

Simultaneous Quantification of Lupeol, Stigmasterol and β -Sitosterol in Extracts of *Adhatoda vasica* Nees Leaves and its Marketed Formulations by a Validated RP-HPLC Method

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ABSTRACT

Background: *Adhatoda vasica* Nees (Acanthaceae) is a well-known medicinal plant used for the treatment of respiratory disorders such as asthma and bronchitis. **Objective:** To develop a simple and precise RP-HPLC method for the simultaneous assessment of lupeol, stigmasterol and β -sitosterol of various extracts of *Adhatoda vasica* Nees. **Materials and Methods:** The compounds were separated on RP-Phenomenex C₁₈ (250mm×4.6mm; 5 μ) column with a mobile phase comprising of 0.1%v/v formic acid in water and methanol (28:82%v/v) splashed at a flow of 0.8mL/min with PDA detector at 208nm. **Results:** The retention time of lupeol, stigmasterol and β -sitosterol was found to be 16.89, 18.26 and 20.72 minutes respectively. The amount of lupeol was abundant in hexane extract (0.952%w/w) and formulation III (23.72ng/g) whereas, stigmasterol (0.285%w/w) and β -sitosterol (8.649%w/w) was highly abundant in chloroform extract and formulation I stigmasterol (2.57ng/g) and β -sitosterol (0.98ng/g). The optimized method was validated for different parameters and all the validated constraints were within the limits as per ICH guidelines. The proposed method was linear over the concentration range of 12.5-200 μ g/mL with correlation coefficients greater than 0.997. The LOD and LOQ values of lupeol, stigmasterol and β -sitosterol were found to be 0.66, 5.64 and 12.8 μ g/mL and 2.01, 17.10 and 36.62 μ g/mL respectively. **Conclusion:** To conclude, the developed method for the simultaneous estimation of lupeol, stigmasterol and β -sitosterol was simple, precise, accurate and thus reliable for the quality control investigations of crude drugs and its herbal formulations.

Key words: *Adhatoda vasica* Nees, HPLC, Lupeol, Simultaneous quantification, β -sitosterol, Stigmasterol.

INTRODUCTION

Standardization is a significant tool for ensuring the quality of herbal drugs. The drug identity, purity, content, chemical and other biological properties determine the quality of the products. Quantitative estimation of chemical markers within the crude drugs is usually troublesome. Separation of markers using optimal separation techniques offer high resolution of the compounds and least interferences.¹ Herbal medicine has been utilized by 85% of the world's population since prehistoric eras for the treatment of a variety of diseases.² Therapeutic efficacy and safety depend upon the quality of herbal medicine. Lack of quality control profiles created obstacles in the approval of ayurvedic medicines for healthcare needs. Establishment of quality control parameters gets affected due to the complexion nature in the chemical compounds and its variability of herbal medicines. Advancement in the modernized analytical methods is increased to subdue these complications by developing an effective separation of marker compounds.³ The approaches in chromatographic separation techniques made quantification of chemical markers in crude drugs

with very limited clean-up.⁴ Mainly, reverse phase-liquid chromatography (RP-HPLC) used for the estimation of various chemical compounds existing in single drug or mixture of drugs and its herbal formulations.⁵

Adhatoda vasica Nees (Acanthaceae), also known as Vasaka in Ayurveda, is a perennial shrub prevalent in the hot regions of Southeast Asia.⁶ The plant is extensively used in Indian system of medicine (ISM) for the medication of respiratory system related ailments such as cough, asthma, allergen-induced bronchial obstruction and it has been used as an herbal remedy for tuberculosis.⁷ The medicinal properties of *A. vasica* characteristic to the presence of active phytoconstituents such as alkaloids, glycosides, flavonoids, coumarins, quinines triterpenes, polysaccharides, polyphenols, vitamin C, proteins and essential oils etc.⁸ *A. vasica* leaves contain tricyclic quinazoline alkaloids (pyrroquinazoline alkaloids) viz. a major alkaloid, vasicine, vasicinone and deoxyvasicine.⁷ Roots of *A. vasica* possess vasicol and vasicinolone, kaempferol and quercetin were present in the plant flowers.⁹ Apart from these, leaves and roots found to the presence of other metabolites including, terpenoids, steroids, saponins, proteins, amino acids, fats, gum resins and sugars.

