ISSN (Print) : 0974-6846
ISSN (Online) : 0974-5645
Indian Journal of Science and Technology, *Vol 9(26), DOI: 10.17485/ijst/2016/v9i26/89180, July 2016*

Liquid Chromatography Coupled to Mass Spectrometry Based Identification of Elite Chemotypes of *Adhatoda vasica* Nees for Profitable Agronomy – A Farmer Centric Approach

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Abstract

In this paper we have identified accessions of a traditional medicinal plant, Adhatoda vasica Nees, from eight eco-geographically distinct nodes of Western Ghats of Malabar area, India. Alkaloids have been extracted from the leaves and analysed qualitatively using spectroscopic techniques. A method has been developed to determine relative amounts of one of the active ingredients – vasicine, using Liquid Chromatography coupled to Mass Spectrometry (LC-MS/MS). The study has revealed samples from Kanjikode and Wadakencherry to have high amounts of vasicine compared to the rest in the study area. Future work should be directed in evaluating samples from similar forests of Western Ghats of Kerala and developing a robust database of metabolite content of various accessions. From such a database, elite chemotypes could be screened and suggested for cultivation by farmers for gaining better income.

Keywords: *Adhatoda vasica,* Alkaloids, LC-MS, Medicinal Plants, Vasicine, Western Ghats

1. Introduction

Apart from the phenomenal phenotypic heterogeneity, plants exhibit rich chemical diversity and complexity[1,2]. Among the plant chemicals, secondary metabolites are of immense interest in the field of phytochemistry and phytomedicine. *Adhatoda vasica* (Malabar nut) contains pyrroloquinazoline alkaloids that have plethora of known uses in traditional medicine. The scope of the present study has been to extract, identify and quantify one of the pyrroloquinazoline alkaloids called vasicine (an active principle) from various isolates representing distinct topogeographic regions of the Malabar area of Western Ghats of India. The aim of this study is to identify elite chemotypes based on qualitative and quantitative analysis of vasicine from the plants. To this end, an extraction protocol is being standardized to obtain maximum alkaloids from the plant material. Based on our empirical results, we nominate two out of the eight screened accessions for commercial cultivation that exhibit comparatively rich vasicine content.

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2. Methods

2.1 Topogeography of the Study Area

Accessions of *Adhatoda vasica* have been selected from eight topogeographically distinct regions of Malabar area of Western Ghats, India shown in Figure 1. These included Kakkayur (elev 2.15 m, 10°39'26.165''N; 76°39'01.357''E), Kanjikode (elev 25.25 m, 10°48'08.079''N; 76°45'02.902''E), Kuzhalmannam (elev -19.89 m, 10°42'06.004''N; 76°35'31.082''E), Kava, Malampuzaha Dam (elev 39.18 m, 10°49'33.948''N; 76°44'06.648''E), Nelliampathi Plateau 1 (elev 906.85 m, 10°32'06.736''N; 76°41'26.022''E), Nelliampathi Plateau-2 (elev 923.04 m, 10°32'21.170''N; 76°41'48.834''E), Malapuram, Perinthalmanna (elev 21.97 m, 10°57'46.114''N; 76°17'21.424''E) and Wadakencherry (elev-27.16 m, 10°34'03.347''N; 76°29'31.569''E). Since the plant accumulates maximum amount of vasicine during the flowering season, the field expeditions have been undertaken in the flowering season of *A. vasica* during late February.

2.2 Sample and Data Collection

Leaves have been collected from the eight sites mentioned above. Leaf materials have been shade dried at room temperature for seven days, ground into a fine powder and sieved through 1 mm mesh. The leaf powder thus obtained has been stored in air tight bottles, protected from light and moisture[3] and used for phytochemical analysis. Trimble Geo Explorer 6000 series, hand held GPS unit which runs with TERRA SYNC software has been used onsite for acquiring the latitude, longitude and elevation of the various sampling sites. The plant voucher specimens collected from all the eight sample spots have been deposited at the herbarium of Botanical Survey of India, Allahabad, for identification and for acquiring the accession numbers.

