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Research Article

Evaluation of the impact of regional variation on β-sitosterol content in *Asteracantha longifolia* Nees. seeds using HPTLC and HPLC technique

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ABSTRACT: **Background:** Seeds of *Asteracantha longifolia* Nees. (Acanthaceae) are an important ingredient in many traditional/herbal formulations used in the management of a wide range of reproductive disorders. They are reported to posses various therapeutically active phytochemical constituents like asteracanthine, lupeol, asterol, β-sitosterol etc. **Materials and Methods:** In this research work, seeds collected from various geographical regions were analyzed using two chromatographic techniques (HPTLC and HPLC). A comparative evaluation of β-sitosterol content in respective samples using these techniques has been carried out and discussed. Both the chromatographic methods have been validated as per ICH guidelines and applied to evaluate the content of β-sitosterol in formulations containing seeds of *A. longifolia*. **Results:** Variation in the β-sitosterol content of *A. longifolia* seeds collected from different geographical regions was observed. Seeds collected from Aligarh were found rich in β-sitosterol while sample from Pen showed minimum content. The methods were also found applicable to evaluate the β-sitosterol content in some traditional/herbal formulations of *A. longifolia* seeds. **Conclusions:** Impact of geographical variation on the content of β-sitosterol in the plant samples was clearly evident from the findings of the current work. There exists a definite impact of climatic and edaphic conditions over the marker content of medicinal plants.

KEYWORDS: *Asteracantha longifolia* Nees., HPLC, HPTLC, regional variation, seeds

INTRODUCTION

Asteracantha longifolia Nees. [Syn. *Hygrophila auriculata* (Schum) Heine, Acanthaceae] is an important medicinal herb used in the Unani system of medicine.[1] It grows naturally in paddy fields or marshy lands and also shows edaphic habit in waste lands.[1] Seeds of *A. longifolia* are an important component of many traditional/herbal formulations because of their scientifically evaluated therapeutic potential.[2] They are useful in the management of hepatic disorders,[2–4] inflammation, fever,[5] treatment of blood disorders, reproductive disorders[1] etc. Seeds of *A. longifolia* have been reported to possess palmitic acid, stearic acid, myristic acid,[1] asterol (I–IV), asteracanthine, asteracanthicine, lupeol and β-sitosterol.[6–7]

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Plants and plant products are subject to wide variation in their phytochemical profile due to their variety, climatic conditions, maturity, post-harvest processing, storage, stability etc. Therefore, it is extremely important to standardize these drugs based on their marker compounds.[8] Chromatographic techniques such as HPTLC[9–12] and HPLC[13–15] are reported to be useful for documentation and quantification of chemical markers form various medicinally important plants to identify morphological and geographical variations.

Based on the reported therapeutic activities of β-sitosterol like anticancer,[16,17] estrogenic,[18] hypolipidemic[19] etc., it was used as a marker to evaluate the quality of *A. longifolia* seeds. In the current research, β-sitosterol content in the seeds collected from various geographical provinces of India were quantitatively evaluated then compared using validated HPTLC and HPLC methods.

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As an application of the method, some traditional formulations of *A. longifolia* seeds were also evaluated for their β-sitosterol content.

MATERIALS AND METHODS

Collection, drying and storage

The plant sample was collected from different geographical regions of India (Maharashtra, Gujarat, Tamilnadu, Rajasthan and Uttar Pradesh) and a representative sample was authenticated from Agharkar Research Institute, Pune (Voucher specimen no. Auth 11-122). Samples were shade dried for a week then subsequently oven dried at 45 °C for another week. The dried raw materials were powdered using a mixer-grinder, sieved (BSS-85) and stored at room temperature in air tight containers. *Sufoof-e-Khaas* (Mahboobia Herbal Products, batch no. 15), Lukol (The Himalaya Drug Company, batch no. A076016B), Speman (The Himalaya Drug Company, batch no. A155013B) and Bruhowin (Bagewadikar Ayurveda Rasashala, batch no. 305) were procured from the local market of Mumbai (India).

Reference standard and reagents

β-sitosterol (98% purity, Figure 1) was procured from Sigma Aldrich chemical company (Steinheim, Germany). AR grade methanol (Batch no. SE1SF61555) and toluene (Batch no. ID1IF61209) while HPLC grade acetonitrile (Batch no. IJ2IF62581) were procured from Merck Specialities Pvt. Ltd. (Worli, Mumbai, India). HPLC grade ethanol (Lot no. 13728) was procured from Commercial Alcohols, Canada.