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Formulation I and II are proprietary Ayurvedic medicine used for cough and jaundice, respectively. Formulation I contain *A. vasica* along with *Curcuma longa*, *Taxus baccata*, *Asparagus racemosus*, *Piper longum*, *Withania somnifera*, *Emblica officinalis*, *Curcuma zedoaria* and *Tinospora cordifolia*. Formulation II contains 15 more ingredients along with *A. vasica*. Formulation III is a versatile combination of medicinal plants having anti-inflammatory property to ease the respiratory tract infection. Apart from *A. vasica*, it contains *Glycyrrhiza glabra*, *Curcuma longa*, *Piper longum* and *Solanum xanthocarpum*.

Various chromatographic methods were reported for the estimation of alkaloids such as vasicine and vasicinone by HPTLC and HPLC in crude drug and its formulations. UHPLC/QqQLIT-MS/MS reported for the simultaneous determination alkaloids and flavonoids in *A. vasica* leaves.¹⁰ Several methods were available for the quantification of lupeol and stigmasterol, lupeol and β -sitosterol by both HPTLC and HPLC.^{11,12} and sterols by HPLC^{13,14} Literature survey revealed that there is no method available for the simultaneous estimation of lupeol, stigmasterol and β -sitosterol.

In the current investigation, we have established a simple, rapid and optimized HPLC method for the simultaneous estimation of lupeol, stigmasterol and β -sitosterol (Figure 1) in *A. vasica* extracts. *A. vasica* containing Ayurvedic formulations were also evaluated for lupeol, stigmasterol and β -sitosterol. The developed method was evaluated for different parameters and validated according to the International Conference on Harmonization (ICH) guidelines.¹⁵ Validation has been carried out based on its linearity, accuracy, precision, limit of detection and limit of quantification. This is the first report on simultaneous quantification of three markers in *A. vasica* leaf extracts.

MATERIALS AND METHODS

Plant material

A fresh matured leaf of *A. vasica* was collected from Allen Road, Tirumani, Chengalpattu district in the month of October 2017 with a latitude of 12.666 and longitude of 79.993. The taxonomic identification of the plants was confirmed by Prof. P. Jayaraman (Botanist), Plant Anatomy Research Centre, Tambaram, Chennai, Tamil Nadu, India. Voucher specimens (PARC/2018/3653) were deposited in the Herbarium of Interdisciplinary Institute of Indian System of Medicine (IIISM), SRM Institute of Science and Technology, Kattankulathur,

Kancheepuram, Tamil Nadu, India. The collected materials were cleaned, shade dried and pulverized in a blender, sieved through 60 mesh sieve and well-preserved in amber coloured airtight container at room temperature.

Standards and reagents

Lupeol (97%), stigmasterol (99%) and β -sitosterol (98%) standards were procured from Natural Remedies Pvt. Ltd, Bangalore, India. Formulation I, II and III were purchased from Ayurvedic pharmacy, Metabolic ward, Interdisciplinary Institute of Indian System of Medicine (IIISM), SRM Institute of Science and Technology, Kattankulathur. Formic acid (analytical grade), methanol (HPLC grade) were procured from Ranchem Private Limited. Mill-Q-water from Millipore was used. Chemicals of analytical grade were obtained from Merck Specialities Private Limited, Mumbai.

Extraction of *A. vasica*

Each 150g of powdered *A. vasica* were extracted in 750ml of hexane, chloroform, ethyl acetate and methanol for 72h at room temperature for non-successive extraction. The extracts were filtered and re-extracted thrice by following the same procedure. The filtrates were combined and concentrated under reduced pressure using a Buchi rotary evaporator.

Preparation of Ayurvedic formulations

Each 5 tablets of Formulation I and II were grinded in a mortar and pestle and 5ml of Formulation III were evaporated to dryness. 100mg of each sample were dissolved in 1mL of methanol and sonicate for 15min. The sample was then filtered and made up to 1mL of methanol.

Instrumentation and chromatographic conditions

Shimadzu LC-20AD HPLC system equipped with CT0-20A controller and the column oven. A Rheodyne 7725 injection valve with 20 μ L loop volume, an SPD-M20A photo-diode array detector and a Lab solution version 7.1 software was used for data acquisition and interpretation. Chromatographic separation was achieved on RP C₁₈ Phenomenex column (250mm x 4.6mm; i.d., 5 μ m pore size). The solvent mixture of 0.1% formic acid in water: methanol (28:82%v/v) at a flow rate 0.8mL/min, the column was maintained at room temperature and the PDA detector was fixed at 208 nm.

