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A Validated HPTLC Method for the Quantification of B-Sitosterol In Leaves, Bark of Putranjiva Roxburghii Wall

Kalyani Abhimanyu Kedar[*1,2], Sanjay R. Chaudhari[3], Avanapu S. Rao[4]

[1]P. E. Society's Modern College of Pharmacy, Nigdi, Pune, Maharashtra, India
[2]Jawaharlal Nehru Technological University (JNTU), Hyderabad, Andra Pradesh, India
[3]Rasiklal M. Dhariwal Institute of Pharmaceutical Education and Research, Pune, Maharashtra, India.
[4]Bhaskar Pharmacy College, Yeknapally, Moinabad (Mandal), R.R. (Dt), Hyderabad, Telangana, India

ABSTRACT

Objective: A simple and sensitive high-performance thin-layer chromatography method was developed and validated for the determination of β-sitosterol in Putranjiva roxburghii Wall leaf and bark
Methods: Analysis of samples was performed on TLC aluminium precoated plate (60F 254) by using mobile phase toluene: ethyl acetate: formic acid (9:1:0.1v/v/v). TLC plate derivatized with vanillin sulphuric acid reagent. The method was validated using International Council for Harmonization (ICH) guidelines, including linearity, precision, accuracy, and robustness.
Results: A good linearity relationship was found to be with correlation coefficient (r2) value of 0.9951 for β-sitosterol, from calibration curve it shows presence of 0.16%w/w for β-sitosterol in leaf extract, 0.07% w/w in bark extract of Putranjiva roxburghii Wall (Family:Euphorbiaceae). Limit of detection and limit of quantitation was found to be 0.04, 0.13 ng spot-1 respectively for β-sitosterol. The interday and intraday precision was found to be 1.33%, 1.99% (%RSD). Accuracy of the method was performed by recovery studies at three different concentration levels and the average percentage recovery was found to be 98.05% for β-sitosterol.
Conclusion: The proposed method for the quantitation of β-sitosterol was found to be simple, specific, accurate and robust in Putranjiva roxburghii Wall.
Keywords: Putranjiva roxburghii Wall; Euphorbiaceae; β-sitosterol; HPTLC; Method validation.

I. INTRODUCTION

Euphorbiaceae family having 220 genera and 4,000 plant species found in various tropical regions of India [1-2]. Following genera of Euphorbiaceae are reported as medicinal plants: *Acalypha, Aleurites. Bridelia, Jatropha, phyllantus, Putranjiva, Ricinus* [2-3,4]. The species commonly seen in India is *Putranjiva roxburghii* Wall which is known as child's amulet tree or child-life tree [5]. *Putranjiva roxburghii* is evergreen tree with drooping branches with corky bark coriaceous leaves, dioeciously flowers [6].

Most frequently recorded folk remedy claims of *Putranjiva roxburghii* Wall mentioned that the plant leaf, bark, seed, nuts are medicinally useful. Paste of seeds of *Putranjiva roxburghii* applied on forehead to check pain. The seeds of this plant species are given daily for one month to women for conception [6]. The bark and the seeds are usefull in antidotal treatment of snake-bite. Its leaves and fruits, stones of this plant have been traditionally used for the treatment of fever, muscle twisting, aphrodisiac, arthralgia and rheumatism [7-9]. It is also used as antinociceptive, antipyretic, anti-inflammatory, antioxidant [10]. This plant has reported various phytoconstituents such as putranjivanonol, putranjic acid, friedelin, putranjivadione, friedelanol and roxburgholone from the trunk bark of *Putranjiva roxburghii* [11-13]. Roxburghonic acid, putraflavone were isolated from the alcoholic extract of *Putranjiva roxburghii* leaves [14].