2.3 Apparatus, Chemicals and Reagents

Glassware and minor equipment: All the glassware used have been of Borosil make. Conical flasks and beakers (500 ml; 250 ml; 100 ml; 50 ml), separating funnels

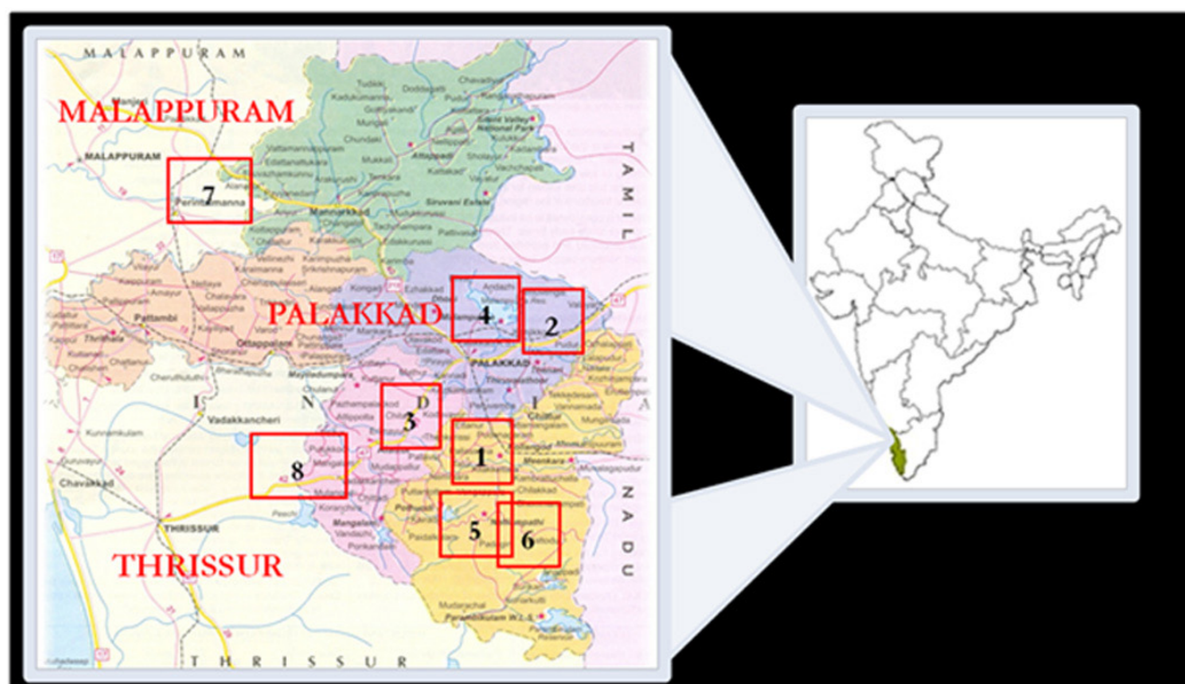

Figure 1. Study area. 1) Kakkayur (Voucher No. 90874); 2) Kanjikode (Voucher No. 90873); 3) Kuzhalmannam (Voucher No. 90867); 4) Malampuzaha Dam (Voucher No. 90879); 5) Nelliampathi Plateau 1 (Voucher No. 90869); 6) Nelliampathi Plateau 2 (Voucher No. 90870); 7) Malapuram, Perinthalmanna (Voucher No. 90871); 8) Wadakencherry (Voucher No. 90876).

(1000 ml; 500 ml), measuring jars (250 ml; 25 ml; 10 ml), pipettes (10 ml), glass rods, porcelain dishes (of varying sizes), amber coloured reagent bottles, transparent reagent bottles and glass containers with air tight caps have been used. Minor equipment's included have been orbital shaker, dark chamber, pH meter, weighing balance (Metler and Sortorious make), hand held room heater with temperature control (local make) test tube stands and holders.

Chemicals and Reagents: All the chemicals used have been of either analytical grade or HPLC grade from sd-fine chemicals, India - methanol, chloroform, ammonia, sulfuric acid, glacial acetic acid, ethyl acetate, Dimethyl Sulfoxide (DMSO), Bismuth carbonate (Merck) and sodium iodide. Stock solution of Dragendorff's reagent has been prepared with bismuth carbonate (5.2 g) and sodium iodide (4 g) in 50 ml of glacial acetic acid and boiled for few minutes. After 12 hr, the sodium acetate crystals upon precipitation have been filtered off. The filtrate (40 ml) has been mixed with 160 ml ethyl acetate and 1 ml double distilled water and stored in amber coloured reagent bottle[4]. 10 ml of the stock solution has been mixed with 20 ml of acetic acid and made upto 100 ml with double distilled water and used as working solution. Miscellaneous**:** Double distilled water, precoated TLC plates, surgical cotton, surgical blade, pH papers, Whatman No. 1 filter papers and tissue papers have been used.