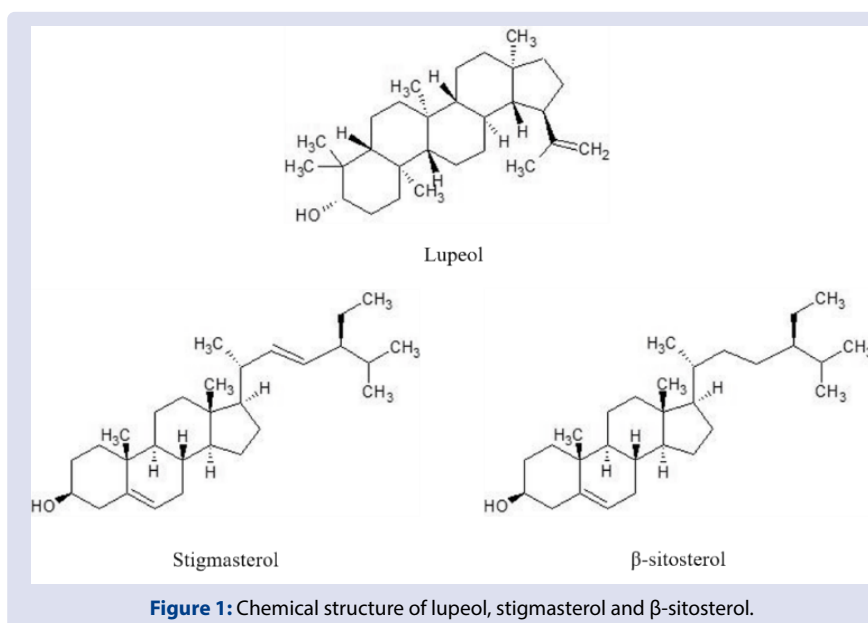


Figure 1: Chemical structure of lupeol, stigmasterol and β -sitosterol.

Preparation of standards and samples

Standard solutions of lupeol, stigmasterol and β -sitosterol were prepared and diluted in the concentration range of 12.5 to 200 μ g/mL in methanol for construction of calibration curve.

Methanolic stock solutions (10mg/mL) of hexane, chloroform, ethyl acetate and methanolic extracts of *A. vasica* were prepared and filtered through a 0.22 μ m PVDF membrane and stored at -20°C.

Development of calibration curve

Calibration was achieved with six dilutions of standards, at concentrations ranging from 12.5 to 200 μ g/mL and triplicate measurement was performed. The linearity of each standard attained by plotting peak areas versus standard concentrations. Identification of peak was attained by comparing both retention time (R_t) and UV absorbance (λ_{max}).

Method validation

According to the ICH guidelines,¹⁵ validation was performed for the developed method for linearity, accuracy, precision, LOD and LOQ. Linearity was performed in the concentration range of 12.5-200 μ g/mL of standards. The accuracy was calculated by means of percentage recovery, which was determined by the standard addition method. Standard solutions of 80%, 100% and 120% were spiked into the samples prior to the extraction and analysed under the optimized method to estimate the spiked recoveries. Precision was determined using repeatability and intermediate precision. Repeatability or intra-day precision was determined by injecting the standards six times on the same day. Intermediate precision was performed with the same sample in the same way on different day. Limit of Detection (LOD) and Limit of Quantification (LOQ) of standards were estimated separately with a standard calibration curve.

Statistical analysis

The data were statistically analysed using the one-way analysis of variance (ANOVA). The results were presented as mean of three replicates \pm standard error of mean (SEM) n=3

RESULTS AND DISCUSSION

Optimization of HPLC chromatographic conditions

HPLC chromatographic conditions were optimized after running with a C_{18} reverse phase column using different mobile phases. All the three marker compounds showed a satisfactory response of detection wavelength at 208nm. Ambient temperature was maintained in the column till the completion of the analysis. A mobile phase of 28%v/v of 0.1% formic acid in water (A) and 82%v/v of methanol (B) used with 0.8mL/min flow rate in an isocratic elution. An HPLC fingerprint was developed for *A. vasica* extract with an optimized chromatographic condition.

