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Research Article

SEPARATION AND QUANTIFICATION OF PHARMACOLOGICALLY ACTIVE MARKERS BOERAVINONE B, EUPALITIN-3-O-β-D-GALACTOPYRANOSIDE AND β-SITOSTEROL FROM *BOERAVIA DIFFUSA* LINN. AND FROM MARKETED FORMULATION

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ABSTRACT

Objective*: Boerhavia diffusa* Linn. which grows abundantly as a weed throughout India has been widely used in Ayurveda for treatment of a number of diseases.

Methods: In the present work a precise, accurate and reproducible HPTLC method is developed and validated for simultaneous quantification of three pharmacologically active markers Boeravinone B, Eupalitin-3-O-β-D-galactopyranoside and β-sitosterol from the whole plant of *Boerhavia diffusa* Linn. and from marketed Punarnava® capsules of Himalaya herbal healthcare. There are no methods reported for simultaneous separation and quantification of these three markers from any plant matrix. These markers were extracted from the whole plant and from Punarnava® capsules using Soxhlet extraction followed by chromatographic separation on HPTLC silica gel 60 F_{254} pre-coated plates using Toluene: Ethyl Acetate: Methanol as mobile phase. Quantification was carried out in binary mode at 342 nm (Boeravinone B and Eupalitin-3-O-β-D-galactopyranoside) and 580 nm (β-sitosterol).

Results: Linear responses for Boeravinone B, Eupalitin-3-O-β-D-galactopyranoside and β-sitosterol were obtained over the concentration ranges of 15-105 µg/mL, 12-84 µg/mL and 12-84 µg/mL.

Conclusion: This HPTLC method can be used as a quality control tool for quantification of these markers simultaneously from raw material as well as marketed formulation.

Keywords: Boeravinone B; Eupalitin-3-O-β-D-galactopyranoside; β-sitosterol; HPTLC.

INTRODUCTION

Boerhavia diffusa Linn. (syn. *B. repens* Linn. var. *procumbens* Hook.f.) belongs to the family Nyctaginaceae and is known as Punarnava in Sanskrit and Hogweed in English[1]. *Boerhavia diffusa* Linn. is an herbaceous plant species growing prostrate as a weed in habitats like grasslands, agricultural fields, fallow lands, wastelands and residential compounds. *Boerhavia diffusa* Linn. is widely used in the traditional medicine of several American, African and Asian countries[2]. In Ayurveda it is used for the treatment of edema, dermatopathies, heart disorders, anaemia, renal disorders, hepatic disorders and inflammatory disorders[1].

Boerhavia diffusa Linn. has drawn a lot of attention for the biological activities the plant possess. Its leaves have been found to possess anti-diabetic[3] and analgesic effects[4], whilst diuretic[2], immunomodulatory[5,6] anti-lymphoproliferative[7] and hepatoprotective[8] properties have been attributed to the roots.

The *Boerhavia diffusa* Linn. plant contains a large number of compounds such as flavonoids, rotanoids, alkaloids, steroids, triterpenoids, quinines, coumarins, proteins[9,10,11,12,13].

The chemical constituents present in the plant include, Punarnavoside[9], Boeravinone A-F[10,11,12], liriodendrin[12], hypoxanthine-9-L-arabinofuranoside[14], Eupalitin-3-O-β-D-galactopyranoside[15], Eupalitin[15], repenone, repenol[16], ursolic acid[2,17], 5,7-dihyroxy-3',4'-dimethoxy-6,8-dimethylflavone[18], β-sitosterol[1,2], stigmasterol, campesterol[10], syringaresinolmono-β-D-glucoside[11], palmitic, heptadecyclic, oleic, strearic, arachidic and behenic acids[12], hentriacontane[2,17], β-ecdysone, triacontanol[1], polysaccharides[19], boerhavisterol, boerhadiffusene, diffusarotenoid, boerhavilanostenyl benzoate[17].

Boerhavia diffusa Linn. is used commercially as an ingredient of several Over the Counter (OTC) herbal formulations, the market samples are often known to be adulterated with *Trianthema portulacastrum* Linn[1,20]. Thus it is important to establish the quality of the plant raw material as well as the finished formulation.

In the present work a precise, accurate and reproducible HPTLC method is developed and validated for simultaneous quantification of three pharmacologically active markers Boeravinone B, Eupalitin 3-O-β-D-galactopyranoside and β-sitosterol from the whole plant of *Boerhavia diffusa* Linn. and from marketed formulation Punarnava® capsules of Himalaya herbal healthcare. There are no methods reported for simultaneous separation and quantification of these three markers from any plant matrix.