2.4 Selective Extraction of Alkaloids

The powdered dry leaf material (100 g) from each of the sample spots has been extracted in duplicates. Methanolic extraction has been undertaken in the initial step for the extraction of vasicine since the extracts obtained from methanolic extraction contained high vasicine content[3,5,6]. The total alkaloid portion has been selectively extracted Figure 2 following the protocol given earlier[3,7] with some minor modifications.

2.5 Qualitative and Quantitative Analysis

TLC analysis: Thin layer chromatography has been carried out by applying 10-20 **µl** of 100 **µg** μl^{-1} concentration of the total alkaloid extract on pre-coated TLC plates (Merck make). The mobile phase consisted of chloroform

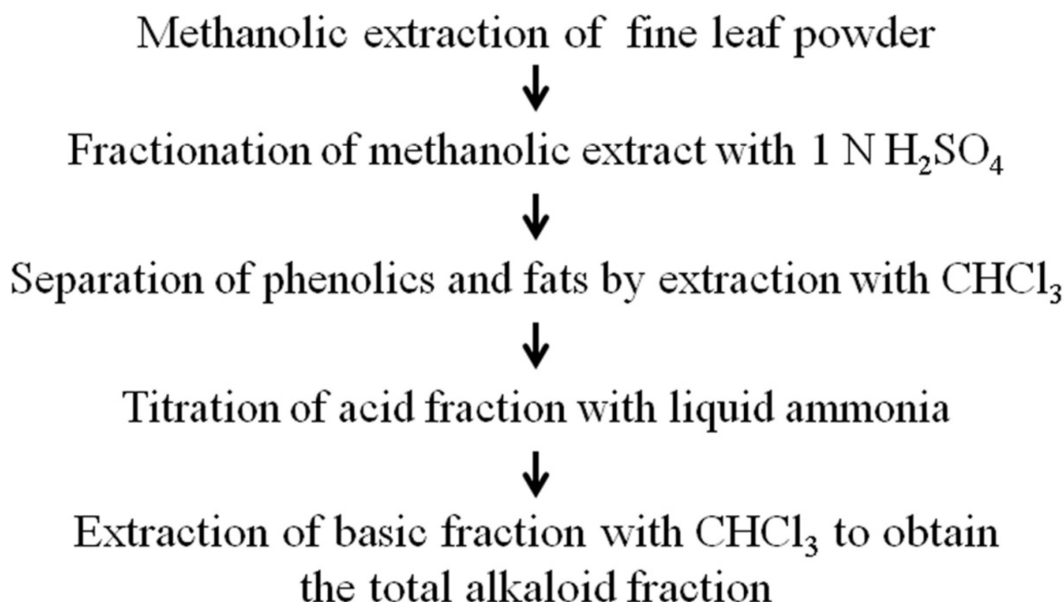

Figure 2. Major steps in the extraction of total alkaloids from *Adhatoda vasica* leaf material.

and methanol (90:10). The developed plates have been viewed under UV light followed by Dragendorff's test for positive confirmation.

UV-Vis spectroscopy: UV-2450 Shimadzu UV-Vis Spectrophotometer has been used to record the UV-Vis spectra of vasicine hydrochloride and the Total Alkaloid Extract (TAE). The solvent used has been DMSO. 1 cm quartz cuvette has been employed as a container for taking the spectral readings. The UV-Vis spectra have been recorded from 200 to 800nm.

Fluorescence spectroscopy: Perkin Elmer LS 55 Fluorescence Spectrophotometer has been used to record the fluorescence spectra of vasicine hydrochloride and The Total Alkaloid Extract (TAE) with DMSO as a solvent. 1 cm quartz cuvette has been employed as a container for taking the spectral readings. The compounds have been excited at 302 nm and the fluorescence emission spectrum was recorded from 320 to 800 nm.

FTIR spectrophotometry: Nicolet iS10 FTIR Spectrophotometer has been used to record the infra-red spectrum of the Total Alkaloid Extract (TAE). The total alkaloid powder has been grinded with potassium bromide and made into a pellet for carrying out FTIR study using regular procedure. The FTIR spectrum has been recorded from 400 to 4000 cm^{-1}.