Quantification of marker compounds in *A. vasica* extracts

The chemical markers were identified and quantified in *A. vasica* extracts and its formulation with respect to lupeol, stigmasterol and β -sitosterol. The chromatograms of lupeol, stigmasterol and β -sitosterol exhibited efficient separation of the three compounds. Hexane extract contained lupeol (0.952%w/w), stigmasterol (0.255%w/w) and β -sitosterol (3.126%w/w), chloroform extract contained lupeol (0.548%w/w), stigmasterol (0.285%w/w) and β -sitosterol (8.649%w/w), ethyl acetate extract contained lupeol (0.487%w/w), stigmasterol (0.105%w/w) and β -sitosterol (1.472%w/w) while methanolic extract contained lupeol (0.105%w/w), stigmasterol (0.113%w/w) and β -sitosterol (5.062%w/w). Each sample was performed in triplicate and the standard and sample chromatograms were shown in Figure 2 and summarized in Table 1.

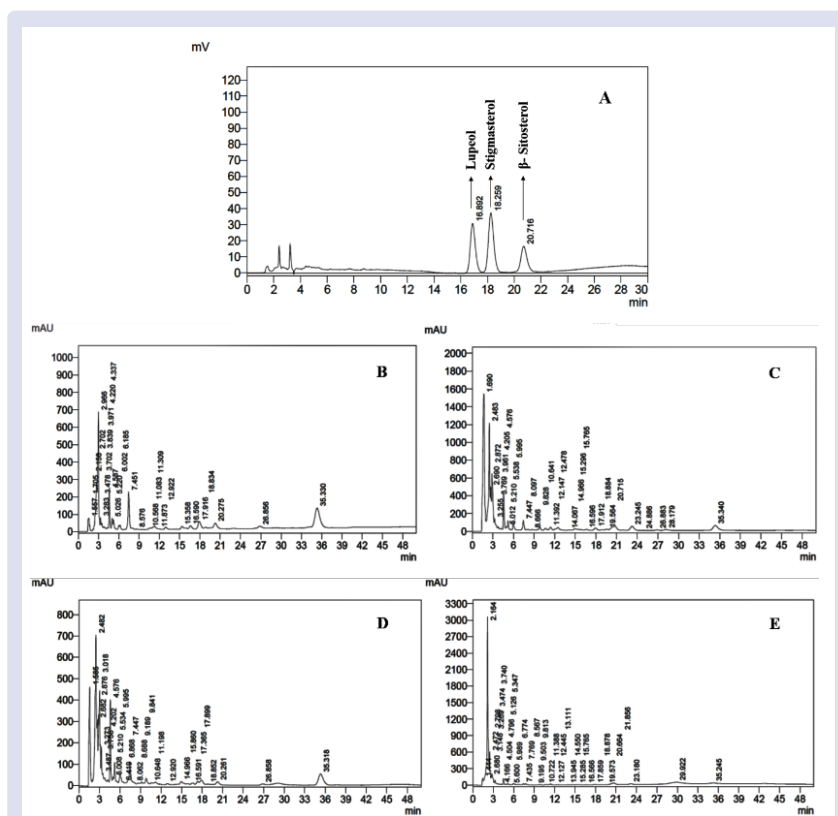


Figure 2: Chromatogram of lupeol, stigmasterol and β -sitosterol standard and extracts of *A. vasica* Nees A. Standard chromatogram, B. Hexane extract; C. Chloroform extract; D. Ethyl acetate extract; E. Methanol extract.

Quantification of marker compounds in marketed formulation

The marker compounds were identified and quantified in the ayurvedic formulations depicted in Figure 3 and Table 2. Formulation III revealed the presence of high content of lupeol (23.72ng/g) whereas stigmasterol (2.57ng/g) and β -sitosterol (0.98ng/g) content were more in Formulation I.

