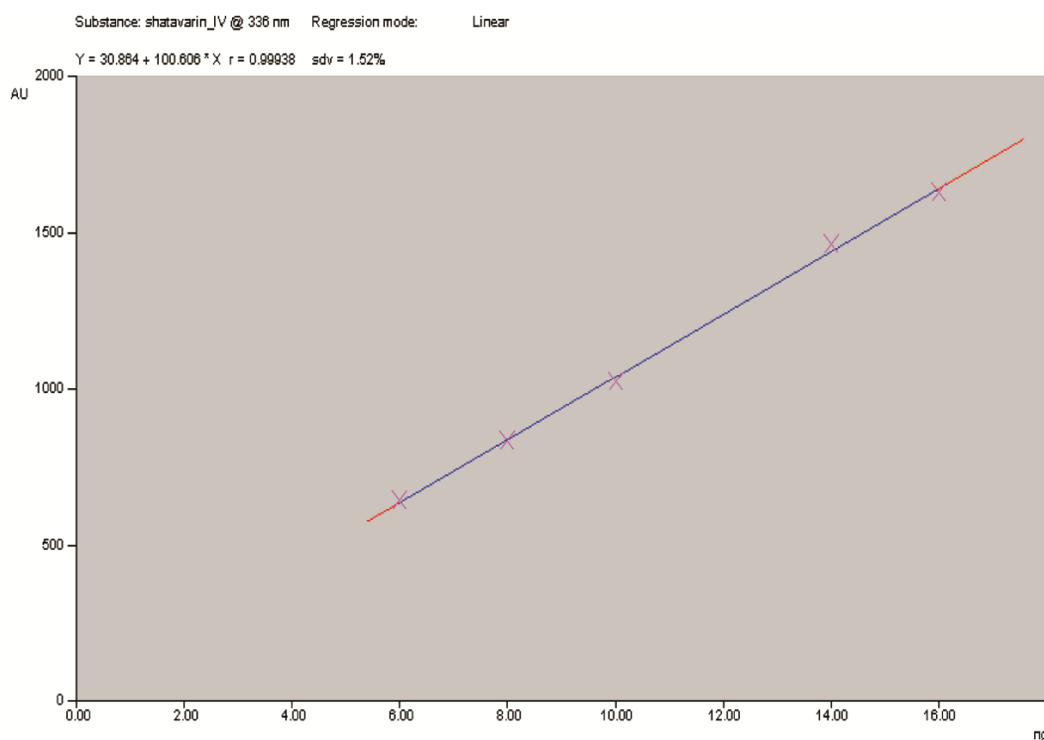


Fig. 3 — Linearity curve of shatavarin-IV

Because of low % RSD value (<2), the method was precise. The calculated precision data are represented in Table 3.

Robustness

Ruggedness or robustness of the method was checked by introducing small changes in mobile phase volume, duration of tank saturation, development distance, relative humidity, time from application to chromatogram and time from chromatogram to scanning; the effects on result were examined. Robustness was done in triplicate at a concentration level of 10 ng spot⁻¹ for shatavarin-IV, and peak areas were calculated. No significant change was observed in R_f or response (area calculated) to shatavarin-IV, indicating that the method was robust and the data

related to these are computed and represented in Table 4.

Specificity

This study was performed by the peak purity of shatavarin-IV and was tested by correlating the spectra of shatavarin-IV extracted from root and shatavarin-IV standard at the peak start (S), peak maxima (M), and at the peak end (E) positions. Correlation between these spectra indicated purity of shatavarin-IV peak (correlation r [S, M] = 0.999, r [M, E] = 0.999).

Table 3 — Precision study of method (n=6)

Precision	Intra-day	Inter-day
Amount per fraction (ng)	10 ng spot ⁻¹	10 ng spot ⁻¹
% RSD range	1.46-1.71	1.78-1.83

Table 2 — Recovery study data (results are average of three determinations at each level)

Sample name	amount of standard in sample	% age of standard spiked	Recovery in %	Average Recovery in %
RS	8.785 ng	80	106	107
	8.785 ng	100	108	
	8.785 ng	120	109	
SS	10.02 ng	80	96	101
	10.02 ng	100	103	
	10.02 ng	120	104	

Table 4 — Robustness study of the method (results are average of three determinations)

Condition	R _f values
Mobile phase volume (±2 mL)	0.11
Development distance (±1 cm)	0.11
Tank saturation time (±5 min)	0.12
Relative Humidity (±10%)	0.11
Time from application to chromatogram (±15 min)	0.11
Time from chromatogram to scanning (±15 min)	0.11