Extraction conditions

Extraction of phytochemical constituents from *A. longifolia* seeds and its formulations were carried out separately for HPTLC and HPLC analysis. For HPTLC, the powdered samples (0.4 g each) were extracted with methanol (10.0 mL), vortex mixed for 1 minute and kept standing overnight followed by filtration through Whatman filter paper No. 1 (E. Merck, Mumbai, India). The filtrates obtained were subjected to HPTLC analysis. For HPLC, the powdered samples (1.0 g each) were extracted in petroleum ether (10.0 mL), vortex mixed for 1 min and kept standing overnight followed by filtration through Whatman filter paper No. 1.[20] The filtrates obtained were evaporated to dryness, reconstituted in equal volume of methanol, vortex mixed for 1 minute and filtered through nylon micro filter paper (0.45 μm) prior to their injection into HPLC system.

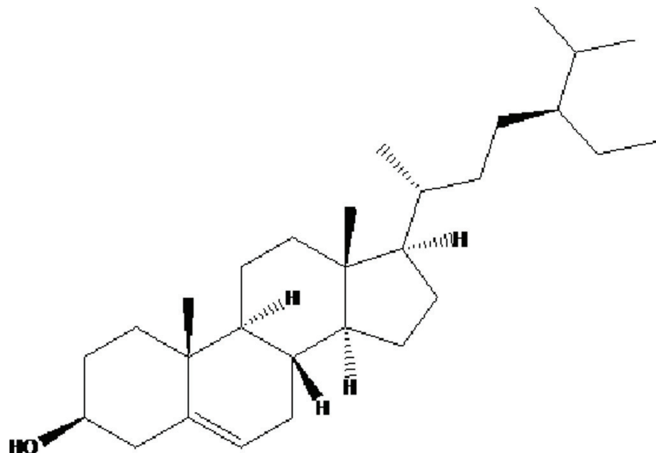

Figure 1. Structure of β-sitosterol.

Chromatographic evaluation

Seeds of *A. longifolia* were subjected to chromatographic evaluation using HPTLC and HPLC techniques wherein the content of pharmacologically active marker β-sitosterol was evaluated from the samples collected from different regions of India.

Optimized instrumental and chromatographic conditions

High performance thin layer chromatography (HPTLC)

Chromatographic analysis was performed on TLC plates pre-coated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with aluminum sheet support. Samples (10.0 μL) were spotted using CAMAG Linomat IV (Switzerland) equipped with syringe (Hamilton, 100.0 μL). Plates were developed in a glass twin trough chamber (CAMAG) pre-saturated with toluene-methanol (8:1, v/v) and scanned with CAMAG TLC Scanner II conjugated with winCATS software. The temperature was maintained at 20 ± 2 °C. For detection of β-sitosterol the plate was derivatized with 10% methanolic sulphuric acid and scanned at 366 nm. CAMAG Reprostar 3 system was used for photo documentation.

High performance liquid chromatography (HPLC)

Chromatographic analysis was performed at room temperature using Jasco's HPLC system comprising of two PU-1580 pumps (HG-1580-31), rheodyne injector (20 μL loop) and photo diode array detector (MD-1510). Chromatograms were recorded by means of Jasco-Borwin chromatography software version 1.50. Separation was achieved on Cosmosil C_{18}-column (150 mm × 4.6 mm, 5.0 μm) using acetonitrile-ethanol [40:60 (v/v)] delivered at a flow rate of 1 mL/min. After a 10 min of equilibration period, the samples were injected into HPLC system. Peaks were recorded at 210 nm.

Preparation of standard solutions

A stock solution of β-sitosterol (1000 µg/mL) was prepared in methanol. Seven calibrant samples [5–60 µg/mL (for HPTLC) and 5–200 µg/mL (for HPLC)] and three quality control samples {Low, mid and high [LQC, MQC, HQC (6.5, 17.5, 48 µg/mL respectively for HPTLC while 6.5, 32.5, 160 µg/mL respectively for HPLC)]} of β-sitosterol were prepared in methanol using the stock solution.