This HPTLC method can thus help to check for the adulteration in the raw material, as well as serve as a quality control tool for quantification of these markers simultaneously from raw material as well as marketed formulation.

MATERIALS AND METHODS

Plant Material

The fresh plant of *Boerhavia diffusa* Linn. was collected from Bhayandar, Thane district, Maharashtra in the month of August 2010. The Herbarium of the plant was prepared and authenticated by Agharkar Research Institute, Pune; its voucher specimen no. is 10-172. The plant material was shade dried for five days and was kept thereafter in hot air oven maintained at 45 ± 5°C for fifteen days. The plant material was then powdered, sieved through 85 mesh and was stored in airtight plastic bottle at room temperature for further analysis.

Boeravinone B (97.3% purity) and Eupalitin-3-O-β-D-galactopyranoside (97.1% purity) were procured from Natural Remedies, Bangalore. β-sitosterol (95.8% purity) was procured from ChromaDex. Methanol, Toluene and Ethyl Acetate all of HPLC grade were procured from E. Merck Specialties Pvt Ltd., Mumbai. Punarnava® capsules (Batch no. F281001G) of Himalaya herbal healthcare were procured from local market.

Preparation of standard stock solutions

Standard stock solutions of pure drugs were prepared separately by dissolving 10 mg of each drug in 10 mL of methanol to get concentration of 1000 µg/mL. This stock was further diluted to 150 µg/mL & 300 µg/mL for Boeravinone B and 120 µg/mL & 240 µg/mL for Eupalitin-3-0-β-D-galactopyranoside and β-sitosterol.

Preparation of calibration and quality levels

For calibration curve, aliquots of 15-105 µg/mL, 12-84 µg/mL and 12-84 µg/mL were prepared from the above stocks for Boeravinone B, Eupalitin-3-0-β-D-galactopyranoside and β-sitosterol respectively.

Also three quality control levels (LQC, MQC, HQC) each of Boeravinone B (105, 60, 30 µg/mL), Eupalitin-3-0-β-D-galactopyranoside (84, 48, 24 µg/mL) and β-sitosterol (84, 48, 24 µg/mL) were prepared for precision, accuracy and ruggedness studies.

Soxhlet Extraction of phytoconstituents

*Plant sample***:** 0.2 gm of accurately weighed whole plant powder was extracted with 200 ml of Methanol in a Soxhlet apparatus for 14 hours, followed by filtration (5µ syringe filter). This extract was concentrated to 5 ml, followed by transferring its contents to 10 ml standard volumetric flask and volume made up to mark with methanol. This filtrate was then used for HPTLC analysis.

Marketed formulation

For analysis of the Punarnava® capsule, contents of twenty capsules were combined and 0.2 gm was accurately weighed was extracted with 200 ml of Methanol in a Soxhlet apparatus for 14 hours, followed by filtration (5µ syringe filter). This extract was concentrated to 5 ml, followed by transferring its contents to 10 ml standard volumetric flask and volume made up to mark with methanol. This filtrate was then used for HPTLC analysis.

Chromatographic procedure

The stationary phase was HPTLC pre-coated silica gel aluminum plate $60F_{254}$ with 250µ thickness, prewashed with methanol. The sample application was performed as per the chromatographic conditions mentioned in table 1. This was followed by detection at 342 nm for Boeravinone B and Eupalitin-3-0-β-D-galactopyranoside (refer Figure 1). This same plate was now derivitised using 1% Anisaldehyde sulphuric acid followed by detection at 580 nm for β-sitosterol (refer Figure 2).

Method validation

ICH harmonized tripartate guidelines Q2 (R1) were followed for the validation of the developed analytical method (ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2 (R1), Nov. 2005). The summary of the validation parameters have been tabulated in Table **[21]**.

Specificity: Specificity was ascertained by analyzing standard compounds and samples. The bands from sample solutions were confirmed by comparing the Rf and spectra of the bands to those of the standards. The peak purity of all the compounds was analyzed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

Inter-Day and Intra-Day Precision*:* Variability of the method was studied by analysing quality control samples of Boeravinone B (105, 60, 30 µg/mL), Eupalitin-3-0-β-D- galactopyranoside (84, 48, 24 µg/mL) and β-sitosterol (84, 48, 24 µg/mL) on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % RSD.

Ruggedness: Ruggedness of the method was assessed by incorporating the small variations in the optimized chromatographic conditions. Effect of change in total volume of the mobile phase from 20 ml to 25 ml and change in chamber saturation time (30 min to 35 min), on the response and Rf of quality control samples was observed.

Stock solution stability: The stability of the master stocks of all the three standards was evaluated by storing the stocks in refrigerator at 2-8°C for 72 hours. This was followed by comparing these concentrations of these stocks against freshly prepared stocks for each standard.