Method validation for HPLC fingerprinting analysis

The developed RP-HPLC methodology was validated and evaluated by determining the range and linearity, accuracy, precision, LOD and LOQ. The technique was also assessed for qualitative analysis by considering the precision and selectivity of eluted chemical marker. Repeatability

was achieved with a high concentration of standards and extracts. Linearity, precision, accuracy, LOD and LOQ were estimated for the quantification of markers. The determination of LOD and LOQ were 0.66 and 2.01 μ g/mL for lupeol, 5.64 and 17.10 μ g/mL for stigmasterol and 12.08 and 36.62 μ g/mL for β -sitosterol, correspondingly. The linearity of three markers was obtained with concentrations versus peak areas in the concentration range of 12.5-200 μ g/mL and R^2 were higher than 0.99 (Table 3). 98.19-102.31% of recovery of the compounds showed an acceptable accuracy. Relative standard deviation (RSD) of all compounds was less than 2%, a representative for high repeatability (Table 4). Precision and intermediate precision have a very low coefficient of variation reveals the method is precise (Table 5). The method validation parameters were shown in Figure 4.

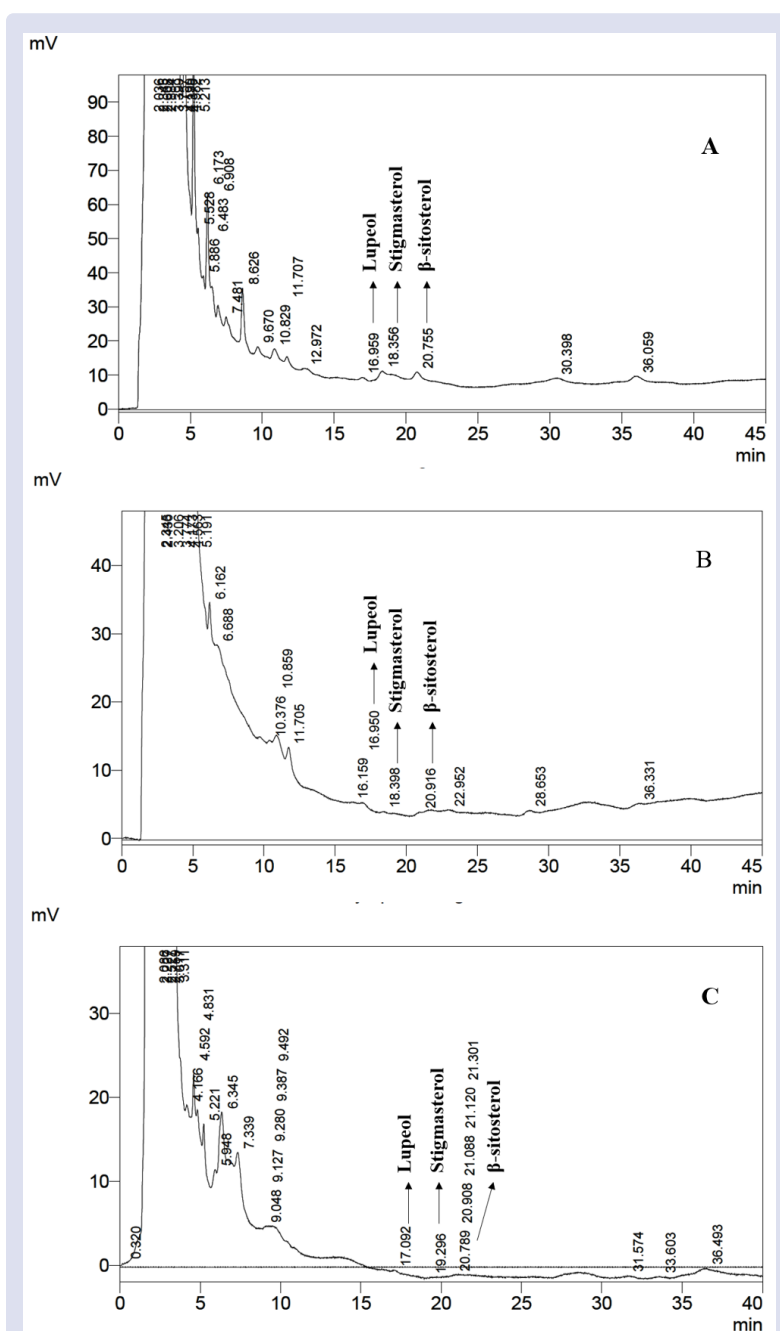


Figure 3: Chromatogram of *A. vasica* containing Ayurvedic formulations A). Formulation I, B). Formulation II and C) Formulation III.