Method validation

The HPTLC and HPLC method for determination of β-sitosterol content was validated according to ICH guidelines.[20] The validation parameters addressed were linearity, sensitivity, precision, accuracy, ruggedness, recovery and stability.

Estimation of β-sitosterol in *A. longifolia* seeds and formulations

Seven calibrant samples of β-sitosterol were analyzed using the optimized chromatographic conditions and the response values for each concentration were obtained (area under curve). These values (response and respective concentration of β-sitosterol) were subjected to the regression analysis in order to obtain a regression equation ($y = mx + c$, where y is response, x is the concentration of β-sitosterol in µg/mL and c is the y-intercept). Similarly, the plant samples were analyzed and the relative response (y) of β-sitosterol in each sample was obtained. The concentration of β-sitosterol (x) in the samples analyzed was determined using the regression equation.

Statistical analysis

Microsoft Excel-2007 was used to determine mean, standard deviation (SD), coefficient of variation (CV) and mean difference during the analysis.

RESULTS AND DISCUSSION

A. longifolia is a promising medicinal plant with great economic potential. The medicinal importance of this plant has been reported in the ancient medical literature.[1] Seeds of the plant contain terpenoids, alkaloids, flavonoids and are traditionally known as an aphrodisiac, renal tonic, and for its health-promoting properties.[7] In this research work, an attempt has been made to compare the content of one of the most common dietary phytosterols (β-sitosterol[21]) in *A. longifolia* seeds collected from different geographical regions of India using validated HPTLC and HPLC technique.

So far, there is no report on the evaluation of the β-sitosterol content in seeds of *A. longifolia*. An HPTLC method for the estimation of β-sitosterol from whole plant of *A. longifolia* has been reported by our group.[22] However, the mobile phase used consisted of three solvents (toluene, ethyl acetate and methanol) which did not resolve β-sitosterol from seeds of this plant satisfactorily. This may be due to the matrix-based variation. Hence, a mobile phase comprising of only two solvents was utilized which resulted in the proper separation of β-sitosterol from the plant matrix. Briefly, the separation of β-sitosterol was achieved from the methanolic extract of *A. longifolia* seeds on TLC plates using toluene: methanol (8:1, v/v) as a mobile phase. Detection of β-sitosterol was carried out by derivatizing the developed plates using 10% methanolic sulphuric acid (Figure 2).

A characteristic band of β-sitosterol was obtained at $R_f = 0.46$ in the matrix of *A. longifolia* seeds and its identity was putatively confirmed by overlaying the densitometric chromatograms and comparing the colour with that of the standard β-sitosterol. The representative HPTLC chromatogram of β-sitosterol and *A. longifolia* seeds is represented in Figure 3.

For the separation of β-sitosterol using HPLC technique, we have used the mobile phase already published by our group[20] which demonstrates the reproducibility and application of a validated method to the other plant matrices. Using this method, β-sitosterol eluted at R_t of

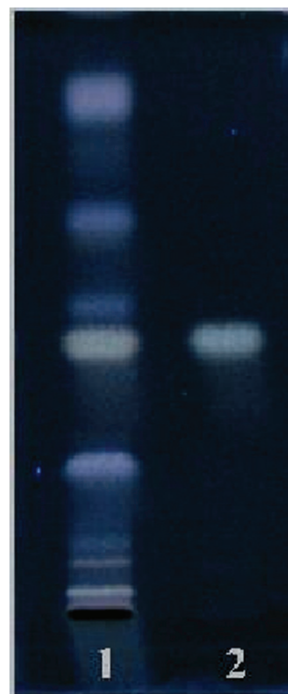

Figure 2. HPTLC analysis of *A. longifolia* seed with β-sitosterol at 366 nm. Track details, 1: methanolic extract of *A. longifolia* seeds, 2: β-sitosterol (25 µg/mL).

