Symposium

Quantification of vasaka alkaloids in *in vitro* cultures and in natural leaves from Indian subcontinents by reversed phase-high performance liquid chromatography

Abstract

Background: The present study was designed to develop an efficient protocol for studying the enhancement of *in vitro* production of secondary metabolites in *Adhatoda vasica* leaves collected from the varied geographical locations of Indian sub-continents by a validated simultaneous high-performance liquid chromatography gradient method for the quantification of vasicine and vasicinone in the developed calli and crude samples. **Materials and Methods:** The analysis was carried out on a reverse phase C_{18} column using 0.1% of orthophosphoric acid: Acetonitrile in gradient manner and carried out the detection at 280 nm wavelength keeping the flow rate of 1.0 mL/min. **Results:** The simultaneous method was found linear with regression coefficient r^2 = 0.991 in a wide range (100–500 μg/mL) precise, accurate, and robust for quinazoline alkaloids in samples. Results clearly showed a significant increase in the concentration of alkaloids in *in vitro* cultures as compared to natural ones. The proposed method was validated as per the International Conference on Harmonization guidelines for accuracy, precision, robustness, limit of detection, and limit of quantitation. **Conclusion:** The developed method was found suitable for quality control of *A. vasica* and for the analysis of vasicine and vasicinone in any type of sample.

Key words:
Adhatoda vasica (L.) nees, callus cultures, high-performance liquid chromatography, validation, vasicine, vasicinone

Introduction

Adhatoda vasica commonly known as vasaka; Malabar nut tree in English and arusa in local Hindi[1] has been used in indigenous systems over last 2000 years.[2] Leaves are administered to clear respiratory passages in the preparations such as cough syrups[3] containing pyrroquinazoline alkaloids mainly vasicine, vasicinone up to 1.3% possessing number of biological activities that is antibacterial,[4] wound healing,[5] abortifacient/oxytocic,[6] antitussive,[7] anti-inflammatory,[8] anti-ulcer,[9] hepatoprotective,[10] and anti-tubercular.[11]

As the large-scale destruction of the natural habitat by it is over exploitation made it endangered hence Food and Agriculture Organization recommended the need for its conservation via *in vitro* production of metabolites.

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Literature survey revealed that the previously reported methods for the quantitative estimation of vasicine and vasicinone in *A. vasica*[12] and Adhatoda *zeylanica*[13] have poor range of linearity and improper separations in an isocratic mode. Therefore, this study was designed for the development of *in vitro* cultures and compared the same to natural ones.

Materials and Methods

Chemicals, standards, samples

Chemicals utilized *viz*., growth hormones and constituents of Murashige and Skoog[14] were of analytical grade obtained from Merck (India). Vasicine ($C_{11}H_{12}N_2O$; assigned purity >99.5%; melting point 212°C) was obtained from Sami Labs Ltd. (Bengaluru, India) as a gift sample and vasicinone from Chromadex, Bengaluru. Methanol, acetonitrile, orthophosphoric acid, and water used were of high performance liquid chromatography (HPLC) grade. The solvents were freshly prepared and degassed before use.

The samples collected in the month of March from the different eco-geographical locations that is Banaras (Uttar Pradesh), Chandigarh, Patiala, Mohali (Punjab), Solan, Nahan, Dalhousie (Himachal Pradesh), Dehradun (Uttrakhand), Hisar, Kurukshetra (Haryana), and Delhi were authenticated by a botanist Dr. Altaf Ahmad, Jamia Hamdard and deposited the voucher specimen in herbarium of BNPL (JH/BNPL/AV1-AV11/2010) Jamia Hamdard, New Delhi, India.

High-performance liquid chromatography instrumentation and conditions

It comprised system quaternary YL9100 pumps (South Korea), a variable wavelength programmable YL9120 ultraviolet (UV) detector and YL9130 column oven, controlled by the YL-Clarity software (Younglin Lin Instrument Co. Ltd, Korea). Standard and sample were filtered through 0.22 µm syringe assisted through rheodyne injector fitted with 0.20 µl fixed loop. The separation was achieved by acetonitrile and 0.1% orthophosphoric acid in a gradient at the flow rate of 1.0 mL/min passing through a C_{18} reverse phase column (Luna) followed by detection at 280 nm under UV. Whole of the analyses were performed in triplicate. Individual peaks were identified from their retention times and calculated the peak areas to obtain the respective concentrations for appropriate solutions by employing the regression equations as obtained from the calibration plots.

In vitro development of static and suspension cultures

The immature leaf explants collected from herbal garden of Jamia Hamdard were subjected for *in vitro* studies. The developed calli showing the best results were further elicited by varying concentration of potassium nitrate (20–35 mM)[15] and NaCl (25–200 mM).[16,17] The concentrations having the highest potential for production were chosen as optimized medium to get maximum amount of vasicine [Figure 1].