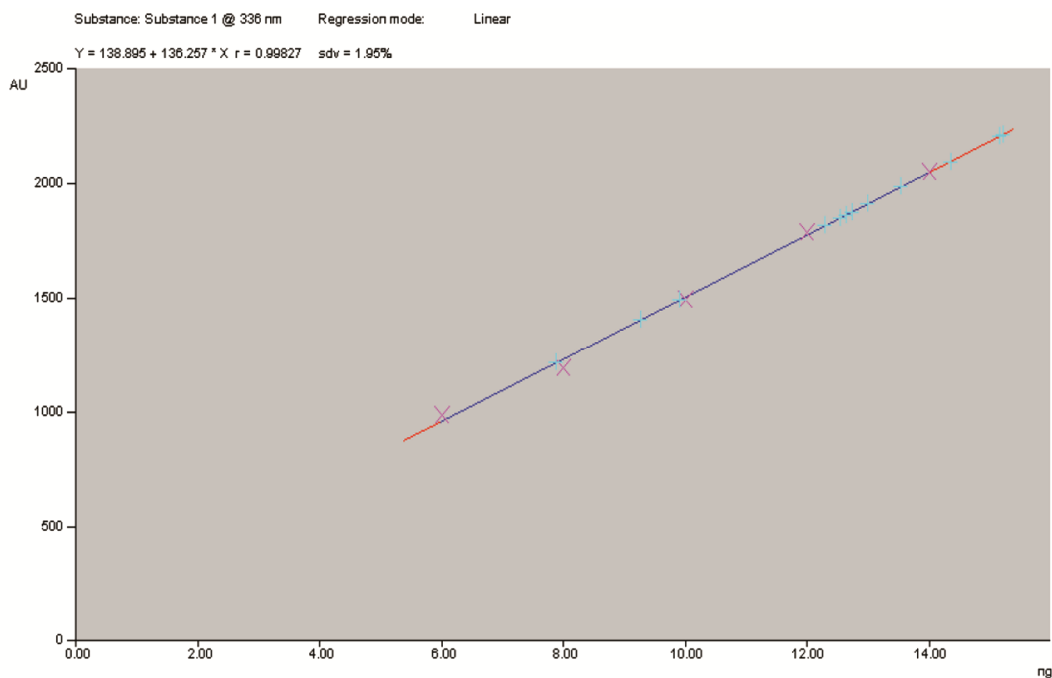


Fig. 4 — Recovery curve of shatavarin-IV

Stability

Stability of analyte was studied both in solvent methanol and the TLC plate. The stability of shatavarin-IV in methanol was performed by applying 10 mg/spot on TLC plate after interval of 24 h for consecutive days and scanning the plate as described above. The stability of shatavarin-IV on TLC plate was performed by rescanning the developed HPTLC plate after interval of 24 h for subsequent days. Spectral comparisons for stability study in solution and on plate are represented in Fig. 5a and Fig. 5b respectively.

Results and discussion

Of the different mobile phases investigated, *n*-hexane: ethyl acetate: methanol 80:10:10 (v/v), resolved shatavarin-IV ($R_f = 0.11$) very efficiently from the other components of the methanolic extracts of roots of RS and SS. The identity of the shatavarin-IV band in the sample extract was confirmed by overlaying the absorption spectrum of the sample with that from the reference standard, obtained by use of the Camag TLC Scanner. The method was validated in terms of precision, reliability, and accuracy. The response to shatavarin-IV was found to be linearly dependent on concentration in the range 6-14 ng absolute quantity on plates, with a correlation coefficient of 0.999. The intraday and interday precisions, expressed as % RSD, were 1.46 and 1.78 respectively, indicating that the proposed method is precise and reproducible. The average recoveries of shatavarin-IV at three different levels were 107% and 101% for RS and SS respectively. The average shatavarin-IV contents of the RS and SS samples, found by the proposed method were 10.9 mg g⁻¹ and 12.5 mg g⁻¹ respectively. The seasonal variation in relation to collection period was significant, the roots (SS) collected in the summer season being enriched with shatavarin-IV. Fig. 6 shows the HPTLC profiles

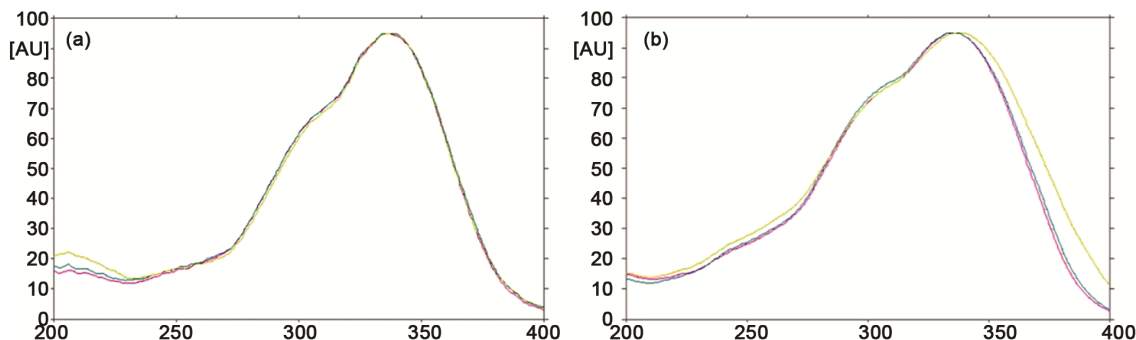


Fig. 5 — Spectral comparisons for stability study (a) in solution and (b) on plate

of a shatavarin-IV standard and the methanol extracts of RS and SS roots, visualized in white light after derivatisation. A typical chromatogram obtained from shatavarin-IV standard and from a methanolic extract of roots of RS and SS represented in Fig. 7.