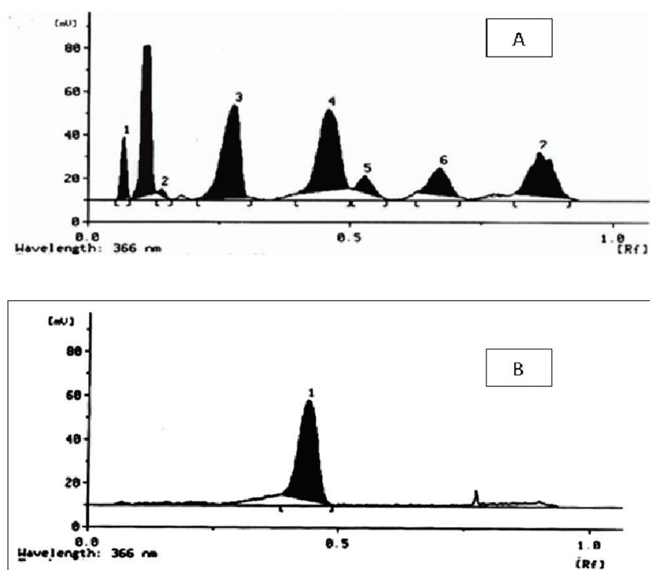

Figure 3. Representative TLC densitometric chromatograms of the methanolic extract of *A. longifolia* seeds collected from Aligarh region (A) and β-sitosterol standard of concentration 25 μg/mL (B). Both the chromatograms were recorded densitometrically at 366 nm using CAMAG TLC Scanner II.

6.8 minute using acetonitrile: ethanol (40:60, v/v) as a mobile phase. The presence of β-sitosterol in the plant matrix was putatively confirmed by comparing its UV absorption spectra at 210 nm with that of the standard β-sitosterol. The representative HPLC chromatogram of β-sitosterol and *A. longifolia* seeds is represented in Figure 4.

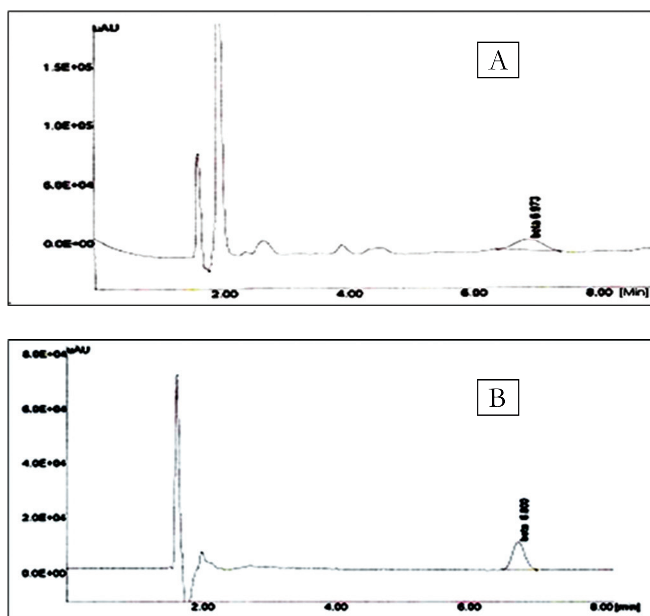

Figure 4. Representative HPLC chromatograms of *A. longifolia* seeds collected from Aligarh region (A) and β-sitosterol of concentration 25 μg/mL (B). Both the chromatograms were recorded with the help of Borwin Chromatography software at 210 nm.

Both the methods were validated as per ICH guidelines and were found to be simple, rapid, specific, precise, sensitive and rugged during the validation experiment which on comparison showed that the HPLC method was more sensitive than HPTLC in estimating β-sitosterol from plant matrix (Table 1). Both the methods were applied to evaluate the impact of regional variation on the content of β-sitosterol in *A. longifolia* seeds collected from different provinces of India. Methods were further used to evaluate β-sitosterol content in some formulations of *A. longifolia* seeds readily available in the local market. Using the regression equation, the exact content of β-sitosterol in the samples was determined.

Variation in the β-sitosterol content was observed in different samples analyzed using both the techniques (Table 2). Samples collected from various regions of Maharashtra showed the β-sitosterol content in the range of 0.22–0.49 mg/g. *A. longifolia* seeds from hilly regions (Khandala and Lonavala) showed comparatively higher β-sitosterol content compared to the seeds from plains of Maharashtra (Thane, Dombivali, Murbad and Pen). β-sitosterol content in the seeds collected from cultivated *A. longifolia* (Baroda, Gujarat) was found almost similar to the samples growing in the hilly regions of Maharashtra. Using both the chromatographic methods, it was observed that the seeds from Uttar Pradesh (Aligarh) and Rajasthan (Udaipur) were found to be the rich source of β-sitosterol while sample from Tamilnadu (Tiruchirappalli) and plains of Maharashtra (Pen and Murbad) showed the minimum content. The possible reasons for such variations may be the significant impact of geographical and environmental factors such as type of soil, exposure to sunlight, temperature, moisture, air, nutrients, etc over the phytoconstituents of medicinal plants.[20,23] Findings of the present work clearly suggests that the there is a definite impact of regional variation on the marker content of medicinal plants and the results were also in compliance with the other published reports.[9–12,20,23] All the samples collected from different localities varied from one another, indicating the possible role of environment in deciding the chemical spectrum of a plant.[24]