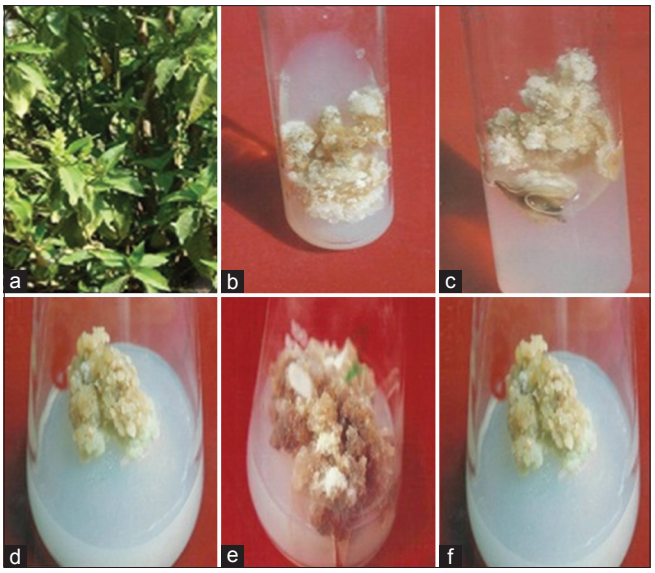

Figure 1: (a) Field growing *Adhatoda vasica* (b) 120 days old callus on Murashige and Skoog + 2,4-D + 6-benzyladenine + indole acetic acid (1 ppm each) (c) 120 days old callus on Murashige and Skoog + 6-benzyladenine + indole butyric acid (1 ppm each) (d) 120 days old callus on Murashige and Skoog + 6-benzyladenine (0.5 ppm) + indole butyric acid (1 ppm) (e) 120 days old callus on Murashige and Skoog + 2,4-D + 6-benzyladenine + indole acetic acid (1 ppm each) treated with 28 mM KNO_3 (f) 120 days old callus on Murashige and Skoog + 2,4-D + 6-benzyladenine + indole acetic acid (1 ppm each) treated with 28 mM KNO_3 + 100 mM NaCl

Calibration and validation of analytical method

Working standards of 500 µg/mL were prepared for vasicine and vasicinone in methanol and obtained the concentrations of 10–500 µg/mL for vasicine and 1–500 µg/mL for vasicinone by appropriate dilutions. The proposed method was validated as per the International Conference on Harmonization guidelines[18] for linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantitation (LOQ).

Preparation of samples

Dried and powdered samples were refluxed with methanol for 2 h. The aliquots obtained were filtered and evaporated to dryness and reconstituted methanol and made up the volume of 10 ml. A similar method was adopted for 1.0 g of dried calli and 0.5 g of suspension cells and adjusted the volume after the filtration with syringe filter of 0.45 µm. The 10 µl of each sample was injected for HPLC analysis.

Results and Discussion

Selection of mobile phase and chromatographic conditions

Number of trials was conducted with different combinations of solvents that are acetonitrile, water, methanol, orthophosphoric acid, and phosphate buffer in the various

ratios. Among all acetonitrile and 0.1% orthophosphoric acid in the ratio of 70:30 (v/v) in gradient mode resulting in sharp peaks with good resolution between them.

Validation parameters

Linearity

The calibration showed good linearity over a wide range of 10–500 and 1–500 μg/mL for vasicine and vasicinone with r^2 = 0.99. The accuracy of the method was evaluated as recovery by standard addition method. The preanalyzed samples were spiked with standard at three different concentration levels and got re-analyzed by the proposed method and mean value was expressed in Table 1.

The precision of the methods was determined by injecting six different injections of the same standard (three concentrations) for repeatability experiment and calculated the assay, relative standard deviation (%RSD) and R_f values.

Robustness was determined by changing the detecting wavelengths and by analyzing the temperature. The %RSD of the experiment was calculated so as to assess the robustness of the method at 10 μg/mL.

The LOD was determined on the basis of signal to noise ratio and found to be 3.0 μg/mL for vasicine and 0.53 μg/mL for vasicinone whereas LOQ as 10 μg/mL for vasicine and 1.0 μg/mL for vasicinone, respectively. The results obtained were summarized in Table 1.

Determination of vasicine and vasicinone in all the samples

The content was analyzed by injecting the samples in triplicates and calculated the peak area by regression equation. The developed mobile phase resulted in the optimal separation of components with well-defined and resolved sharp peaks in both standard and samples [Figure 2].

