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Research Article

IMPACT OF REGIONAL VARIATION ON β-SITOSTEROL CONTENT IN *VITEX NEGUNDO* LINN. FRUITS: EVALUATION USING HPTLC

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ABSTRACT

Vitex negundo L. (Verbenaceae) fruits are reported to possess a wide range of therapeutic properties. The present work is an attempt to evaluate the impact of regional variation on the content of β-sitosterol in *V. negundo* fruits using validated HPTLC method. Chromatographic separation was achieved on TLC plate pre-coated with silica gel 60 F_{254} using toluene: ethyl acetate: methanol: glacial acetic acid (8:1:0.5:0.3, v/v/v/v) as a mobile phase. Detection of β-sitosterol was carried out by derivatizing the plate with 10% methanolic sulphuric acid reagent followed by densitometric scanning using CAMAG TLC scanner 4 at 366 nm. The method was validated as per ICH guidelines. Statistical analysis of the data reveals that the content of marker in the samples from different geographical regions varied significantly.

Keywords: β-sitosterol, HPTLC, *Vitex negundo,* regional variation.

1. INTRODUCTION

Vitex negundo L., (Verbenaceae) commonly known as *Nirgundi*, is a woody aromatic shrub, distributed throughout India. Various parts of *V. negundo* such as leaves, flowers, roots, fruit pulp and seeds have been extensively used as medicine for a wide range of ailments since ancient times [1].

V. negundo fruits are reported to possess nervine, cephalic, estrogenic and emengogue properties and also have been found effective on premenstrual water retention [2]. Fruits are extensively used in various Ayurvedic, Unani and herbal formulations. Fruits of this plant are reported to possess various phytoconstituents like luteolin, n-hentriacontane, nonacosane, p-hydroxybenzoic acid, 5-oxyisophthalic acid, β-sitosterol etc. β-sitosterol (Figure 1) has been reported to possess a wide range of therapeutic activities such as anticancer [3], lipid-lowering [4], estrogenic and anti-diabetic [5] etc.

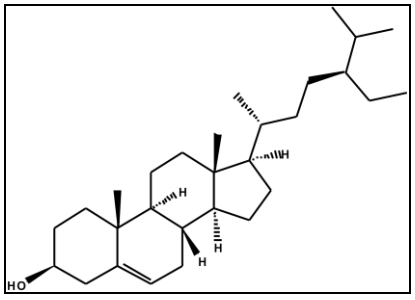


Fig. 1: Structure of β-sitosterol

HPTLC technique is reported to be useful for identification of morphological and geographical variations in terms of chemical markers from various medicinally important plants. The literature revealed that, so far, chromatographic characterization of *V. negundo* fruits in terms of β-sitosterol content has not been carried out using validated HPTLC technique. Thus, the present investigation describes the development and validation of HPTLC method for the estimation of β-sitosterol from *V. negundo* fruits collected from different geographical regions of India. As an application of the method, β-sitosterol content was also determined from an Ayurvedic formulation of *V. negundo* fruits, *Anu Taila*.

2. MATERIAL AND METHODS

2.1. Collection, drying and storage

Plant material collected from Mumbai, India was authenticated by Agharkar Research Institute, Pune (Auth 08-72) and a voucher specimen was deposited for future reference. Fruits were also collected from different geographical regions of India like Vadodara, Udaipur, Lonavala and Malvan in order to study the impact of regional variation on β-sitosterol content. Samples were oven dried at 45°C, powdered, sieved through BSS sieve (85 mesh) and stored in air-tight containers. An Ayurvedic formulation of *V. negundo* fruits, *Anu Taila* was purchased from the local market.

2.2. Reference standard and reagents

β-sitosterol standard (95% purity) was procured from Sigma Aldrich Chemical Company, (Steinheim, Germany). Solvents of analytical grade were procured from Merck Specialities Pvt. Ltd., India.

2.3. Chromatographic characterization

2.3.1. Extraction of phytochemical constituents from V. negundo samples

Accurately weighed plant sample (1.0 g) was extracted with methanol (10.0 mL). The mixture was vortexed for 1 min, kept standing overnight and then filtered through Whatman filter paper No. 1 (E. Merck, India). *Anu Taila* (1.0 mL) was extracted with methanol (9.0 mL). The mixture was vortexed for 1 min, kept on shaker for 2 h, subjected to refrigeration at 4°C overnight and filtered through Whatman filter paper No. 1 (E. Merck, India). The filtrates were subjected to chromatographic analysis.

2.3.2. Preparation of standard stock solution

A stock solution of β-sitosterol (1000.0 µg/mL) was prepared in methanol. Seven calibrant samples ranging from 4.0-60.0 µg/mL and three quality control samples of β-sitosterol namely low, mid and high (5.0, 15.0, 50.0 µg/mL respectively) were prepared in methanol using the stock solution.

2.3.3. Optimized chromatographic conditions for estimation of β -sitosterol from V. negundo samples

The HPTLC system used consisted of CAMAG TLC Scanner 4 supported by winCATS software version 1.4.7 equipped with CAMAG Linomat 5 sample spotter and CAMAG Reprostar 3 system for photo-documentation. A Denver analytical balance (Goettingen, Germany) was used to weigh the standard. Chromatographic separation of the phytochemical constituents was achieved on TLC plate (E. Merck) pre-coated with silica gel 60 F_{254} (0.2 mm thickness) on aluminium sheet support.