LC-MS/MS analysis: The extracted alkaloid fraction has been analyzed by LC-MS/MS technique for identification and relative quantification of vasicine. A purified vasicine has been purchased from Natural Remedies, Bangalore. Reverse Phase (RP) separation of metabolites using a Luna Amino Column (Phenominex) followed by tandem analysis on a triple quadrupole mass spectrometer (QQQ, Agilent Technologies, Santa Clara, CA) has been achieved. The alkaloids have been quantified using Multiple Reaction Monitoring (MRM).

3. Results and Discussion

Adhatoda vasica accessions from eight topogeographically distinct regions of the Malabar area of Western Ghats have been subjected to methanolic extraction. The methanol extract has been subsequently processed to separate total alkaloids. The crude methanolic extract and Total Alkaloid Extract (TAE) have been concentrated till they reached a constant weight. Dry weights of the extracts have been quantified shown in Figures 3a and 3b using an electronic balance.

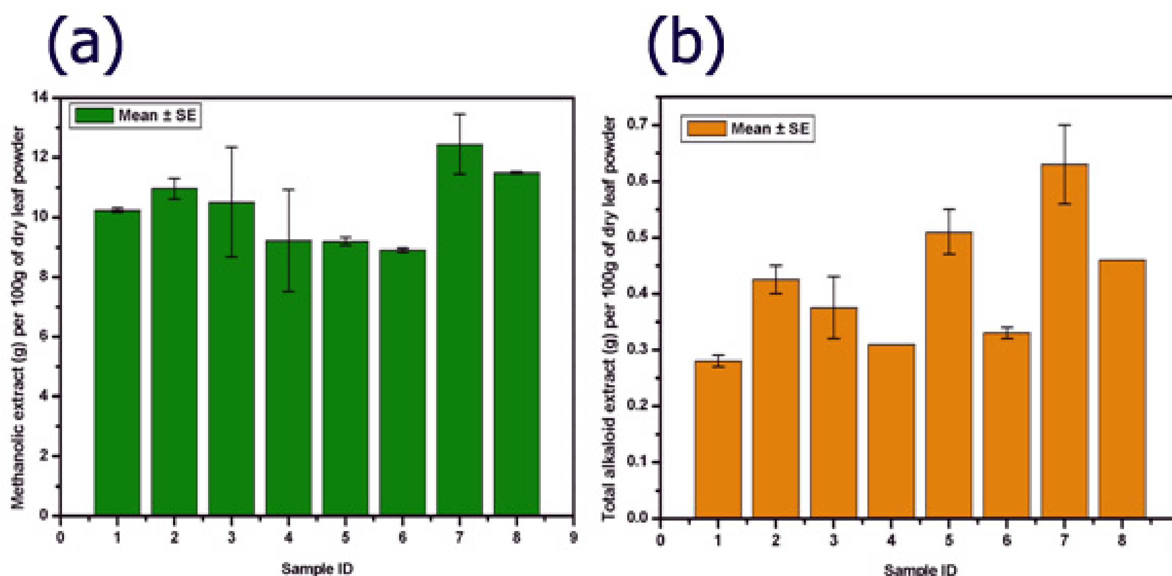

Figure 3. (a) Weight of crude methanolic extract and (b) Total Alkaloid Extract (TAE) of *Adhatoda vasica* from eight topogeogrphically distinct regions of the study area.

The methanolic extracts which contained acidic, basic and neutral components have been qualitatively tested for the presence of various secondary metabolites. The total alkaloid extract has been subjected to thin layer chromatographic analysis for the detection of alkaloids. Two distinct spots have been identified on the developed TLC plates under UV light corresponding to the Rf values in the literature for vasicine and vasicinone. After UV detection a positive confirmation has been achieved with Dragendorff's test. The alkaloids have been precipitated with Dragendorff's reagent and browinish yellow spots are visualized. The total alkaloid extract and vasicine hydrochloride (pure compound) in Dimethyl Sulfoxide (DMSO) have been then subjected to UV-Visible spectrophotometry. The UV-Vis spectra shown in Figure 4 of both the analytes have showed overlapping peaks at 251 nm. The λ_{max} for vasicine hydrochloride and TAE has been 302 nm and 305.2 nm respectively. The UV spectra from the present study have been very close to the values obtained with methanol as a solvent (298 nm) from the past work[3,7]. There have been other small peaks at 433.2 nm and 463.6 nm with low absorbance in the spectrum for TAE that would suggest the presence of compounds other than vasicine. Also the broadening of the peaks of the UV-Vis spectrum of TAE could be due to the presence of other quinazoline alkaloids apart from vasicine.