β-Sitosterol is a dietary supplements, found in a variety of plants and plant oils. Phytosterols are similar in structure to cholesterol except some minor structural differences [15]. β-Sitosterol was estimated by HPLC in

Ampelocissus latifolia (Roxb.) species [16] Based on literature β-Sitosterol has promised antidiabetic activity and it shows antioxidant effects and specifically useful for hypercholesterolemia and radioprotective acivity [17-20]. The was also studied Some reports are available on plant sterols and plant stanols use full in the management of hyperlipidaemia [21]. β-sitosterol isolated from *Syzygium cumin* (L.) inhibits the activity of α-amylase enzyme and thus slows down the glucose release in the blood stream. Previous analytical work includes that amentoflavone, β-amyrin and stigmasterol determined from *Putranjiva roxburghii* by HPTLC method [22, 23]. So an attempt has been made to carry out chromatographic analysis of leaves and bark of *Putranjiva roxburghii* Wall.

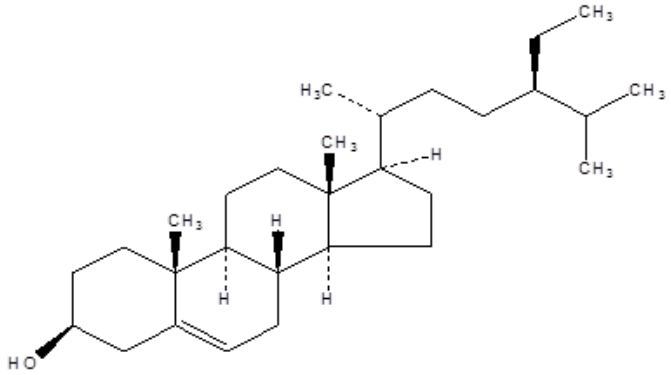


Figure 1. Chemical structure of β-sitosterol

II. MATERIALS AND METHODS

Plant material

The leaves and trunk bark material of fully grow tree of *Putranjiva roxburghii* Wall was collected from Khadaki region of Maharashtra, India in June 2014. The taxon is authenticated from Botanical Survey of India, Pune dated 18/08/2014 with Voucher number BSI/WRC/Cert./2014 and collection no.KKA 01. The herbarium specimen is deposited in the Modern college of pharmacy, Nigdi, Pune.

Chemicals and reagents

β-sitosterol was purchased from Sigma-Adrich (USA). All other solvents, reagents and Silica gel 60 F_{254} precoated HPTLC Plates (20 × 20 cm) were purchased from Merck (Germany).

HPTLC instrumentation and experimental conditions

Method development parameters includes sample and test solution preparation, HPTLC instrumentation condition, preparation of developing chamber, derivatization reagents were carried out as per guideline mention in USP (Ch.203). According to this chromatographic analysis was done on 10×10 cm HPTLC Silica gel F_{254} plates. Samples of extracts, formulations and standards were applied as band length 8 mm wide and 8mm apart by Camag Linomat 5 sample applicator. The application rate of sample on plate was $150nl^{-1}$. The plate was developed in previously saturated 10×10 cm twin-trough glass chamber at room temperature. Initially different mobile phases were use for chromatogram development from this best resolution was observed in the composition of toluene: ethyl acetate: formic acid (9:1:0.1v/v/v) for β-sitosterol. Dry TLC plate derivatized with vanillin sulphuric acid reagent, heat plate at 105^{o}C and observed separation of bands it helps in analysis of β-sitosterol in leaf and bark Extracts [24]. Analysis was done at 540nm in absorbance remission mode by win CATS Chromatography software.

Preparation of standard solution

A stock solution of β-sitosterol (100µg/ml) was prepared by dissolving 10mg of accurately weighed β-sitosterol in 100ml chloroform. For calibration 0.2-1.2µl standard solution was applied to HPTLC plate in the range 20-120 ng per band.