Table 1: Quantification of lupeol, stigmasterol and β -sitosterol in *A. vasica* by RP-HPLC method.

| Extracts | Lupeol (%w/w) | Stigmasterol (%w/w) | β -sitosterol (%w/w) |
|---------------|------------------|---------------------|----------------------------|
| Hexane | 0.952 \pm 0.02 | 0.255 \pm 0.02 | 3.126 \pm 0.02 |
| Chloroform | 0.548 \pm 0.01 | 0.285 \pm 0.01 | 8.649 \pm 0.04 |
| Ethyl acetate | 0.487 \pm 0.02 | 0.105 \pm 0.03 | 1.472 \pm 0.01 |
| Methanol | 0.105 \pm 0.05 | 0.113 \pm 0.01 | 5.062 \pm 0.02 |

Data are represented as mean \pm SD (n=3)

Table 2: Quantification of lupeol, stigmasterol and β -sitosterol in *A. vasica* containing formulations by RP-HPLC method.

| Formulations | Lupeol (ng/g) | Stigmasterol (ng/g) | β -sitosterol (ng/g) |
|-----------------|------------------|---------------------|----------------------------|
| Formulation I | 4.20 \pm 0.03 | 2.57 \pm 0.01 | 0.98 \pm 0.05 |
| Formulation II | 8.09 \pm 0.04 | 0.36 \pm 0.02 | 0.27 \pm 0.06 |
| Formulation III | 23.72 \pm 0.02 | 1.10 \pm 0.02 | 0.63 \pm 0.03 |

Data are represented as mean \pm SD (n=3)

Table 3: Regression parameter, linearity, limit of detection (LOD) and limit of quantification (LOQ) of the proposed RP-HPLC method.

| Compounds | Concentration range (μ g/mL) | R _t (min) | Regression equation | R ² | LOD (μ g/mL) | LOQ (μ g/mL) |
|---------------------|-----------------------------------|----------------------|----------------------|----------------|-------------------|-------------------|
| Lupeol | 12.5-200 | 16.811 \pm 0.08 | y = 4197.1x - 841.56 | 0.9997 | 0.66 | 2.01 |
| Stigmasterol | 12.5-200 | 18.150 \pm 0.11 | y = 5387.9x - 9212.1 | 0.9994 | 5.64 | 17.10 |
| β -sitosterol | 12.5-200 | 20.588 \pm 0.12 | y = 2667.4x - 9767.9 | 0.9976 | 12.08 | 36.62 |

Data are represented as mean \pm SD (n=6)

Table 4: Recovery study of three markers present in *A. vasica*.

| Compounds | Amount added (mg/g) | Recorded amount (mg) | Recovery rate (%) | % RSD |
|---------------------|---------------------|----------------------|-------------------|-------|
| Lupeol | 80 | 1.001 \pm 0.03 | 100.13 \pm 3.48 | 1.48 |
| | 100 | 0.996 \pm 0.02 | 99.64 \pm 1.77 | 1.77 |
| | 120 | 1.014 \pm 0.03 | 101.44 \pm 3.05 | 1.95 |
| Stigmasterol | 80 | 1.012 \pm 0.04 | 101.18 \pm 4.23 | 1.23 |
| | 100 | 0.982 \pm 0.02 | 98.22 \pm 2.39 | 1.39 |
| | 120 | 1.023 \pm 0.02 | 102.31 \pm 2.26 | 1.76 |
| β -sitosterol | 80 | 0.982 \pm 0.05 | 98.19 \pm 5.14 | 1.94 |
| | 100 | 1.004 \pm 0.03 | 100.42 \pm 2.96 | 1.96 |
| | 120 | 1.005 \pm 0.05 | 100.51 \pm 5.25 | 1.25 |

Data are represented as mean \pm SD (n=3)

