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Geographical classification of *Adhatoda vasica* based on HPLC fingerprint analysis coupled with multivariate analysis

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Abstract
Adhatoda vasica (Nees) of family acanthaceae is a shrub growing throughout the Indian cape and is valued for having alkaloids with bronchodilation activity. Both seasonal and geographical variation is witnessed for the chemical constituents of the plant. A steadfast and comprehensive method for classifying the origin and assessing the quality of *Adhatoda vasica* has been developed. The method is based on analysis of HPLC fingerprints, combined with principal component analysis (PCA). Sixteen samples of *Adhatoda vasica*, collected from different origin of India, were used to establish the fingerprints and 15 peaks were selected for the analysis. PCA classified all the 16 samples into two groups. This method was successfully used to identify the geographical origin of the *Adhatoda vasica* samples whether from Gujarat or not and to evaluate their quality.

Keywords: *Adhatoda vasica*, HPLC, fingerprint, principal component analysis

Introduction
Adhatoda vasica (Nees) is a shrub having an imperative medicinal value globally. This plant has been used commonly in Ayurvedic System of medicine (Singh *et. al*, 2017) [1]. It is a well known plant by its Sanskrit name Vasaka. The plant is valued for containing bronchodilator alkaloids, mainly vasicine. So, all the parts of the plants are widely used for respiratory treatment like cold, cough, whooping cough and chronic bronchitis and asthma as sedative expectorant. Pyrroquinazoline are alkaloids the main constituents of *Adhatoda vasica* (Lone *et al.*, 2013) [2]. *Adhatoda vasica* may play a vital role as an important source for the discovery of new and potential drug molecules and can be used as a novel medicinal herb (Kapgate, 2018) [3]. Raw material which is manufactured should be of good quality and effective. Apart from this, In Charak Samhita it is clearly mentioned that, effectiveness of drug will also create impact whether it is collected from proper *desh* i.e. place or in proper season when it is completely grownup and properly stored. Other than charaka Samhita, the collection and storage of crude drug described in Ayurvedic texts like, Sushrut Samhita, Ashtang hridaya, Sharangdhar Samhita etc. can also be useful (Shailesh, 2015) [4]. Chemistry of plant drugs may vary with factors such as growing stage, harvest time, locality, storage condition, processing and manufacturing procedures, but they can yield robust information to address adulteration issues for quality assurance in herbal industry (Hegde *et al*., 2017) [5]. In one study, Highest amount of chemical constituents found to be present in the inflorescence and in the month July to September (Ranade and Acharya, 2015) [6]. In another study about 125 individual plant accessions collected from five different geographical locations estimated for vasicine concentration showed a significant variation in the amount of the alkaloid present (Varma *et al*., 2011) [7]. So, In case of *Adhatoda vasica* such a geographical and seasonal chemical variation entertain towards quality control. For the authentication of Indian herbal medicines one or two markers in herbs or herbal mixtures are used. As this strategy does not evaluate all chemical components present in the chromatographic profile, it has been proved to be insufficient for the quality control as the whole components in crude herbs is responsible for their beneficial medicinal effects. With the development of analytical technique, chromatographic fingerprints have been widely used for the authentication and quality control of medicinal plants (Gong *et al*., 2003 and Liang *et al*., 2004) [8, 9]. Although the differences between chromatograms can be visually discriminated, the process is subjective and not quantitative. In addition, the fingerprint chromatograms consist of complex multivariate data sets due to the complexity of herbal medicines, so minor differences between very similar chromatograms might be missed (Donno *et al*., 2016) [10]. Thus, the chemometric methods, such as clustering analysis (CA) and principal component analysis (PCA), etc., should be

taken into consideration for reasonable pattern recognition, further for quality control of these herbal medicines (Duan *et al*., 2012) [11]. In this study, a simple, sensitive and reliable HPLC method was developed for establishing the chemical fingerprints of 16 samples of *Adhatoda* v*asica* from various locations in India. *Adhatoda* v*asica* from different locations were efficiently discriminated when combined with chemometric method, such as PCA. The aim was to establish an efficacious method and development of strategies for conservation, utilization and domestication of *Adhatoda* v*asica*.