Preparation of sample solution

Extract of leaves and bark were prepared by weighing 50gm of dried powdered drug of leaves and bark of *Putranjiva roxburghii* and extraction was carried out by soxhlet extraction assembly for 6hrs. Solution was filtered, concentrated and use for HPTLC analysis. From this weigh 10mg leaf and bark extract and transferred to a 10ml volumetric flask. Chloroform was added to volumetric flask to make final concentration (1000µg/ml)

Method validation

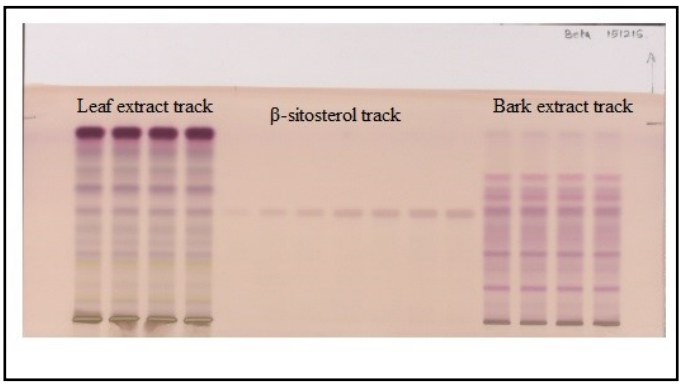
The analytical method was validated for linearity, precision, accuracy, specificity, limit of detection (LOD) and limit of quantitation (LOQ) according to ICH guidelines (ICH, 2005). The linearity was carried out by

applying different concentration of standard β-sitosterol. Quantitation of marker in sample was carried out by calibration curve. LOD, LOQ were carried out according to formula {LOD= 3.3(SD/S) and LOQ= 10 (SD/S)}. Precision studies include, repeatability and system precision. Accuracy by recovery studies were carried out by spiking known concentration of standard to pre-analyzed samples. The robustness was carried out by making small variation in optimized method parameters such as variation in composition of mobile phase, chamber saturation time etc. The specificity of the method was determined by comparing R_f values.

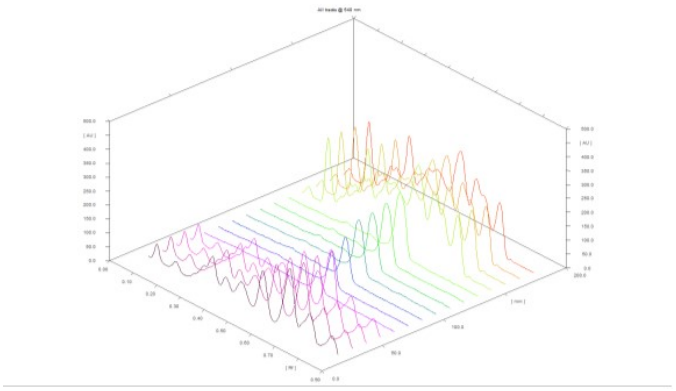
III. RESULTS AND DISCUSSION

Solvent system optimization

For optimization of solvent system various compositions of mobile phases were use. When mobile phase consisting toluene: ethyl acetate (10:1) component in samples not get resolved. In ordered to improve resolution in between peaks mobile phase in composition of toluene: ethyl acetate: formic acid (9:1:0.1v/v/v), gives compact peak of standard and standard in samples. Observation shows the same R_f value (0.5) (Fig. 3) for β-sitosterol in standard and samples.



(a)



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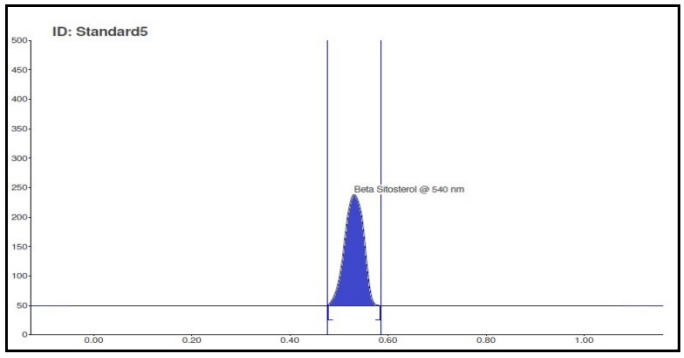

