Rapid, sensitive, and validated UPLC/Q-TOF-MS method for quantitative determination of vasicine in *Adhatoda vasica* and its *in vitro* culture

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ABSTRACT

Background: *Adhatoda vasica* a perennial herb has been used in Ayurvedic and Unani system of medicines since last 2000 years and has been employed for the treatment of respiratory tract ailments. **Objective:** To develop and validate new, rapid, and highly sensitive high throughput ultra-performance liquid chromatography/quadrupole-time-of-flight mass-spectrometry (UPLC/Q-TOF-MS) method for the quantitative estimation of vasicine in the leaves and to establish *in vitro* cultures of *Adhatoda vasica* for production of vasicine. **Materials and Methods:** The chromatographic separation was achieved on a Waters ACQUITY UPLC™ BEH C8 (100.0 × 2.1 mm; 1.7 µm) column packing using isocratic mobile phase consisting of acetonitrile: 20 mM ammonium acetate (90:10; v/v) in a multiple reactions monitoring mode using the transitions m/z 189.09 → 171.08 for vasicine. **Results:** The vasicine was eluted at 2.58 ± 0.05 min and established a dynamic range of linearity over the concentration range of 1–1000 ng/ml (r^2 = 0.999 ± 0.0005). The lower limit of detection and quantification was 0.68 and 1.0 ng/ml, respectively. There was no significant difference observed in the content of vasicine (0.92-1.04%w/w) among the eleven samples collected from different locations of India. The *in vitro* cultures developed showed that addition of extra 28 mM KNO_3 and 100 mM NaCl in MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) + benzyladenine (BA) + indole acetic acid (IAA) (1 ppm each) produces faster biomass and higher amount of quinazoline alkaloids. **Conclusion:** Rapid, efficient, and sensitive UPLC/Q-TOF-MS method was developed for the estimation of vasicine and an efficient protocol for development of *in vitro* cultures was proposed, which can be used at large scale for industrial production of vasicine using bioreactors.

Key words: *Adhatoda vasica*, *in vitro* cultures, UPLC/Q-TOF-MS, validation, vasicine

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INTRODUCTION

Adhatoda vasica, commonly known as vasaka, as Malabar nut tree in English and arusa or adulsa in local Hindi language[1] is a primary herb of Ayurvedic system of medicine and has been used in indigenous systems of medicines in India.[2] The plant is widely employed for the treatment of various disorders of respiratory tract.[3] The leaves of the plant were reported to contain the quinazoline alkaloids–vasicine, vasicinone, and deoxyvasicine.[4] Vasicinolone, vasicol, and peganine have also been reported in the roots, whereas bioflavonoid namely quercetin and kaempferol are reported in flowers.[5] The leaves of plant have been employed in Ayurveda for the treatment of respiratory disorders since long time. Lower concentrations of vasicine induce bronchodilation and relaxation of tracheal muscles whereas higher concentrations offered significant protection against histamine-induced bronchospasms in guinea pigs. Uterine stimulant effects have also been reported for vasicine.[6]

It was also noted from literature that the content of vasicine in *A. vasica* analyzed using different analytical methods were varying a lot (0.05-2.08% w/w) employing HPLC[7-9] technique which needs reevaluation using a very robust and sensitive latest analytical techniques like ultra-performance liquid chromatography/quadrupole-time-of-flight mass-spectrometry (UPLC/Q-TOF-MS) and that in turn has been proved to be an extremely sensitive and specific technique for the analysis of basic drugs.

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Chromatographic methods like thin layer chromatography (TLC)/high performance thin layer chromatography (HPTLC).[10] HPLC[7,9,11,12] and gas chromatography-mass spectrometry (GC-MS)[13] have also been reported for quantification of vasicine, but these methods were found to suffer from low resolution, lower sensitivity/selectivity, and longer analytical times. HPLC method employing ultraviolet (UV) detector led to lack of baseline separation of peaks, eventually resulted in impure peaks. Moreover, the method showed a poor range of linearity and improper separations.[7]

The acquity UPLC is specially designed to resist higher back pressures with the advantages of fast injection cycles, low injection volumes, negligible carryover, and temperature control (4-40°C), which collectively contributes to speedy and sensitive analysis. Furthermore, acquity UPLC columns contain hybrid XTerra sorbent, which utilizes bridged ethylsiloxane/silica hybrid (BEH) structure that ensures the column stability under high pressures and over wide pH range (1-12). In addition to UPLC, the use of orthogonal quadrupole time-of-flight mass spectrometry (Q-TOF-MS) with low and high collision energy full scans acquisition simultaneously performed, offers more possibilities in screening and identification resulting in valuable fragmentation information.[14] Consequently, UPLC/Q-TOF-MS has been proved to be a powerful hyphenated technique for analytical investigations.[15]