Materials and Methods

Plant material collection

The leaves of *Adhatoda* v*asica* were collected from different locations of India in month of July to September. Total 16 samples of *Adhatoda* v*asica* were collected. The plant was identified by a taxonomist and a herbarium specimen was deposited in the Pharmacognosy department of our institute.

Chemicals and reagents

Deionized water was used from in-house Milli-Q system (Millipore, USA). All the solvents Methanol (Merck, Mumbai, India), and Acetonitrile (RANKEM, RFCL Ltd., Haryana, India) used were of high-performance liquid chromatography (HPLC) grade. Potassium dihydrogen phosphate and triethylamine was obtained from Sigma–Aldrich. All the solutions were filtered through a 0.22 mm membrane.

Sample preparation

1 g Powdered sample was extracted by heating on water bath in 25 mL methanol for 1 hr at 45° C and filtered through Whatman No. 1 filter paper. Final volume was makeup to 25 mL. All test samples were passed through 0.20 µ nylon filter (Sartorius, Germany) before injecting into HPLC.

Instrumentation and chromatographic conditions

HPLC analysis was performed using a Waters system (Waters Corp., Milford, MA, USA), equipped with a 600 series binary pump, a vacuum degasser, a 2707 auto sampler and a 2487 dual λ absorbance detector. Chromatographic separations were achieved using gradient elution on a Thermo scientific BDS HYPERSIL C18 column (250 mm*4.6 mm, 5 µm). Mobile phase A 0.5M Phosphate buffer (pH: 5) and Mobile phase B was Acetonitrile. The gradient elution program was as follows: 0% (B) for 0–10 min, 10% (B) for 10–20 min, 15% (B) for 20–30 min, 20% (B) for 30–40 min, 25% (B) for 40–50 min, 40% (B) for 50–60 min, 50% (B) for 60–61 min, and 0% (B) for 60–61 min. with injection volume of 50 µL. The flow rate was 1 mL/min and the detection wavelength was set 273 nm with 70 min run time and and Empower software (Waters Corp.) was used for data collection and processing.

Chemometric analysis for quality evaluation

Principle component analysis

For further discriminating the investigated samples, PCA, a sophisticated technique widely used for reducing the dimensions of multivariate problems, was carried out based on the differences in the samples. This technique can reduce the dimensionality of the original data set by explaining the correlation amongst a large number of variables in terms of a smaller number of underlying factors (principal components or PCs) without losing much information (Pierce *et al*., 2005) [12]. In this study, PCA was performed on the common peak areas from the HPLC fingerprints using Minitab 17 software.

Result and Discussion

Optimization of HPLC condition

To give the most chemical information and best separation in the chromatograms, the mobile phase and its flow rate, conditions for elution, column temperature and detection wavelength were investigated in this study. The chromatographic conditions were optimized for 16 samples. The different ratio of Phosphate buffer (pH: 5) to Acetonitrile in the mobile phase could elute different components, so the gradient elution was selected. From the results of comparative study of column temperature of 20° C, 25° C and 30° C and flow rate of 0.8, 1.0 and 1.2 mL/min, column temperature was kept 25° C and the flow rate was set at 1 mL/min. Furthermore, the UV absorbance was monitored at the range of 210–400 nm; and by comparison, 273 nm was selected as the detection wavelength. Under the optimal conditions, the components in the extracts of these 16 samples (Table 1) were well separated.

Table 1: Different samples collected from different location of India tested in this work.

Sr. No	Samples Collected from different Origin
1	Valsad, Gujarat
2	Veraval, Gujarat
3	Vadodara, Gujarat
4	Banaskatha, Gujarat
5	Gandhinagar, Gujarat
6	Rajkot, Gujarat
7	Banglore, Karnataka
8	Mohali, Punjab
9	Indore, Madhya Pradesh
10	Ahmedabad, Gujarat
11	Karnal, Haryana
12	Thane, Maharashtra
13	Bhubaneswar, Odisha
14	Ernakulam, Kerala
15	Surendranagar, Gujarat
16	Bhavnagar, Gujarat

HPLC fingerprints of *Adhatoda* v*asica*

To obtain the standard fingerprint, 16 batches of *Adhatoda* v*asica* samples were analyzed. Chromatograms of these samples are shown in Figure 1. All the peaks that existed in all samples were initially utilized for the identification of the plant.