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 *A. longifolia* seeds. On the basis of the concentration of β-sitosterol, seeds of *A. longifolia* can be selected from a region which gives maximum content which may be supported by its efficacy. Multiple samples must be analyzed from a region showing maximum content of bioactive marker in order to study the intra-regional variations.

Table 1: Comparative account on the results of method validation parameters for β-sitosterol using HPTLC and HPLC technique

Parameters	Results	
	HPTLC	HPLC
LOD (µg/mL)	1	0.5
LOQ (µg/mL)	3	1
Linearity (µg/mL)	5–60	5–200
Regression equation	y = 39.76x + 194	y = 3570x – 7704
Mean coefficient of determination (r^2)	0.993	0.996
System suitability (% CV, n = 5)		
R_f/R_t	1.38	0.15
Area	0.83	0.11
Precision (% CV, n = 3)		
Within-Batch	0.67–0.99	0.02–0.63
Between-Batch	0.54–1.45	0.10–0.40
Recovery (%, n = 7)		
LQC	97.14	98.32
MQC	99.33	99.68
HQC	98.40	96.54
Stability		
Long-term stability		
Standard stock solution stability (For 10 days)	Stable at (4 ± 1 °C)	Stable at (4 ± 1 °C)
Short-term stability		
Bench top stability (For 6.00 hours)	Stable at (25 ± 2 °C)	Stable at (25 ± 2 °C)
Auto sampler stability (For 12.00 hours)	Not applicable	Stable at (4 ± 1 °C)
Ruggedness	Rugged	Rugged

Table 2: Comparative assay results and method application

Samples	Concentration of β-sitosterol in mg/g	
	HPTLC	HPLC
Regional variation		
Aligarh (Maharashtra)	0.67 ± 0.01	0.69 ± 0.02
Dombivali (Maharashtra)	0.31 ± 0.01	0.34 ± 0.01
Baroda (Gujarat)	0.47 ± 0.01	0.49 ± 0.02
Khandala (Maharashtra)	0.49 ± 0.02	0.50 ± 0.01
Lonavala (Maharashtra)	0.44 ± 0.03	0.45 ± 0.01
Murbad (Maharashtra)	0.26 ± 0.04	0.28 ± 0.02
Pen (Maharashtra)	0.22 ± 0.04	0.23 ± 0.01
Tiruchirappalli (Tamil Nadu)	0.23 ± 0.02	0.23 ± 0.01
Thane (Maharashtra)	0.39 ± 0.04	0.38 ± 0.02
Udaipur (Rajasthan)	0.62 ± 0.01	0.63 ± 0.03
Formulations		
Sufoof-e-Khaas (powder)	0.15 ± 0.03	0.17 ± 0.01
Lukol (tablet)	0.23 ± 0.02	0.28 ± 0.05
Speman (tablet)	0.28 ± 0.07	0.31 ± 0.03
Bruhowin (tablet)	0.21 ± 0.02	0.24 ± 0.03

Values are expressed as mean ± SD of 3 analyses.

Chromatographic methods developed in the current research work can be useful as routine quality control tools for the seed samples of *A. longifolia* and its formulations. These methods can also be applied to various plant matrices and polyherbal formulations containing β-sitosterol.

CONCLUSIONS

As single marker-based quantitative methods would be complementary approaches for the quality control and stability assessment of herbal preparations, the results of the current study could be used by industries for the characterization of *A. longifolia* seeds and its formulations in order to check their uniformity. *A. longifolia* grows naturally in paddy fields or marshy lands and also shows edaphic habit in waste lands and therefore, seeds can be collected easily. Using such validated methods *A. longifolia* seeds with precise quality can be encouraged in herbal industries.

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