Table 1: Results of method validation for vasicine and vasicinone

Parameters	Vasicine	Vasicinone	Limits
System suitability tests			
Theoretical plates	8354	37,121	$n > 2000$
Asymmetry	3.33	0.875	< 3
Resolution	-	6.645	> 2
Tailing factor	2.75	0.95	< 3
Validation			
Linearity range, regression co-efficient	0.993	0.995	$r^2 > 0.99$
Accuracy (%)	95.4-102.4	97.3-104.3	95-105
Repeatability (%RSD)	1.5	2.2	< 2.5
Intra-day precision (%RSD)	1.1	1.4	< 2.5
Inter-day precision (%RSD)	0.8	2.4	< 2.5
Inter-system precision (%RSD)	0.7	1.1	< 2.5
Robustness (%RSD)	1.7	1.5	< 2.5
LOD (μg/mL)	3.0	0.53	-
LOQ	10	1.0	-

LOD – Limit of detection; LOQ – Limit of quantitation; RSD – Relative standard deviation

Comparative analysis of vasicine content in different sample of natural leaves

The analysis of samples showed the presence of vasicine ranging from 0.64% to 0.88% and vasicinone from 0.56% to 0.43% containing highest amounts in Delhi ones, which in turn was not a significant variation among the different collections [Figure 3].

Development of *in vitro* cultures and analysis of vasicine content

The Delhi sample [Figure 1] containing highest content of vasicine was subjected to tissue culture studies as the leaves of plant were available throughout the year. The analysis of all the cultures showed significant content of vasicine, whereas cultures treated with 28 mM KNO_3 and 100 mM NaCl showed significant increase as compared to control [Figure 3].

Conclusion

The content of vasicine analyzed in optimized suspension cultures showed manifold increase in comparison to natural leaf led to conclude that it possess a great scope for the production of pharmaceutically important quinazoline alkaloids using bioreactors at industrial level.

Tissue culture of the highest yielding explants showed a higher increase whereas suspension cultures showed the better ability for their production. The method developed was found to be accurate, reproducible, specific, and precise and well enough to separate the desired components from the immediate constituents present in the samples. The method is suitable for the routine analysis of the components in crude as well as *in vitro* samples.

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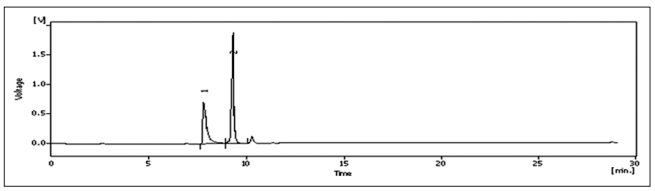

Figure 2: A typical high-performance liquid chromatography chromatogram of standard vasicine (1) and vasicinone (2) eluting at the R_t 7.81 and 9.3 min

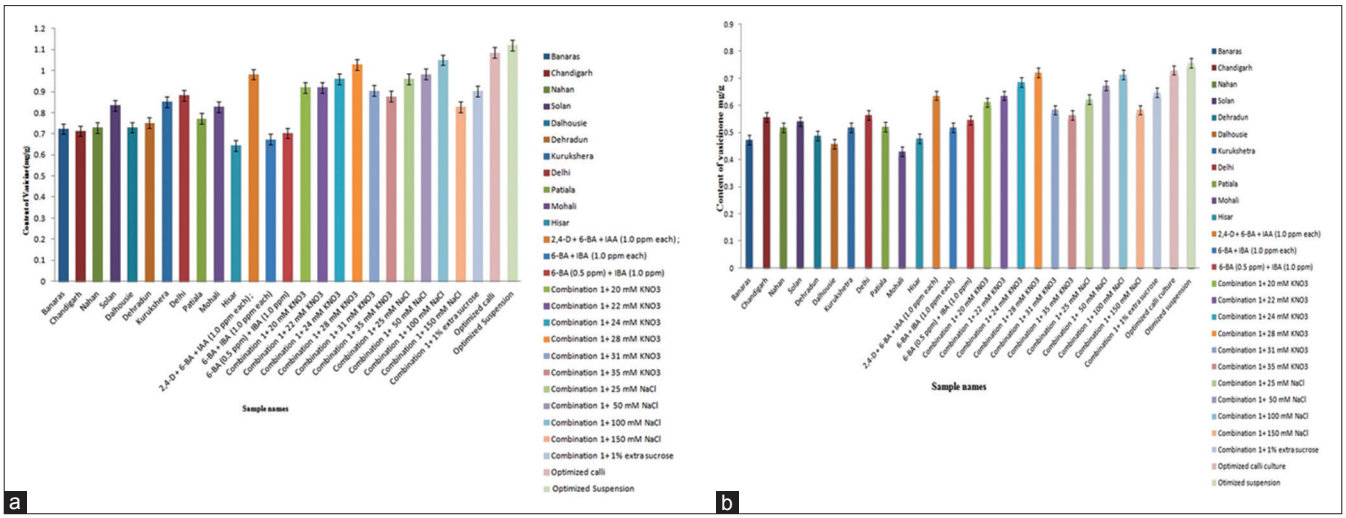

Figure 3: Content of vasicine (a) and vasicinone (b) (mg/g) in the samples of different locations/*in vitro* cultures by high-performance liquid chromatography

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Conflicts of interest

There are no conflicts of interest.

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