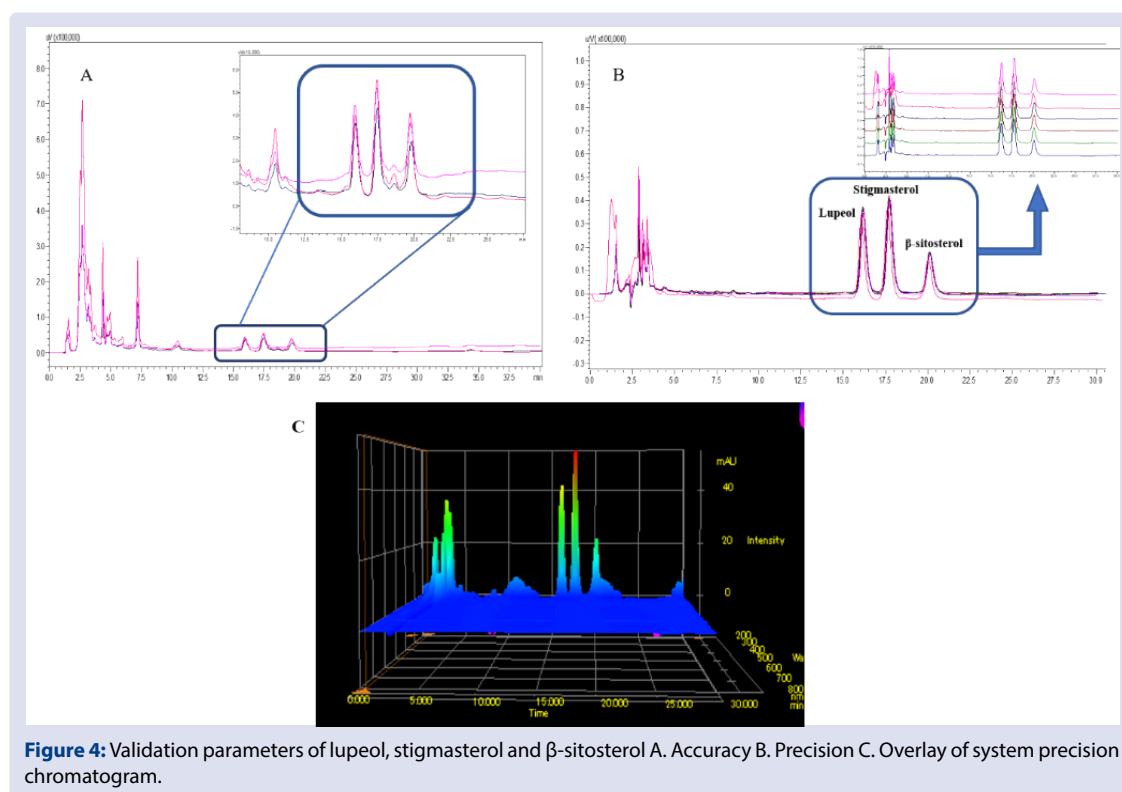


Table 5: Precision of the intra-day and inter-day HPLC measurement for lupeol, stigmasterol and β -sitosterol in *A. vasica*.

| Compounds | Intra-day | | Inter-day | |
|---------------------|------------------|---------|------------------|---------|
| | Contents (%w/w) | RSD (%) | Contents (%w/w) | RSD (%) |
| Lupeol | 1.195 \pm 0.01 | 1.22 | 1.149 \pm 0.01 | 1.01 |
| Stigmasterol | 4.159 \pm 0.07 | 1.64 | 4.210 \pm 0.06 | 1.31 |
| β -sitosterol | 0.326 \pm 0.01 | 1.71 | 0.317 \pm 0.01 | 1.82 |

Data are represented as mean \pm SD (n=6)

CONCLUSION

Standardization of ayurvedic plants possesses problem in the identification and quantification of chemical markers. The proper identification and estimation require optimized method development and validation. The crude drugs and polyherbal formulation need special attention for the method development since incompatible sample preparation can cause significance in the recovery of the compounds. Taking all factors into consideration, we have reported a unique RP-HPLC method for the identification and simultaneous quantification of lupeol, stigmasterol and β -sitosterol in *A. vasica* extracts and its Ayurvedic formulations. The developed method provides optimum separation in such a way that gives maximum resolution and avoids merging of compounds. Further, the developed method was validated for different parameters and found accurate, reproducible, specific and precise. The proposed method is suitable for the routine analysis of lupeol, stigmasterol and β -sitosterol in different crude plants as well as polyherbal formulations.

CONFLICTS OF INTEREST

None.