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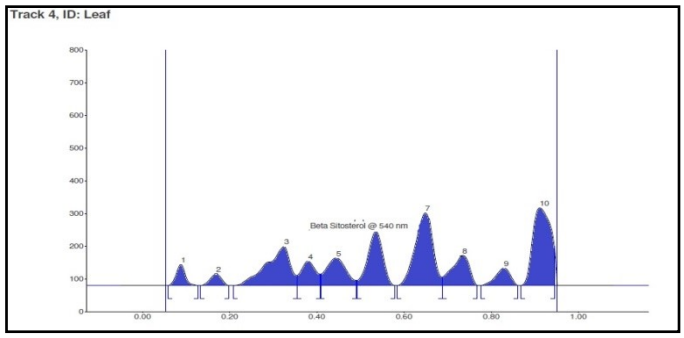

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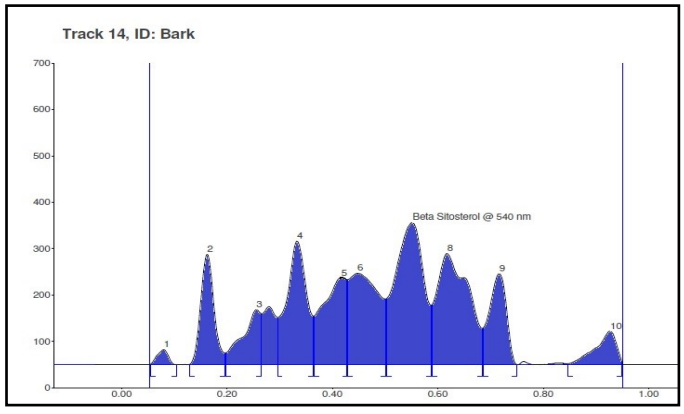

(e)

Figure 3. HPTLC Chromatogram of leaf, bark with β-sitosterol (a), 3D display of leaf, bark with β-sitosterol chromatogram (b), HPTLC Chromatogram of standard β-sitosterol (c), leaf extract (d), Bark extract (e) at 540nm

IV. METHOD VALIDATION

Linearity

For determining the linearity range of standard β-sitosterol, a series of spots of different volumes (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 µl) were applied so as to get 20-120 ng quantity of standard per band, respectively. Linearity was evaluated in triplicate. The plate was scanned at 540nm and curve was prepared with respect to area *vs.* amount per spot (Figure 2). A good linearity relationship was found to be with correlation coefficient (r^2) value of 0.9951 for β-sitosterol (Table 1 and Figure 2)

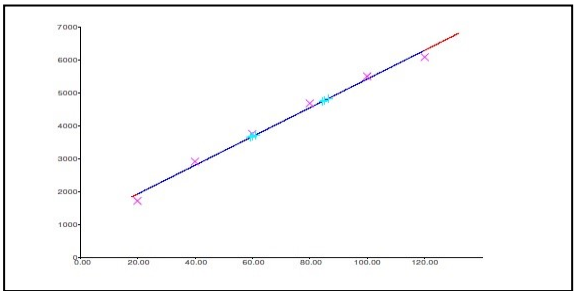

Figure 2. Calibration curve of Standard β-sitosterol at 540nm

Quantification of β-sitosterol

0.6µl of the plant leaf, bark extract were applied to HPTLC plate in triplicate and the amount of β-sitosterol in samples were determined by using calibration curve of standard.

This method shows the presence of 0.16%w/w for β-sitosterol in leaf extract, 0.07% w/w in bark extract.

Limit of Detection and quantitation

In order to determine limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on standard deviation (SD) and slope (S) of the calibration curve at levels approaching to the LOD according to formula { LOD= 3.3(SD/S) and LOQ= 10 (SD/S)}. LOD and LOQ calculated and found to be 0.04, 0.13 ng $spot^{-1}$ for β-sitosterol (Table 1).

Table 1. Method validation parameters for the quantitation of β-sitosterol by HPTLC.