Limit of Detection(LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated using the formula

$$LOD = \frac{3.3\,\sigma}{S} \qquad LOQ = \frac{10\,\sigma}{S}$$

Where σ = Standard deviation of the responses of calibration curve

S = Slope of the calibration curve

Recovery

The accuracy of the method was assessed by performing recovery study at three of different levels (80, 100 and 120%, spiking Boeravinone B, Eupalitin-3-O-β-D-galactopyranoside and β-sitosterol in plant matrix and market formulation). The percent recovery and the average percent recovery for each were calculated.

Instrumentation and Chromatographic Conditions - Table 1

Mobile phase	Toluene **:** Ethyl acetate : Methanol (7 : 1 : 2)
Plate size	10 X 10 cm, 20 X 10 cm
Band length	8 mm
Sample app vol.	5µl (CAMAG LinomatV applicator)
Chamber saturation time	30 min (25±2°C)
Development distance	80 mm
Scanning wavelength	342 nm & 580 nm(CAMAG Scanner IV)
Slit dimensions	6.0 X 0.45 mm
Scanning speed	20 mm/sec
Derivitising reagent.	1% Anisaldehyde sulphuric acid

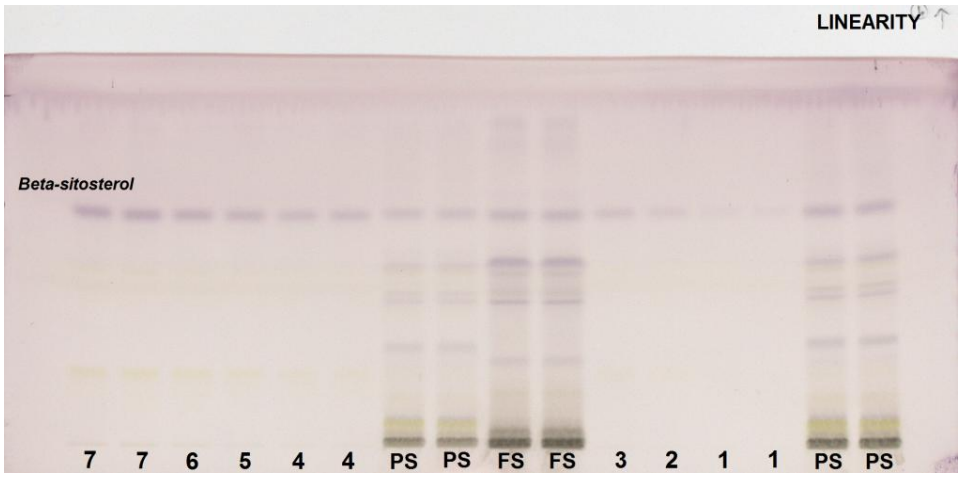

Fig. 2: HPTLC plate under visible light post derivitisation for the Linearity experiment with bands of β-sitosterol in Calibration standards, Whole plant and Marketed formulation

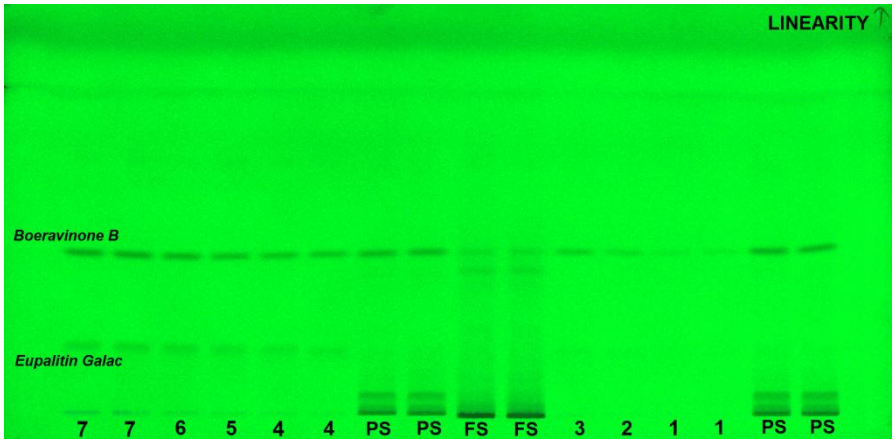

Fig. 1: HPTLC plate at 254 nm for the Linearity experiment with bands of Boeravinone B and Eupalitin-3-O-β-D-galactopyranoside in Calibration standards, Whole plant and Marketed formulation.