Fig. 6 — HPTLC profiles of the methanol extracts of roots of rainy season (RS) and summer season (SS) and standard shatavarin-IV (S4) visualised at white light after derivatisation

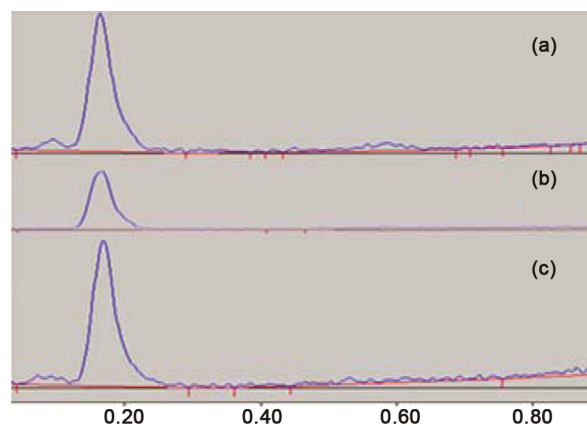


Fig. 7 — TLC chromatograms obtained from methanol extract of RS roots (a), standard shatavarin-IV (b) and methanol extract of SS roots (c)

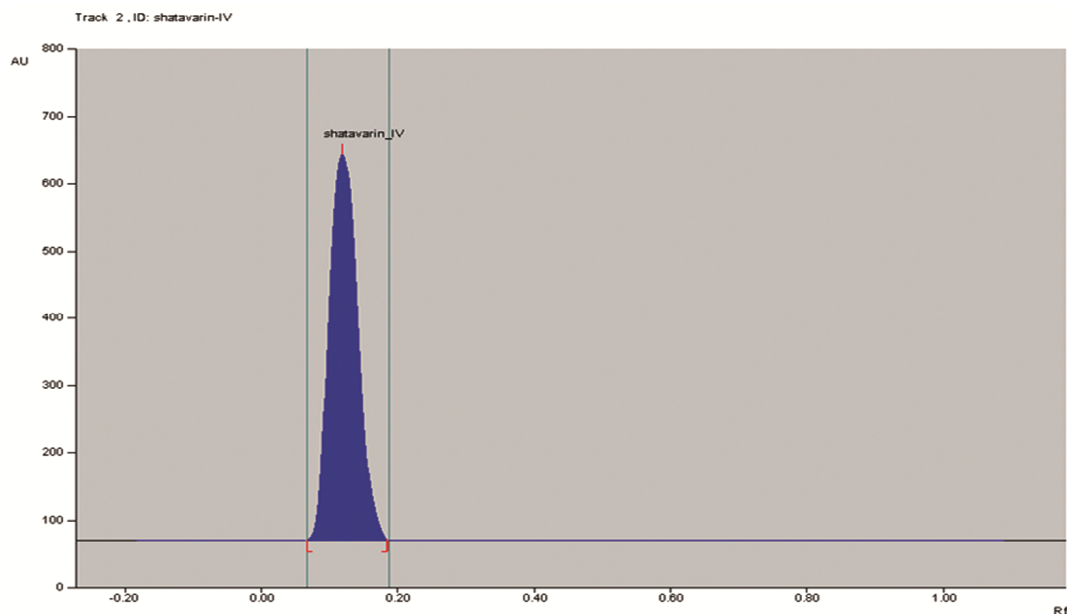


Fig. 8 — Chromatogram obtained from shatavarin-IV, (R_f 0.11) on silica gel 60F₂₅₄ with *n*-hexane: ethyl acetate: methanol 8:1:1 (v/v) as the mobile phase.

Chromatogram obtained from shatavarin-IV, (R_f 0.11) on silica gel 60F₂₅₄ with *n*-hexane: ethyl acetate: methanol, 8:1:1 (v/v) as the mobile phase is represented in Fig. 8.

Conclusion

Shatavarin-IV is reported to be one of the major constituents of *A. racemosus* roots. HPTLC is a suitable tool for quantification of such steroidal saponins. We developed a simple, precise and accurate HPTLC method for estimation of shatavarin-IV from the *A. racemosus* roots collected at 2 different seasons. The validated method revealed that the quantity of shatavarin-IV is more in the roots collected in summer than that collected in the rainy seasons. The authors reached a conclusion that these findings provide an evidence to support the ancient text claim in respect to the quantity of the biomarker present in the plant.

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