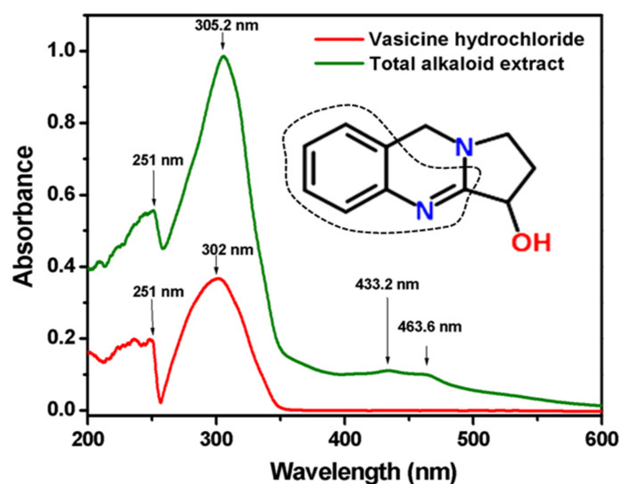

Figure 4. UV-VIS spectra of vasicine hydrochloride and total alkaloid extract showing the presence of aromatic groups in the Ultra-violet range.

The UV-Vis spectra of both the analytes showed the presence of aromatic groups in the compounds which have been detected in the UV region. The total alkaloid extract and vasicine hydrochloride in DMSO have been excited at 302 nm and observed the emission spectra using fluorescence spectrophotometer. Both the analytes showed overlapping emission maximum at 359 nm shown in Figure 5. Vasicine hydrochloride has had another peak at 380.1 nm while TAE showed a peak at 414 nm which

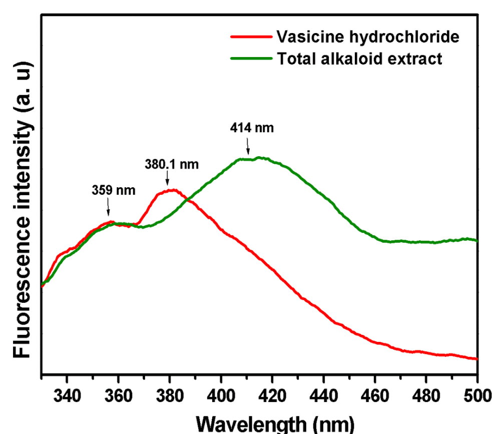

Figure 5. Fluorescence emission spectra of vasicine hydrochloride and total alkaloid extract.

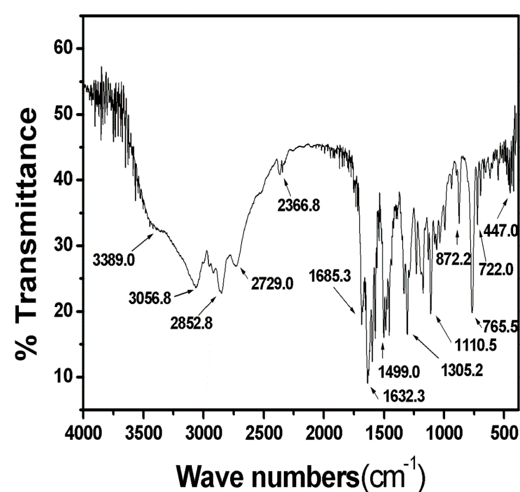

Figure 6. Fourier Transform Infrared (FTIR) spectrum of the total alkaloid extract.