In view of interesting biological activities associated with vasicine despite the extensive use of vasaka, tissue culture studies for production of vasicine have been limited. The present study was therefore focused on initiation and establishment of tissue cultures of *A. vasica* to compare the potential of *in vitro* cultures for production of secondary metabolites. The large variation obtained using different analytical methods in vasicine content of different samples[7-9] created havoc for herbal drug industries and even for government agencies in export of *A. vasica* and its formulations. Hence, it urgently needs a highly sensitive, advanced, and high throughput analytical method for correct quantification and revalidation of vasicine content among different locational samples of the Indian subcontinent, which are used by herbal drug industries. Therefore, a very rapid, more sensitive, and high throughput UPLC/Q-TOF-MS method was developed and validated for the first time to determine content of vasicine in natural leaves collected from different locations of north India to determine its exact amount. The proposed method was also applied for quantification of vasicine in developed *in vitro* cultures.

MATERIALS AND METHODS

Chemicals, standards, and samples

Vasicine ($C_{11}H_{12}N_2O$; assigned purity > 99.5%; melting point (mp) 212 °C) was obtained from Sami Labs Ltd (Bangalore, India) as a gift sample. LC-MS grade acetonitrile (assigned purity 99.9%) was purchased from Sigma-Aldrich, USA. MS grade ammonium acetate and ammonium formate were obtained from Fluka Analytical, Sigma-Aldrich, Netherland. Formic acid (assigned purity > 98%) was obtained from Fluka Analytical, Sigma-Aldrich, Germany. Water used in the entire analysis was of LC-MS grade. Other chemicals used were of analytical grade obtained from commercial sources. Growth hormones (utilized for tissue culture studies) and constituents of MS[16] (Murashige and Skoog) medium for media preparation were of analytical grade obtained from Merck (India). The leafy samples were collected at their flowering stage in the month of March from the different ecogeographical locations and varying altitudes in India, that is, Banaras (Uttar Pradesh), Chandigarh, Patiala, Mohali (Punjab), Solan, Nahan, Dalhousie (Himachal Pradesh), Dehradun (Uttrakhand), Hisar, Kurukshetra (Haryana), and Delhi [Table 1]. The samples collected were identified by a botanist Dr. Altaf Ahmad, Department of Botany, Jamia Hamdard and authenticated in Bioactive Natural Product Laboratory (BNPL), using microscopy and by running TLC marker of all the samples and voucher specimen were deposited in herbarium of BNPL (JH/BNPL/AV1-AV11/2010), Department of Pharmacognosy and Phytochemistry, Jamia Hamdard, New Delhi, India.

UPLC conditions

UPLC was performed with a Waters ACQUITY UPLC™ system (Serial No# F09 UPB 920M; Model Code# UPB;

Table 1: *Adhatoda vasica* accessions sampled for UPLC/Q-TOF-MS analysis collected from different ecogeographical locations

Place of collection	Latitude (N°)	Longitude (E°)	Altitude (m)
New Delhi	28°63'	77°22'	216
Hisar	29°9'	75°43'	215
Kurukshetra	29°58'	76°53'	260
Nahan	30°33'	77°21'	932
Chandigarh	30 °43'	76°47'	321
Banaras	25°22'	83°00'	81
Solan	30°90'	77°09'	1,580
Dehradun	30°19'	78°04'	557
Patiala	30°20'	76°24'	252
Dalhousie	32°38'	75°58'	2040
Mohali	30°68'	76°72'	295

UPLC/Q-TOF-MS: Ultra performance liquid chromatography/quadrupole-time-of-flight mass-spectrometry

Waters Corp., MA, USA) equipped with a binary solvent delivery system, an autosampler, column manager, and a tunable MS detector (Serial No# JAA 272; Synapt; Waters, Manchester, UK). Chromatographic separation was performed on a Waters ACQUITY UPLC™ BEH C8 (100.0 × 2.1 mm; 1.7 μm) column at 40 ± 5°C. The mobile phase employed for UPLC analysis consisted of acetonitrile; 20 mM ammonium acetate (90:10; v/v) in a gradient mode, which was degassed, previously. The flow rate of the mobile phase was kept at 0.50 ml/min and 10 μl of sample solution was injected in each run. The total chromatographic run time was 5.50 min. The column and autosampler were maintained at 40 ± 5 and 4 ± 5°C, respectively and pressure of the system was set to 15,000 psi.