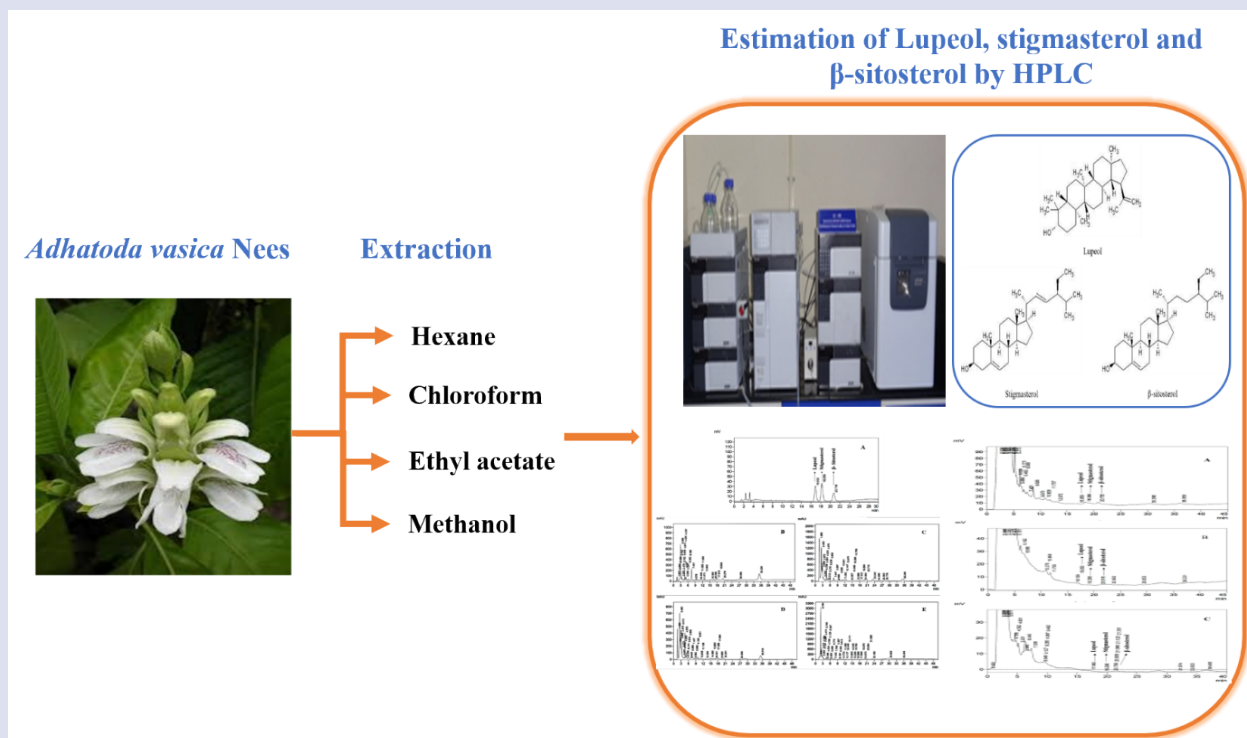
ABBREVIATIONS

ISM- Indian System of Medicine; RP-HPLC-PDA- Reverse phase high performance liquid chromatography-photodiode array; *A. vasica*-*Adhatoda vasica*; PARC- Plant anatomy research centre; PVDF- Polyvinylidene fluoride; LOD- Limit of Detection; LOQ-Limit of Quantification; UV-Ultraviolet; RSD- Relative standard deviation; ANOVA- Analysis of Variance; SEM- Standard error of mean.

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GRAPHICAL ABSTRACT



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Dr.K.ilango received his Ph.D from Sri Ramachandra Institute Higher education and Research, Chennai and his Master's degree from Birla Institute of Technology and Science, Pilani, Rajasthan and graduated from Annamalai University, Chidambaram. His academic journey in SRM began in 1994. Since then, Dr. K. Ilango's involvement in Academics and Research had been remarkable. Currently he is working as Professor, SRM College of Pharmacy & Dean, Interdisciplinary Institute of Indian System of Medicine, SRM Institute of Science and Technology, his contribution and sharing of knowledge has benefited several aspiring students.



Ms.S.Nandhini has completed her Bachelors of Pharmacy and Masters of Pharmacy (Pharmacognosy) from Madras Medical College, Chennai. She is currently pursuing PhD under the supervision of Dr.K.ilango at IIISM, SRM Institute of Science and Technology.

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