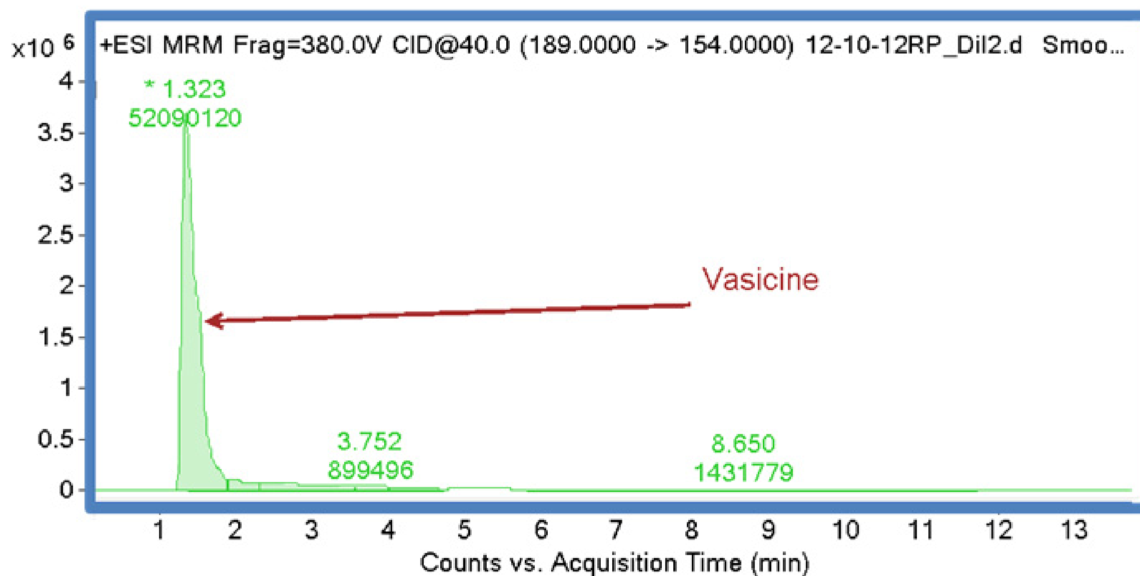

Figure 7. LC-Chromatogram for vasicine measured by Multiple Reaction Monitoring.

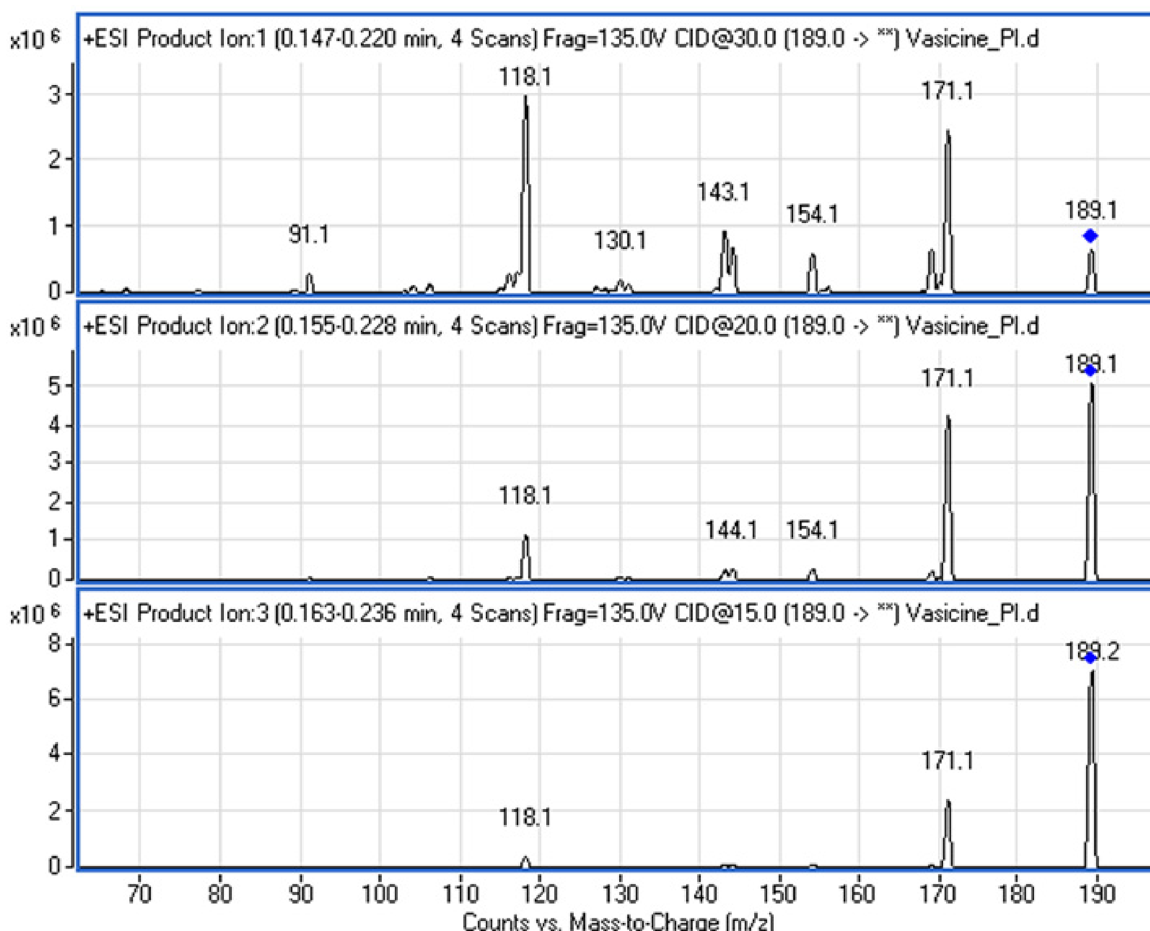

Figure 8. Product ion spectra for vasicine obtained from mass spectrometry showing the exact mass of the analyte.