Q-TOF-MS conditions

The mass spectrometry was performed on a quadruple orthogonal acceleration time-of-flight tandem mass spectrometer (Waters Q-TOF Premier™). The nebulizer gas was set to 500 l/h, the cone gas to 50 l/h, and the source temperature at 100°C. The capillary voltages were set to 3.0 kV and sample cone voltages were set to 35 V. Argon was employed as the collision gas at a pressure of 5.3×10^{-5} torr. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions of m/z 189.09 → 171.08 for vasicine with a scan time of 1.0 and 0.02 s interscan per transition. The optimum values for compound-dependent parameters like trap collision energy (Trap CE) and transfer collision energy (Tran CE) were set to 29.0 and 1.0 V, respectively for fragmentation information.

The Q-TOF Premier™ was operated in V mode with resolution over 8,500 mass with 1.0 min scan time and 0.02 s interscan delay. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the Mass Lynx V 4.1 software installed in the instrument.

In vitro development of static and suspension cultures

The immature young leaf explants of *A.vasica* collected from herbal garden of Jamia Hamdard, New Delhi were washed thoroughly under running tap water with few drops of detergent and finally with double distilled water thrice. The washed explants were kept in Bavistine® solution (1%, w/v) for 10 min to remove fungal contamination and then treated with Savlon® (1%, v/v) for 1 min. The Savlon® treated explants were again washed thrice with double distilled water and transferred to alcohol (70%, v/v) for 30 s. After that, explants were disinfected with 0.1% w/v mercuric chloride for 3 minutes and finally washed with double distilled sterile water for five to seven times to ensure complete removal of sterilant under aseptic conditions. Treated explants were placed in sterile Petri plates for inoculation. The sterile leaves were injured all over the surface for rapid induction of calli using sterile needle.

The sterile explants were aseptically inoculated on 250 × 150 mm culture tubes containing 15-20 mL of Murashige and Skoog (MS) basal medium supplemented with various concentrations/combinations of different growth regulators like indole acetic acid (IAA), 6-benzyladenine (BA), 2,4-dichlorophenoxy acetic acid (2,4-D), and indole butyric acid (IBA). Cultures were incubated at 25 ± 2ºC under warm florescent light with intensity varied from 2000-3000 lux and provided with 16/8 h of light/dark period. A total of 7-21 days were required for the induction of calli and it was first noticeable along the midrib of the leaf, young calli obtained were light yellow to yellowish brown in color. Regular subculturings were done at every 3 weeks for proper growth of calli. The initiated calli on MS medium supplemented with growth hormones showing best results were chopped and transferred aseptically into flasks containing MS medium prepared afresh with same hormonal combination and varying concentration of potasium nitrate (20-35 mM)[17] and NaCl (25-200 mM)[18,19] to check their effect on biomass and vasicine production. All the cultures were maintained for 120 days with regular growth monitoring and subculturing after every 3 weeks, which were harvested and analyzed for vasicine contents. Further, *in vitro* cultures treated with different concentrations of KNO_3 and NaCl having the highest potential for production of vasicine was used as optimized culture medium for production of biomass as callus culture (up to 120 days) and suspension culture (up to 28 days) to get highest amount of vasicine. Suspension cultures were developed by transferring fresh and friable calli (2-3 week old; approximately 0.5 g each) into MS basal media (without agar) supplemented with growth regulators and optimized content of KNO_3 and NaCl. The cultures were kept on a rotating shaker (Macro Scientific Works, New Delhi) and agitated at 100 rpm with an amplitude of 1.0 cm in a culture room at 25 ± 2°C maintaining the photoperiod of (16/8 h light/dark cycles) with a relative humidity (RH) of 70%. Subculturings were carried out at a regular interval of every 7 days for rapid growth and development of suspended cells. Growth kinetics studies were also carried out to monitor the growth of cells at 7th, 14th, 21st, and 28th day. The cultures were maintained for 28 days and vasicine content was determined in harvested cells on last day.

Calibration standards and quality control sample preparation

The standard stock solution containing 1000 μg/ml of vasicine were prepared by dissolving requisite amount

in LC-MS grade methanol (sonicated: 44 kHz, 250 W at 25°C for 20 min). The stock solutions were appropriately diluted to prepare a series of standard working solutions and stored at 4°C. The solutions were brought to room temperature and filtered through 0.22 μm membrane filter before UPLC/Q-TOF-MS analysis.

Calibration curve standards consisting of a set of ten non-zero concentrations (A–J) were prepared by dissolving vasicine in methanol to yield a concentration range of 1-1000 ng/ml (1, 5, 10, 20, 50, 100, 200, 400, 800, and1000 ng/ml). However, quality control (QC) samples were prepared at three levels; 800 ng/ml (HQC, high quality control), 400 ng/ml (MQC, middle quality control), and 5.0 ng/mL (LQC, low quality control). All the solutions were stored at 2-8°C until use.