Parameters	Results
Range of linearity (ng $spot^{-1}$)	20-120
Regression of equation	Y = 1048 + 43.54 * X
Slope	43.54
Correlation coefficient (r^2)	0.9951 ±0.003
LOD	0.043 ng
LOQ	0.13 ng

Precision

Precision studies were carried out to show the reproducibility of the proposed developed method. Intraday precision study was carried out by applying six times 60ng per band of same concentration. It can be analyzing at three different times in a day for intraday precision and the same procedure was followed for three different days to determine interday precision. The results were reported as SD (%RSD) (Table 2). The % RSD was found to be 1.33 %, 1.99% for interday and intraday precision. The low %RSD indicated the method is precise for the analysis (Table 2).

Table 2. Interday and intraday precision for quantitation of β-sitosterol by HPTLC densitometric method.

Concentration (ng $spot^{-1}$)	β-sitosterol			
	Interday precision		Intraday precision	
	Mean peak area ± SD*	%RSD**	Mean peak area ± SD	%RSD
60	3775.182±50.49	1.33	2781.45±55.41	1.99

* SD-standard deviation, ** RSD-relative standard deviation.

Specificity

The specificity of the method was determined by analysing standard drug and sample. The presence of β-sitosterol in leaf, bark were confirmed by comparing R_f of sample with standard.

Recovery studies (Accuracy)

Accuracy of method was studied by performing recovery studies at 3 levels of β-sitosterol. The pre-analyzed samples were spiked with 80%, 100% and 120% of the standard β-sitosterol and analyzed by the proposed HPTLC method. The experiment was conducted six times the percentage recovery at three different levels of β-sitosterol was found to be 98.14, 99.01, 97% respectively (Table 3).

Table 3. Accuracy (recovery study) determined for the TLC-densitometric method.

% of standard spiked to the sample	Theoretical content (µg $spot^{-1}$)	Experimental content (µg $spot^{-1}$±SD*)	Recovery (%)	RSD* (%)
80	0.108	0.106	98.14	0.94
100	0.12	0.1188	99.01	0.43
120	0.13	0.1261	97.00	1.36

* SD-standard deviation, ** RSD-relative standard deviation.

Robustness

Robustness was studied in triplicate at 60ng $band^{-1}$ by making small variation in optimised method parameters

such as variation in composition of mobile phase, chamber saturation time. The results were examined in terms of relative standard deviation (%RSD) and standard error of peak area (Table 4). Mobile phase prepared by solvent system such as Toluene: Ethyl acetate: Formic acid in composition (9: 1:0.1 v/v/v), (9.1:0.5: 0.1 v/v/v), (8.9:1.1:0.1 v/v/v) etc. Duration of saturation time change during chromatograph development (15, 20 and 25 min) respectively. The plate was activated at 110^0C for 20 min and analysed at 540nm. By introducing small changes into TLC method % RSD was obtained less than 2% proved the robustness of proposed method.

Table 4. Robustness study

Parameters				
Mobile phase (Toluene: Chloroform)				
Actual (v/v)	Used(v/v)	Level	Mean peak area ± SD*	%RSD**
Toluene: Ethyl acetate: Formic acid (9:1:0.1v/v/v)	9.1:0.5:0.1	+1	3291.34±36.34	1.11
	9:1:0.1	0	3387.7±55.04	1.62
	8.9:1.1:0.1	-1	3281.94±47.94	1.46
Saturation Time (Minutes)				
Actual				
20	15	-5	3358.73±54.22	1.61
	20	0	3331.34±46.63	1.39
	25	+5	3267.89±1.43	1.43

* SD-standard deviation, ** RSD-relative standard deviation.

V. CONCLUSION

In present study HPTLC method was developed and validated for the determination of β-sitosterol in *Putranjiva roxburghii* Wall leaf and bark extracts, which shows 0.16, 0.07% w/w respectively. The developed method found to be simple, accurate, specific and robust for the analysis of β-sitosterol in crude drug sample. Based on these results leaves of this plant contains higher β-sitosterol may be use full for analysis of various biological activity. So this proposed method may be useful in analysis of β-sitosterol containing plant species and polyherbal formulations.

VI. ACKNOWLEDGMENTS

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VII. CONFLICTS OF INTEREST

There are no conflicts of interest.

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