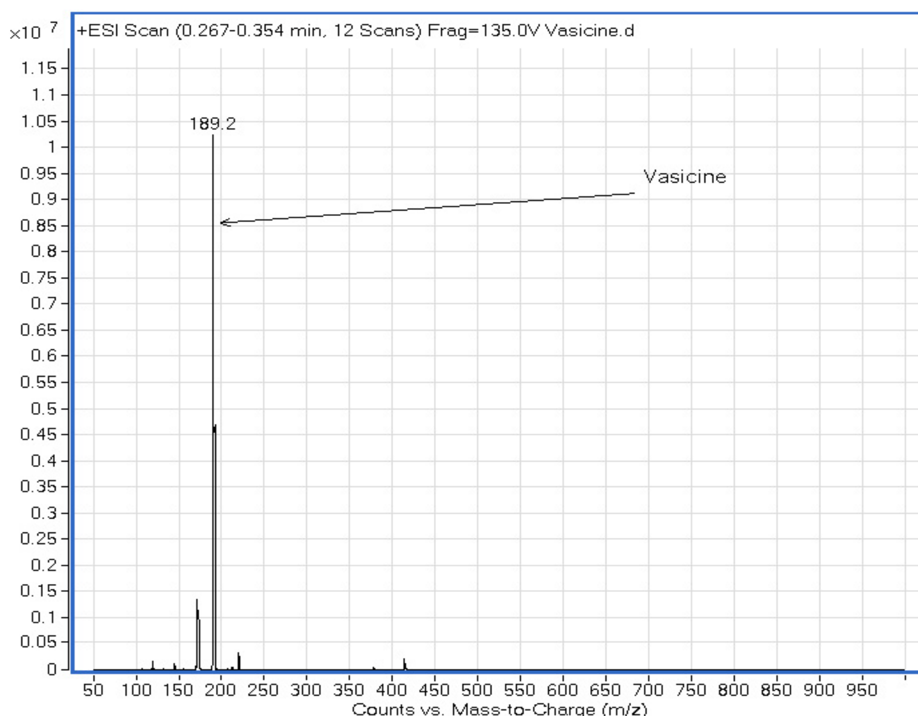

Figure 9. Mass of vasicine analyzed by mass spectrometry.

could be due to the presence of compounds other than vasicine in the TAE. However, since there has been an emission maximum at 359 nm in both the spectra we presume the presence of vasicine in the total alkaloid extract.

Fourier transform infrared spectra of the total alkaloid extract showed similarity in the peaks to vasicine. The broad peak at 3500 to 3100 cm^{-1} corresponds to hydroxyl groups in the sample and the other at 1635 cm^{-1} is due to the imine functional group in the extract is shown in Figure 6. The peaks at 1575, 1499 and 765 cm^{-1} are due to the aromatic ring. The above data has been in consonance with the past literature[8,9] where various functional groups have been identified for vasicine via >C=N group at 1629 cm^{-1}, aromatic groups at 1579, 1498, 1406, 933, 903, 767, and 722cm^{-1} and hydroxyl groups at 3389 cm^{-1}.

After having identified the presence of pyrroloquinazoline alkaloids in the total alkaloid extracts from various sample spots, an attempt has been made to quantify the relative amount of vasicine. LC-MS/MS technique has been used for the quantification of vasicine from the total alkaloid extract. Reverse Phase (RP) separation of metabolites using a Luna Amino Column (Phenominex) has been achieved.

In Figure 7 we present the chromatogram obtained for vasicine. Liquid chromatography coupled to mass spectrometric analysis has yielded the identification and quantification of vasicine in TAE shown in Figure 8.

