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ORIGINAL ARTICLE

HPTLC Method Development and Validation for Quantification of Biomarker β-Sitosterol and Betulinic Acid in the Leaves, Stem and Roots of *Spermadictyon suaveolens* Roxb.

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ABSTRACT
A simple method has been established for the, β-Sitosterol and Betulinic Acid biomarker in the Leaves, Stem and Roots of Spermadictyon suaveolens Roxb belonging to the Rubiaceae family. Chromatography was performed on Silica gel 60 F254 precoated plates using mobile phase solvents of n-Hexane: Ethyl Acetate 8:2 (v/v) for both the biomarkers, thereafter derivatized with Anisaldehyde Sulphuric Acid (ASA) and then scanned, quantified at 540 nm. A clear band for β-Sitosterol at RF 0.21 ± 0.03 and for Betulinic Acid at RF 0.15 ± 0.03 was observed. The intra-day and inter-day precisions with mean % RSD value (n=6) was found to be 1.902% and 1.186% and for Betulinic Acid was 2.980% and 2.117% which proves that the method created is in fact specific and reproducible. The Quantity of β-Sitosterol found in leaf, stem and root were 0.1518µg, 0.1582µg and 0.1014µg and for Betulinic Acid were, 0.0043µg, 0.0592µg and 0.1012µg of plant part respectively. The Recovery has been seen to be >95% for β-Sitosterol and >80% for Betulinic Acid in the root of the plant. Thus, quality assurance of crude drugs as well as the determination of dosage may be decided by referencing to this method.
Keywords*: Spermadictyon suaveolens Roxb., β-Sitosterol, Betulinic Acid, HPTLC, Quantification, Validation.*

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INTRODUCTION

Many Phyto-constituents from plants act as marker compound to determine the authenticity of the part of the plant or plant itself. Well established and validated protocol may be used for the profiling of the phytochemical and quantification of standard compounds in medicinal plants.

Spermadictyon suaveolens Roxb with synonym of *Hamiltonia suaveolens,* Forest Champa, is a woody shrub, 2-3 meters in height, from the family Rubiaceae mostly found in the Indo-Pak subcontinent [1]. Among the plethora of medicinal properties, its used for, particular usage in, viral infections, diabetes and wound healing are of significance [2]. Leaves and bark have shown anti-oxidant and anti-microbial properties [3] while the stem has been used by traditional practitioners to cure Herpes zoster virus known as 'Nagin'[4]. Roots are applied to relieve joint pain [5] as well as in rheumatoid arthritis and bloody dysentery in animals [2].

β-Sitosterol and Betulinic Acid found in the leaves, stem and root of *Spermadictyon suaveolens* Roxb can be further utilized for authentication of the plant and its parts, as a biomarker. β-Sitosterol is a phytosterol which has numerous medicinal properties [6]. Found in most plants like nuts, legumes, olive oil etc. shows diverse nutraceutical benefits like anticancer, anti- inflammatory, anti-diabetic, antioxidant, wound healing, lipid lowering etc.[7]–[10].

Betulinic Acid is a Pentacyclic Triterpenoid that has proved to show anti-retroviral, anti-diabetic, anti-inflammatory, anti-malarial as well as anti-cancer and anti-obesity properties [11] . Found in dried fruits like raisins and trees like birch eucalyptus and plane trees [12].

HPTLC is a comprehensive analytical method by which large number of samples may be analyzed at the same time and has been in use since the last two centuries helping in phytochemical identification and quantification[13]. This method makes use of very less quantity of sample extracts and solvents, also it takes much less time as compared to other documented methods[14]. The main purpose of such an endeavor was to develop and create a HPTLC valid method for β-Sitosterol and Betulinic acid biomarkers in the plant chosen here. Also, literature survey shows not much work has been done on these biomarkers and on this plant.

MATERIAL AND METHODS

The whole plant of *Spermadictyon suaveolens* Roxb. were collected from 'Go Green' Nursery at Karnala, Panvel. The specimen was authenticated from; St. Xavier's College Blatter's herbarium, and was found to have the accession number of 23598.

Apparatus

Instrument Camag Linomat V sample applicator, Camag Twin trough glass chamber and Camag TLC Scanner IV equipped with Vision Cats 3.2.22308.1 version.

Reagents

99.8% purity reagents like n-Hexane, Ethyl acetate, methanol used were of analytical quality and procured from Merck Chemicals. Class A grade glassware like Pipettes, Standard volumetric flasks were used. β-sitosterol Standard and Betulinic Acid were procured from Yucca Enterprises.

Preparation of Stock Solution

Stock solutions -A

10.0 mg of standard β-sitosterol was dissolved with 10.0 mL of methanol in a standard volumetric flask and sonicated to prepare 1µg/µL.