For separation of β-sitosterol from *V. negundo* samples, the samples (10.0 µL each) along with the standard β-sitosterol (10.0 µg/mL) were spotted on TLC plate as bands (8.0 mm wide) at a distance of 15.0 mm from the edges under similar instrumental conditions. Plate was developed up to a distance of 85.0 mm in CAMAG twin trough glass chamber pre-saturated with mobile phase toluene: ethyl acetate: methanol: glacial acetic acid (8:1:0.5:0.3, v/v/v/v) for 15 min. After development, the plate was dried in a current of air at room temperature. The plate was derivatized using 10% methanolic sulphuric acid reagent and dried in an oven preset at 110°C for 10 min. For densitometric scanning, the source of radiation used was mercury lamp (366 nm). All measurements were performed at 22 ± 1°C. Plate was photodocumented at 366 nm.

2.3.4. Estimation of β -sitosterol from V. negundo samples

Relative response for the characteristic band of β-sitosterol in *V. negundo* fruit samples and formulation was obtained. The content of β-sitosterol in each sample was determined using the regression equation obtained through regression analysis of the calibration curve.

2.4. Method validation

The HPTLC method was validated as per ICH guidelines [6] in terms of its specificity, system suitability, sensitivity, linearity, precision, stability, recovery and ruggedness.

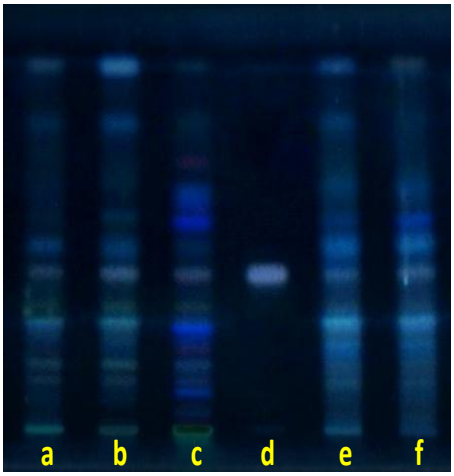
2.5. Statistical analysis

Microsoft Excel-2007 was used for the statistical evaluation of results.

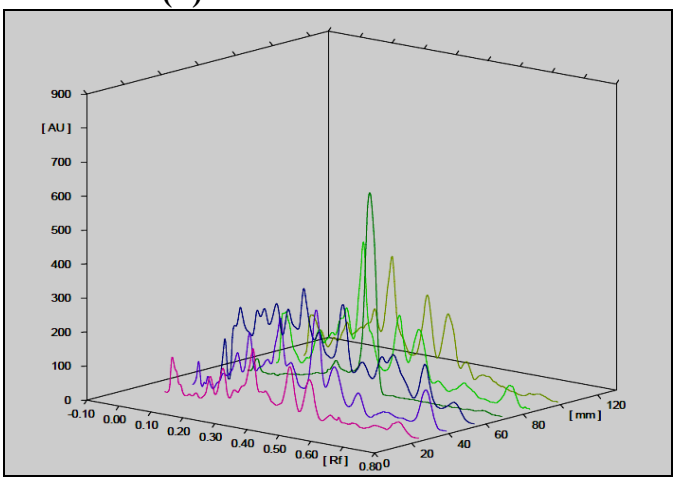
3. RESULTS AND DISCUSSION

Fruits of *V. negundo* are extensively used in traditional systems of medicine and are used to treat a wide spectrum of health disorders. Hence, there is a concern about its authenticity and quality control. HPTLC methods are commonly applied for the identification, assay or content uniformity of herbal raw materials and their formulations [6-8]. In the present work, quality of *V. negundo* fruits was evaluated on the basis of common phytoestrogen β-sitosterol using a validated HPTLC method. Samples collected from different regions in India were also subjected to the estimation of β-sitosterol using HPTLC.

For chromatographic separation of β-sitosterol from *V. negundo* fruits, we employed the mobile phase reported by our group for chromatographic characterization of some other medicinally important plants [9, 10]. This demonstrates the reproducibility and application of a validated method to other plant matrices. Briefly, the separation of β-sitosterol was achieved from the methanolic extract of the samples on TLC plate using toluene: ethyl acetate: methanol: glacial acetic acid (8:1:0.5:0.3, v/v/v/v) as a mobile phase and visualization of the bands was made possible using 10% methanolic sulphuric acid as a derivatizing reagent. β-sitosterol was detected at R_f = 0.37 and its identity in the matrix of *V. negundo* fruits and *Anu Taila* was confirmed by comparing the R_f values and colour of characteristic band with that of the standard β-sitosterol [Figure 2 (A and B) and Figure 3 (A and B)].