Validation of UPLC/Q-TOF-MS method

The method validation of vasicine was performed according to United States Food and Drug Administration (USFDA) guidelines.[20] The linearity of the method was determined by analysis of six standard plots containing non-zero concentrations. Peak area vs concentration of analytes were utilized for construction of calibration curves using weighted ($1/x^2$) linear least squares regression of the vasicine. The lower limit of quantitation (LLOQ) is the lowest concentration of the calibration curve, which was determined based on the signal to noise ratio of 10:1. The extraction efficiency (recovery) of vasicine was performed at LQC, MQC, and HQC levels. It was evaluated by comparing the mean area response of six replicates of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC levels. For determining the intraday accuracy and precision, replicate analysis of all samples of vasicine was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ, LQC, MQC, and HQC samples. The interday accuracy and precision were assessed by analysis of six precision and accuracy batches on three consecutive validation days. For evaluating ruggedness of the method, one batch of precision and accuracy was run using a different column (same type) by a different analyst employing the same instrument. The six replicates were run for LLOQ, LQC, MQC, and HQC samples.

Preparation of samples

Leaves of *A. vasica* collected from different geographical locations were shade dried, powdered, and passed through sieve # 60 so as to obtain uniform powder. For carrying out the analysis, 2.0 g of powdered leaves were refluxed with methanol for 2 hours. The aliquots obtained of different samples were filtered through Whatmann filter paper and evaporated to dryness. The residues obtained were reconstituted in LC-MS grade methanol and further transferred to 10 ml of volumetric flask to finally make up the volume. Dried and powdered 120 days old calli and 28 days old suspension cells obtained from suspension culture, respectively were subjected to extraction with methanol under reflux conditions using 1.0 g of dried calli and 0.5 g of dried suspension cells as per the above discussed method. The samples were filtered through 0.22 μm membrane filter and kept in an autosampler. The 10 μl of each sample was injected for UPLC/Q-TOF-MS analysis.

RESULTS AND DISCUSSION

Selection of column and optimization of chromatographic conditions

The objective of this study was to quantify vasicine in leafy samples collected from different geographical regions, their calli, and in the suspended cells. Since vasicine (MW; 188.23) [Figure 1] contains a secondary amine in its structure and presence of basic nitrogen in the molecule, exhibited favorable sensitivity in positive ion mode detection so UPLC/Q-TOF-MS was selected for analysis. For the analysis, two analytical columns, Chirobiotic V2 (25 × 4.6 mm, 5 μm) and UPLC™ BEH C8 (100.0 × 2.1 mm; 1.7 μm) were shortlisted for method development. Baseline chromatographic resolution could not be achieved on the Chirobiotic V2 column with a mobile phase ammonium formate buffer (10 mM, pH 4.00-5.50): acetonitrile (70:30, v/v). However, a good resolution was observed on UPLC™ BEH C8 column using ammonium acetate buffer: acetonitrile (1:9, %v/v). Baseline separation of vasicine was obtained within run time of 5.50 min without any interference. Methanol, acetone, and isopropyl

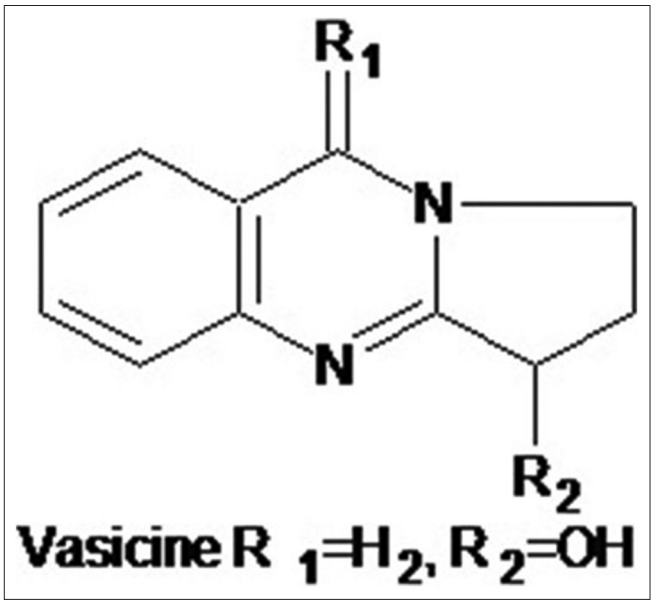

Figure 1: Chemical structure of vasicine

alcohol have been tried for instant elution; but they did not afford chromatographic separation. In order to undertake successful quantification of vasicine, tuning parameters for ESI + were optimized for protonated precursors and product ions of analytes. Finally, a sharp peak of vasicine was found at retention time of 2.58 ± 0.05 [Figure 2]. The MS full scan spectra for vasicine showed protonated precursor $[M + H]^+$ ions at m/z 189.09 and most abundant product ions at m/z 171.08 [Figure 3]. The optimum collision energies employed were 13.0 eV for vasicine. Quantification was carried out on the basis of main product ions by keeping the identical capillary voltage of 3.0 kV for monitoring the product ions.