7 : Calibration standard Level -7

6 : Calibration standard Level -6

5 : Calibration standard Level -5

4 : Calibration standard Level -4

3 : Calibration standard Level -3

2 : Calibration standard Level -2

1 : Calibration standard Level -1

PS: Plant sample

FS: Formulation sample

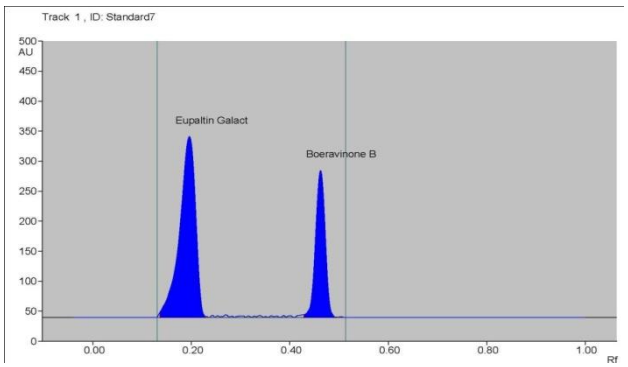

Fig. 3: Densitogram obtained from Standard level-7 for Boeravinone B, Eupalitin-3-O-β-D- galactopyranoside

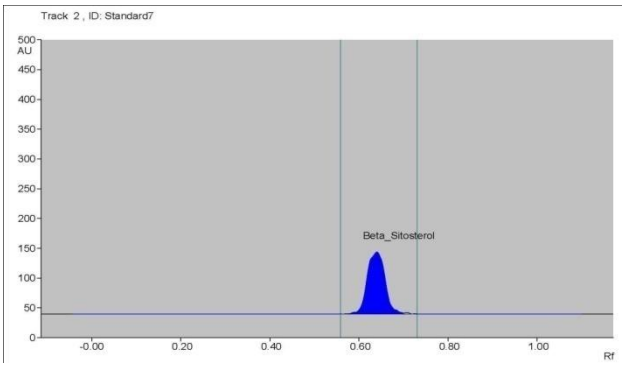

Fig. 4: Densitogram obtained from Standard level-7 for β-sitosterol

RESULTS AND DISCUSSIONS

Different mobile phases containing various ratios of toluene, n-hexane, ethanol, methanol, ethyl acetate, formic acid and acetone were tried. Finally the mobile phase consisting of Toluene: Ethyl acetate: Methanol (7: 1: 2) was selected for obtaining well resolved peaks. The optimum wavelength for detection and quantification used was 342 nm for Boeravinone B and Eupalitin-3-O-β-D-galactopyranoside and 580 nm for β-sitosterol. The retention factors for Boeravinone B, Eupalitin-3-O-β-D- galactopyranoside and β-sitosterol were found to be 0.46 ± 0.01, 0.19 ± 0.01and 0.64 ± 0.01 respectively, refer Figures 3 and 4.

The validated HPTLC method was found to be linear from 15-105µg/ml for Boeravinone B and 12-84µg/ml for Eupalitin-3-O-β-D-galactopyranoside and β-sitosterol. The correlation coefficient was found to be ≥0.99. The LOD and LOQ values calculated from the standard curves were below the linearity range. The precision (%RSD) of the method was found to be ≤ 2%. The recoveries values were found to be within 90-110%. The assay value for whole plant was found to be 0.4%, 0.09% and 0.23% for Boeravinone B, Eupalitin-3-O-β-D-galactopyranoside and β-sitosterol respectively, while for formulation was 0.1% and 0.22% for Boeravinone B and β-sitosterol respectively.

Summary of the Validation parameters - Table 2

Parameter		Boeravinone B	Eupalitin-3-O-β-D-galactopyranoside	β-sitosterol
Linearity(µg/ml)		15 - 105	12 - 84	12 - 84
Correlation coefficient		0.99840	0.99917	0.99899
LOD (µg/ml)		4.34	3.93	4.07l
LOQ (µg/ml)		11.84	10.72	11.10
Precision (RSD)		≤ 2 %	≤ 2 %	≤ 2 %
Recovery	Plant	97.93%	108.13%	101.44%
	Formulation	94.45%	-	92.11%
Assay	Plant	0.4%	0.09%	0.23%
	Formulation	0.1%	-	0.22%
Stock solution stability (2-8°C)		Stable till 72 hours	Stable till 72 hours	Stable till 72 hours
Specificity		Specific	Specific	Specific
Robustness		Robust	Robust	Robust

CONCLUSION

A precise, accurate and reproducible HPTLC method is validated for simultaneous quantification of three pharmacologically active markers Boeravinone B, Eupalitin-3-O-β-D-galactopyranoside and β-sitosterol. This HPTLC method can aid in confirming adulteration in the raw material as well as serve as a quality control tool for quantification of these markers simultaneously from raw material as well as marketed formulation.

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