A tandem analysis has been undertaken on a triple quadrupole mass spectrometer assisted by electrospray ionisation of the compounds. Vasicine content has been quantified using Multiple Reaction Monitoring (MRM). The product ion spectra showed intense parent ion peak of vasicine as a function of decrease in the Collision Induced Dissociation (CID) energy is shown in Figure 8. On the top panel the CID energy supplied has been 30 where the parent ion of vasicine got fragmented into several daughter ions. With the decrease in the energy supply to 20 there has been a reduction in the fragmentation represented on the panel two. With further decrease in the energy supply, on the third panel, there has been an increase in the intensity of the parent ion and disappearance of the daughter ions. Electron Impact (EI) ionisation technique has been used to find out the mass of vasicine in the literature whereas in our current study we used Electrospray Ionisation (ESI) which causes minimal fragmentation of the compounds analysed than in EI

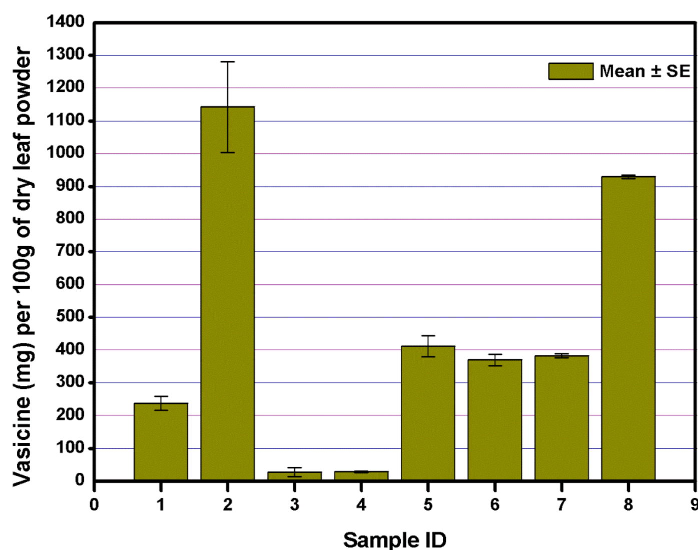

Figure 10. Relative vasicine content across the sample spots quantified using LC-MS/MS.

technique. The mass (m/z = 189) obtained for vasicine is shown in Figure 9 in the current study has been in accord with earlier reports with EI mass spectrometry[9,10].

The relative yields of vasicine using LC-MS/MS is shown in Figure 10 across the sample spots of the study area suggested high vasicine content from the samples of Kanjikode (Sample ID-2) followed by Wadakencherry (Sample ID-8), whereas samples from Malampuzaha (Sample ID-3) and Kuzhalmannam (Sample ID-4) showed low vasicine content. According to Muralidhar et al., extraction of dried leaf powder in methanol over a hot water bath and HPLC quantification showed least amount of vasicine (0.59%) in Palakkad sample compared to Bangalore, Shimoga, Trichy, and Kolhapur samples[11]. In our current study we have achieved extraction of vasicine in good amount (>0.90%) from dried leaf powder without heating the solution on a water bath during extraction. Furthermore, percentage of vasicine obtained in our study has been tenfold more than the amount obtained by any solvent extraction from the literature with simple conventional procedures [5].

4. Conclusions

Qualitative and quantitative analysis of vasicine from leaf materials using spectroscopic and chromatographic methods has been achieved. Liquid chromatography coupled to mass spectrometry based identification and relative quantification of vasicine present in the various isolates of *Adhatoda vasica* from the study area has been achieved. Accessions of *A. vasica* from Kanjikode (Sample ID-2) and Wadakencherry (Sample ID- 8) have exhibited relatively high vasicine content than rest of the sample spots. Based on our experimental results, we suggest these accessions for commercial cultivation by farmers for better income. Future efforts should be directed in cataloguing the metabolite profile of *A. vasica* accessions across similar pockets of Western Ghats for identification of elite chemotypes that could be suggested for cultivation by farmers.

5. Acknowledgements

The authors offer their heartfelt gratitude to Bhagawan Sri Sathya Sai Baba, the Founder Chancellor of Sri Sathya Sai Institute of Higher Learning for His constant motivation. Authors cordially thank Dr. Arun Sreekumar and Dr. Nagireddy Putluri (Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, US.) for their help in conducting LC-MS/MS experiments. The authors thank Sri. Aswath Narayan for his valuable inputs. The financial support provided

by the University Grants Commission under the SAP-DRS Level-II programme is gratefully acknowledged by the authors. R.S. Sai Murali would like to thank the administration of Lovely Professional University for their encouragement and facilities for analyzing the data and drafting the manuscript.

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