Validation parameters

The method for quantification of vasicine was validated as per the USFDA guidelines.[21] Linearity of vasicine was established over a concentration range of 1-1000 ng/ml with respect to peak area, which showed good correlation ($r^2 > 0.999$) using least squares linear regression model. The limit of quantification (LOQ) in the present method was 1.00 ng/ml [Table 2]. Four precision and accuracy batches were run to check intraday, interday precision, and accuracy. The results for % relative standard deviation (%RSD) and accuracy are summarized in [Table 3]. The %RSD and accuracy ranged from 1.368 to 3.424 and 99.00 to 99.92 for intraday and from 0.910 to 4.778 and 99.76 to 102.00% for interday precision, respectively.

The recovery for vasicine was calculated by comparing peak areas of samples, which were prespiked with analytes at low (25%), medium (50%), and high concentration (75%) levels; with peak areas of analytes representing 100% extraction of samples at low, medium, and high concentration levels. The mean extraction recovery of vasicine was noted as 98.57. There were no significant interferences observed at the retention times of analytes.

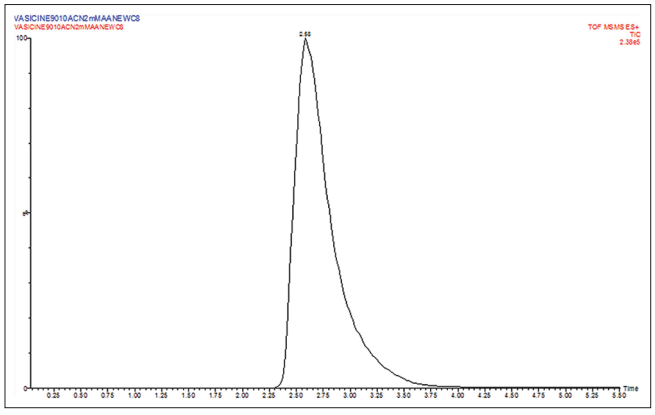

Figure 2: A typical chromatogram of standard vasicine, showing RT at 2.58 min by selective reaction monitoring scan mode (m/z 189.09)

Comparative analysis of vasicine content in different sample of natural leaves

The present work investigated the comparative analysis of vasicine in different samples of leaves of *A. vasica* collected

Table 2: Results of validation parameters of the method

Analyte (vasicine)	Results
Linear range	1−1000 ng/ml
Regression equation	17.51x−2.678 (r^2=0.999±0.0005)
LOD	0.68 ng/ml
LOQ	1 ng/ml
Intra precision	RSD=3.424% (*n*=6)
Inter precision	RSD=4.776% (*n*=6)
Recovery	96.939% (*n*=6)

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

Table 3: Intra-and inter day precision and accuracy for determination of vasicine in vasaka samples by UPLC/Q-TOF-MS

Sample	Mean[a] (ng/ml)	SD	%CV	%Accuracy
Intraday				
LLQQC	0.99	0.034	3.424	99.00
LQC	4.96	0.137	2.769	99.20
MQC	399.69	7.022	1.757	99.92
HQC	797.24	10.90	1.368	99.66
Interday				
LLQQC	1.02	0.049	4.778	102.00
LQC	5.01	0.145	2.899	100.20
MQC	399.05	4.254	1.066	99.76
HQC	801.54	7.295	0.910	100.10

[a]*N*=6. LLQQC: Lower limit of quantitation; LQC: Low quality control; MQC: Middle quality control; HQC: High quality control; SD: Standard deviation

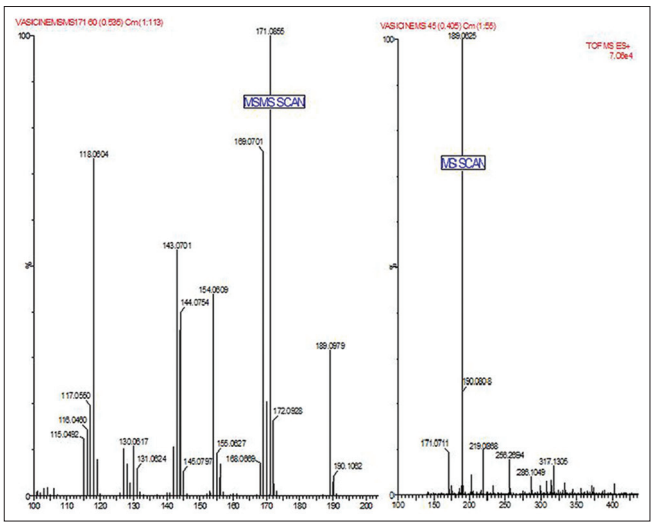

Figure 3: (a) The mass spectrum (MS) of vasicine showing precursor ion spectra (protonated precursor $[M + H]^+$ ions at m/z 189.06) along with fragmentation transitions. (b) The mass spectrum (MS) of vasicine showing product ion spectra (major fragmentized product ion mass spectra at m/z 171.08) showing fragmentation transitions precursor ion spectra at m/z 189.09