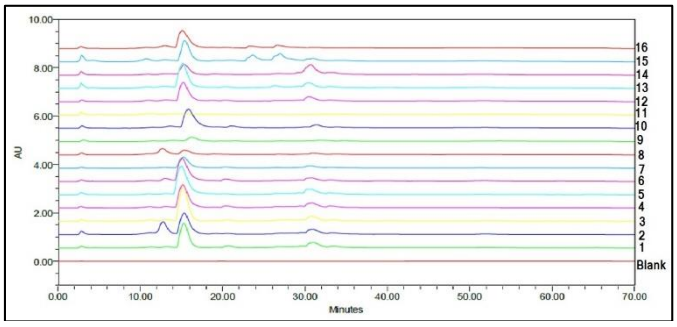

Fig 1: Overlain chromatogram of HPLC fingerprints of 16 samples (1–16)

Quality evaluation by PCA

Samples can be easily distinguished and discriminated using dimensionality reduction method, PCA. PCA was carried out

and after using covariance type of matrix; all data were displayed as scores and loadings in a coordinate system of principal components resulting from data dimensionality reduction. Based on PCA analysis variables (retention time) reduced to 15. Out of 41, 15 important retention times are 2.8, 10.5, 11.3, 13.2, 15.3, 16.3, 20.7, 22.4, 26.1, 26.9, 29.1, 29.5, 30.4, 31.1, and 39.1. As result, the first 5 Principal components as shown in Figure 2 (A) account for most of the total variability in the data. A two dimensional PC score plot clearly classifies the *Adhatoda vasica* samples into two groups as shown in Figure 2 (B). Group 1 is showing the samples from outside Gujarat and group 2 is showing the samples from Gujarat. So, the above results suggested that the authenticity of *Adhatoda vasica* samples could be determined through HPLC analysis coupled with PCA.

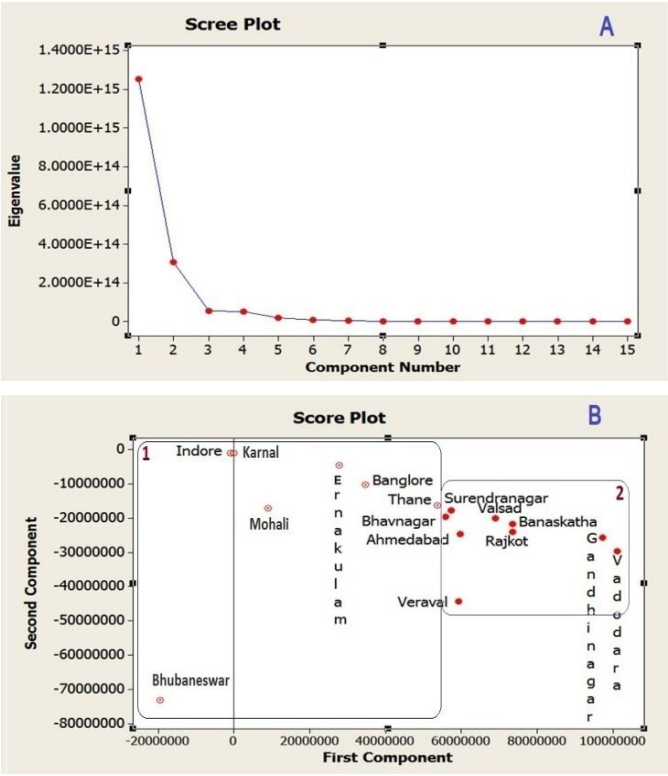

Fig 2: (A) Screes plot of 16 samples. (B) Scores plot of PCA for different *Adhatoda vasica* samples on the first two PCs

Conclusion

In this study, a simple, reliable and accurate HPLC fingerprint profile was developed. The fingerprint of the *Adhatoda vasica* sample showing 15 important peaks represented the characteristics of the herb's constituents and provided an accurate method for the quality control of *Adhatoda vasica*. Chemometric methods should be applied with the HPLC fingerprint techniques for the geographical classification of the *Adhatoda vasica* samples. In conclusion, to improve the current quality insurance technology of *Adhatoda vasica*, HPLC coupled with multivariate analysis were developed. Integrated HPLC and multivariate analysis supplies qualitative determination so our method could be a critical complement for current quality control studies of *Adhatoda vasica*.

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