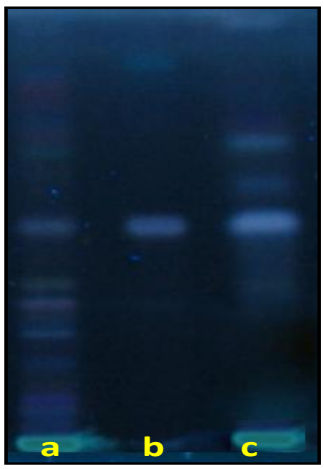


(A)

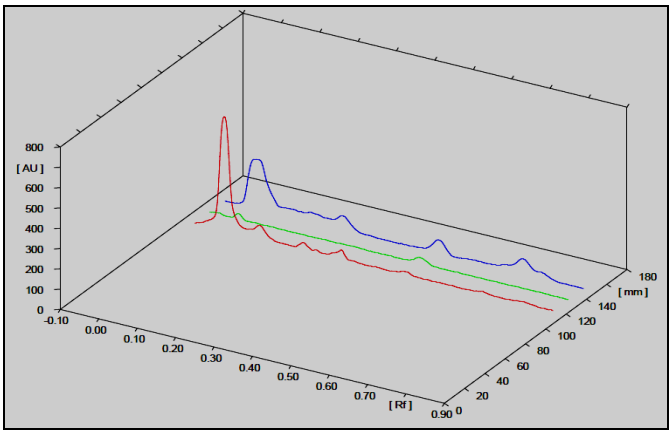


(B)

Fig. 2: HPTLC plate photo (A) and overlay of the chromatograms (B) of *V. negundo* fruits collected from different geographical regions with standard β-sitosterol at 366 nm. Track details: a) Vadodara b) Udaipur, c) Lonavala, d) β-sitosterol (50 µg/mL), e) Mumbai, f) Malvan



(A)



(B)

Fig. 3: HPTLC plate photo (A) and overlay of the chromatograms (B) of *V. negundo* fruits from Mumbai and *Anu Taila* with standard β-sitosterol at 366 nm. Track details: a) *V. negundo* fruits from Mumbai, b) β-sitosterol (50 µg/mL) c): *Anu Taila*

The method was validated as per ICH guidelines and it was found linear and precise. The method was found sensitive as limit of detection (LOD) and limit of quantification (LOQ) values were 2.0 µg/mL and 4.0 µg/mL respectively. The average recovery for quality control samples of β-sitosterol from the matrix of *V. negundo* fruits was 101.91 ±1.05%. The method was also found rugged for the parameters like change in analyst, change in mobile phase composition and change in spotting volume. The results of method validation experiment for β-sitosterol are depicted in Table 1.

Table 1: The results of method validation parameters for β-sitosterol using HPTLC technique

Parameters	Results
LOD and LOQ (µg/mL)	2.0 and 4.0
Linearity (µg/mL)	4.0 – 60.0
Regression equation	y = 31.804 x + 4.327
Mean coefficient of determination (r^2)	0.997
System suitability (% CV, n = 6)	
R_f	0.01
Response (area)	0.01
Precision (% CV, n = 3)	
Within-Batch	0.01 – 0.03
Between-Batch	0.01 – 0.06
Recovery using QC samples (% Mean ± S.D., n = 7)	
Low	97.35 ± 2.30
Mid	103.24 ± 1.45
High	105.13 ± 3.23
Stability	
Standard stock solution stability (for 30 days)	Stable at (4 ± 1^{0}C)
Bench top stability (For 6 h)	Stable at (22 ± 2^{0}C)
Ruggedness	Rugged

Using the regression equation (y = 31.804 x + 4.327), the exact content of β-sitosterol was determined in *V. negundo* fruits collected from different regions and its Ayurvedic formulation. The impact of regional variation on the β-sitosterol content of *V. negundo* fruits was clearly evident from HPTLC analysis and the results were in compliance with the other published reports [6-8]. Samples collected from Mumbai showed the maximum β-sitosterol content while sample from Lonavala had minimum (Table 2). Method was also found applicable to evaluate the β-sitosterol content from an Ayurvedic formulation *Anu Taila* containing *V. negundo* fruits. The β-sitosterol content in *Anu Taila* formulation was found to be 0.43 ± 0.11 mg/mL.

Table 2: β -sitosterol content in V. negundo fruits collected from different geographical regions of India

Place of collection	β-sitosterol content in mg/g (Mean ± S.D., n=7)
Mumbai	0.49 ± 0.18
Udaipur	0.25 ± 0.04
Malvan	0.30 ± 0.13
Vadodara	0.38 ± 0.18
Lonavala	0.13 ± 0.09

Although β-sitosterol is not a plant specific marker, it was chosen for its proven therapeutic efficacy against various ailments for the quality evaluation of *V. negundo* fruits. On the basis of the concentration of β-sitosterol, *V. negundo* fruits can be selected from a region having maximum content which may be supported by its efficacy. Thus, the developed method can be used as a powerful quality control tool for botanical identification in terms of their β-sitosterol content.

4. CONCLUSION

Results of the present study could be used by industries for the characterization of *V. negundo* fruits and its allied formulations in order to check their uniformity. *V. negundo* fruits with precise quality can be encouraged in herbal industries using such validated methods.

5. ACKNOWLEDGEMENT

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