from different parts of India by employing UPLC/Q-TOF-MS. The method was employed in the present investigation to confirm content of vasicine in all samples collected from different ecogeographical locations as large variations (0.05-2.8%) have been noted in the previous reports by several authors.[7-9,11-13] The UPLC/Q-TOF-MS analysis of sample collected in month of March showed the presence of vasicine ranging from 0.92-1.005%, which in turn was not a significant variation amongst the samples collected from different regions [Figure 4].

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

The *in vitro* callus and suspension cultures were developed as an alternative source of medicines for higher *in vitro* production of pharmaceutically important secondary metabolites. The Delhi sample [Figure 5a] containing 9.74 ± 0.168 mg/g of vasicine was subjected to plant tissue culture studies for the development of calli as the leaves of plant were conveniently available throughout the year for carrying out tissue culture studies and moreover, UPLC/Q-TOF-MS analysis of leaves also revealed significant content of vasicine in sample as evident from Figure 4; after surface sterilization and inoculation in MS medium containing different hormonal combinations. The hormonal combinations showing the best results of initiation in less than 21 days were:

1. MS basal medium supplemented with 2,4-D + BA + IAA (1.0 ppm each): Profused growth with soft creamy colored calli [Figure 5b]
2. MS basal medium supplemented with BA (0.5 ppm) + IBA (1.0 ppm): Slow growth with soft yellowish colored calli [Figure 5c]
3. MS basal medium supplemented with BA + IBA (1.0 ppm each): Medium growth with soft greenish yellow colored calli [Figure 5d].

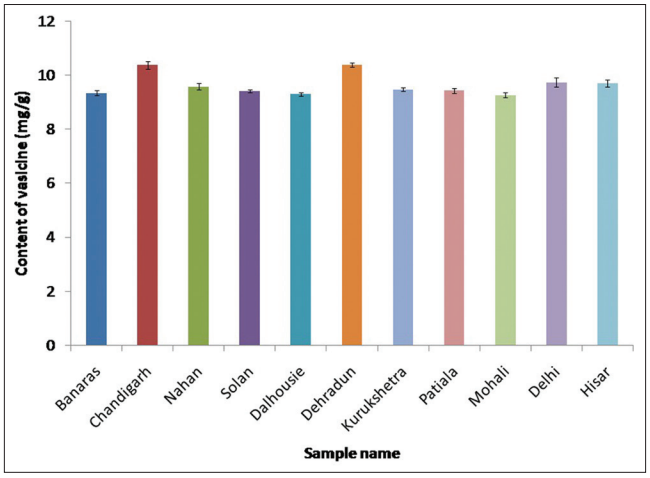

Figure 4: Content of vasicine (mg/g) in samples of different locations of the Indian subcontinent as obtained using ultra performance liquid chromatography/quadrupole-time-of-flight mass-spectrometry (UPLC/Q-TOF-MS) method

All the cultures were developed and maintained on same medium up to 120 days with monitoring of growth kinetics and subculturing at an interval of 21 days. Growth kinetic studies showed the continuous rapid growth of culture from 42nd day to 105th day and after that it showed senescence in all three cultures, whereas the cultures grown on MS basal medium supplemented with 2,4-D +BA+ IAA (1.0 ppm each) showed the best biomass production [Figure 6a].

The soft creamy colored fast growing calli with hormonal combination 2,4-D + BA + IAA (1.0 ppm each) were chopped after 21 days and approximately 500 mg each were transferred to culture tubes containing MS basal medium with same hormonal combination and different concentration of KNO_3 and salt (NaCl) as elicitors to check their effects on biomass and secondary metabolite production as per earlier reports.[21,22]

It was observed that the addition of KNO_3 increased growth of biomass up to 28 mM [Figure 5e] which was similar to growth at 24 mM KNO_3, whereas the addition of KNO_3 above 28 mM reduced biomass as evident from growth kinetics [Figure 6b] and supported by earlier reports.[21,22] Addition of NaCl in medium also showed positive effect on production of biomass up to 150 mM,

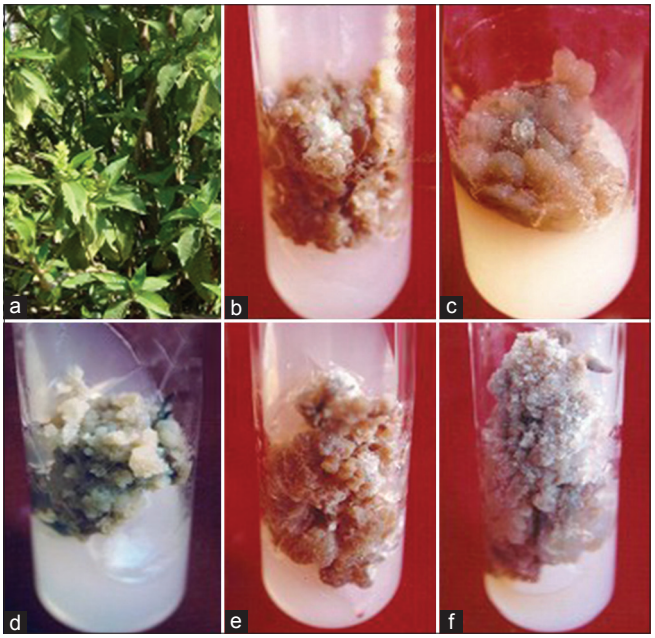

Figure 5: (a) Field growing plants of Adhatoda vasica, (b) 120 days old callus on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) + benzyladenine (BA) + indole acetic acid (IAA) (1 ppm each), (c) 120 days old callus on MS medium supplemented with BA + indole butyric acid (IBA) (1 ppm each), (d) 120 days old callus on MS medium supplemented with BA (0.5 ppm) + IBA (1 ppm), (e) 120 days old callus on MS medium supplemented with 2,4-D + BA + IAA (1 ppm each) treated with 28 mM KNO3, and (f) 120 days old callus on MS medium supplemented with 2,4-D + BA + IAA (1 ppm each) treated with 28 mM KNO3 + 100 mM NaCl

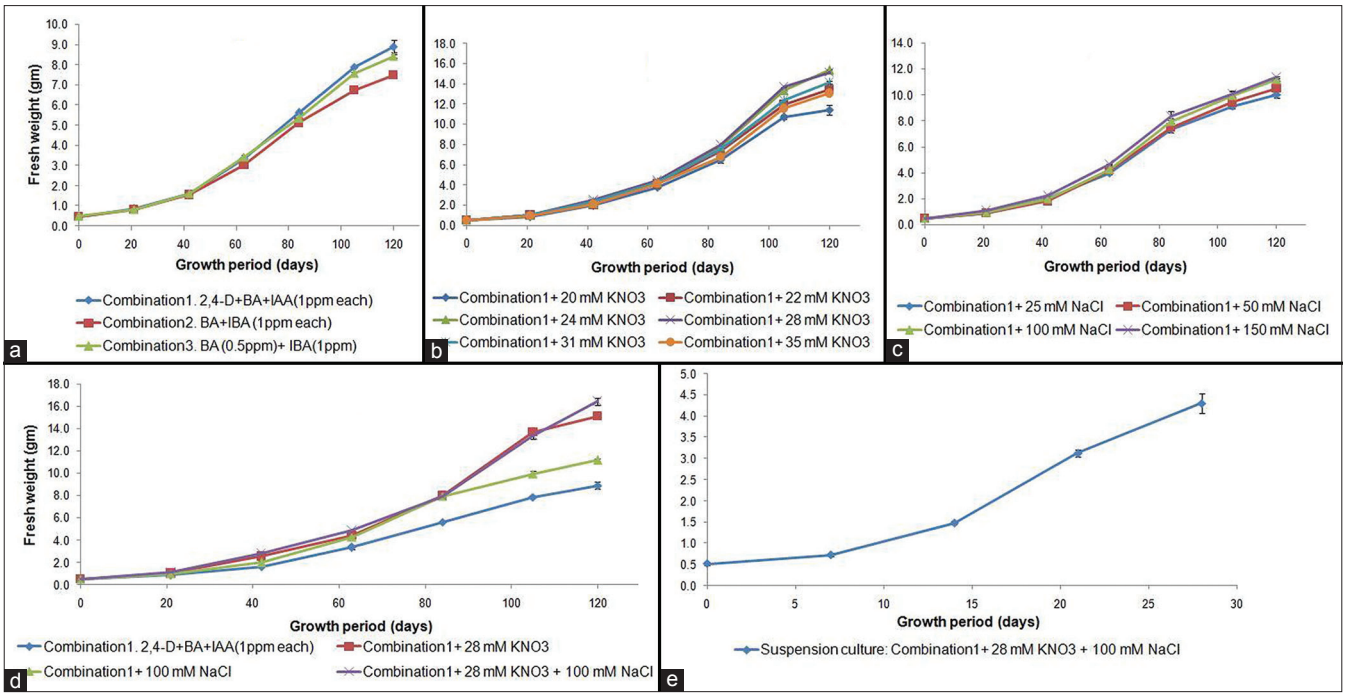

Figure 6: (a) Growth kinetics (GK) of callus culture (cc) of A. vasica (Av) upto 120 days of growth period. (b) GK of cc of Av supplemented with 2, 4-D, BA, IAA, and different concentrations of KNO3. (c) GK of cc of Av supplemented with 2, 4 D, BA, IAA, and different concentrations of NaCl. (d) GK of cc of Av supplemented with 2, 4-D, BA, IAA, and 100 mM NaCl + 28 mM KNO3 in comparison with other treatments showing improved biomass production. (e) GK of suspension culture of Av supplemented with 2,4-D, BA, IAA, and with 2,4-D, BA, IAA, and with 100 mM NaCl + 28 mM KNO3 (optimized culture conditions)

whereas above 150 mM blackening of tissues was found, which did not survive more than 80 days at 200 mM NaCl. The growth of biomass at 100 mM NaCl was similar to that at 150 mM NaCl. The results obtained with NaCl treatment [Figure 6c] were found in accordance to the earlier reports.[17-19,21-23]

The content of vasicine was analyzed by the proposed method in all the treated cultures after the growth period of 120 days and it was found that treatment of cultures with 28 mM KNO_3 and 100 mM NaCl showed highest vasicine production, which in turn was selected for the further studies. The 21 days old calli grown in MS basal medium supplemented with 2,4-D +BA+ IAA (1.0 ppm each) was subcultured in the tubes containing fresh medium with similar hormone combinations with extra 28 mM KNO_3 and 100 mM NaCl, which showed that the calli were in continuous growth phase even after 120 days of culturing [Figure 5f] and found to be best for biomass production of vasaka [Figure 6d]. Growth remained continuous till the plateau was achieved resulting in a sigmoid curve as evident from their kinetics curve.

All the cultures were analyzed for content of vasicine [Figure 7]. The optimized callus culture showed 123.3% increase in vasicine content as compared to control (callus without KNO_3 and NaCl). The cell suspension cultures of callus of optimized medium was

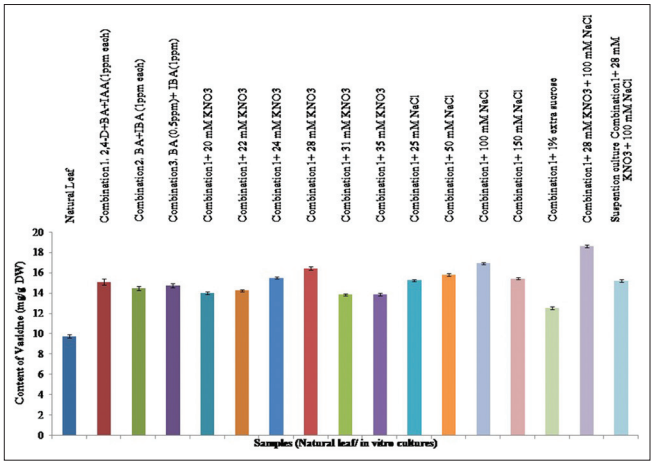

Figure 7: Content of vasicine (mg/g) in natural leaf, 120 days old dried leaf calli developed on MS basal medium supplemented with various hormonal combinations with or without treatments of KNO3 +NaCl and 28 days old suspension culture on MS basal medium with optimized culture conditions

developed and maintained up to 28 days, which showed fast growth of biomass in liquid and on an average cells were duplicating in approximately 7 days as compared to 21 days of solid medium [Figure 6e]. The content of vasicine was analyzed by proposed method, which showed 155% increase of vasicine content as compared to natural leaf. The content of vasicine obtained in 28 days suspension culture was slightly lesser as compared to content of

120 days old callus with the same treatment of elicitors, whereas the duration required for production of similar amount of biomass was reduced to one-third. Hence, it can be concluded that the suspension culture of *A. vasica* with optimized culture conditions possess a great scope for production of pharmaceutically important quinazoline alkaloids using bioreactors at industrial level.

CONCLUSIONS

UPLC with Q-TOF-MS offers improved quality data in terms of increased detection limits and chromatographic resolution with greater sensitivity. In the present investigation, a validated UPLC/Q-TOF-MS method for determination of vasicine in natural leaves and *in vitro* culture was developed and validated, which was found sensitive enough to monitor lower concentrations of vasicine with short analysis time (5.50 min) and simple extraction procedure. The assay was successfully employed for quantification of vasicine in natural samples of vasaka collected from different regions of India, which did not show any significant variation among collected samples, unlike to earlier reports using HPTLC or HPLC.[7-12] The optimized tissue culture protocol developed for quinazoline alkaloids can be used at industrial level using bioreactors for large scale production and constant supply of vasicine.

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