Preparation of 0.1 µg/µL

Stock solution -B

stock solution A was initially dissolved by sonication in 5.0 mL of methanol, then mixed additional methanol was added in a 10 ml volumetric flask up to the mark. Thus, stock solution-B of β-sitosterol of 0.1 µg/µL was prepared.

Stock solution -C

Betulinic Acid (0.5µg/µL) was prepared in methanol by mixing 5.0 mg of standard Betulinic Acid with 5.0 mL of methanol in a standard volumetric flask at first. Then further addition of methanol to make up the 10 mL.

Stock solution-D

Further to prepare (0.05 µg/µL) solution, 0.1 mL of the stock solution was initially dissolved by sonication in 5.0 mL of methanol, then mixed additional methanol was added in a 10 ml volumetric flask up to the mark. In this way, stock solution of Betulinic Acid of 0.05µg/µL was prepared

Plant Sample Preparations

Methanol was used for extraction of β-Sitosterol and Betulinic acid from plant powder. Plant extracts of the concentration 100 µg/µL were prepared by taking shade dried leaf, stem, root powder of *Spermadictyon suaveolens* Roxb, 1 gm separately and further extraction with 10.0 mL of methanol. It was first sonicated for 20 min and then kept for 24 hours and then filtered with Whatmann filter paper No. 41. The filtrate was used for quantification. The amount of the plant leaf, stem and root sample solution which was applied for quantification was 20 µL.

Method Development Procedure

After several trials of using solvents for both biomarkers, in varying proportions the mobile phase was selected as n-hexane: Ethyl Acetate (8:2) (v/v). As both the biomarkers are from the same class of compound, the same mobile phase was found to be apt for both. This mobile phase showed the best resolution and so was selected as the solvent phase. To observe the distinct bands, the developed plate was dried, derivatized by ASA- anisaldehyde sulphuric acid reagent and heated to 110 ºC. The optimized saturation time was observed to be 20 min. Development distance of 70 mm along with each band length of 8 mm and a distance of 13 mm between the bands were applied. The bands were observed at 540 nm using tungsten lamp in absorbance–reflectance mode. The conditions in HPTLC are given in Table 1.

Method Validation

The validation studies of Recovery, Precision, Polynomial linearity, limit of quantification (LOQ), limit of detection (LOD) and quantification studies were performed as per the ICH guidelines for the Method Validation Process [15].

Quantification was done by the external standard method [16]. A chromatogram was developed using standards and with different concentration ranging from 1 µL-7 µL of stock solution A for β-sitosterol and

0.5 µL- 3.5 µL of Stock solution D for Betulinic acid. leaf, root, stem extract with concentration of 20 µL, plotted on the same HPTLC plate and the calibration curve was obtained by plotting standard peak area against concentration.

For determining the **linearity** range, a series of 7 spots of different volumes ranging from 1 µL-7 µL of stock solution A and 0.5 µL- 3.5 µL of Stock solution D was applied on the same HPTLC plate for β-Sitosterol and Betulinic acid respectively. After this the plate was scanned at 540 nm, and a curve was prepared with respect to peak area vs. Concentration per spot.

As per the ICH guidelines, the limit of detection **(LOD)** and limit of quantitation **(LOQ)** were determined at a signal to noise ratio of 3:1 and 10:1 respectively. Standard deviation (SD) of response and slope was calculated for LOD (DL=3.3xSD/S) and LOQ (DL=10xSD/S).

Intra-day **precision** was performed with in a single day by application of the six bands to a HPTLC plate (each 2µL) of standard β-sitosterol of stock solution B and (each 1µL) of standard Betulinic Acid Stock Solution C of 0.5 µg/µL, the densitograms and peak areas were recorded. The peak areas for each applied concentration β-sitosterol and Betulinic acid were recorded for the Inter-day precision on three consecutive days.

For **specificity** studies, assay and impurity method was performed using methanol, solvent system of n-hexane:Ethyl Acetate (8:2) (v/v), standards of β-sitosterol and Betulinic acid along with the leaf, stem and root samples on a single HPTLC plate with chamber saturation of 20 minutes with filter paper Whatmann No.1.

RESULTS AND DISCUSSION

Method development

An effective method for the separation of constituents present in the leaf, stem and leaf of *Spermadictyon suaveolens* Roxb was developed exhibiting precise peaks of standard β-sitosterol and Betulinic acid. High resolution bands of β-sitosterol and Betulinic acid were obtained at Rf = 0.21 $\pm$ 0.03 and for Betulinic Acid at Rf = 0.15 $\pm$ 0.03

Quantification

Quantification of β-sitosterol and Betulinic acid was done in leaf, stem and root parts of *Spermadictyon suaveolens* Roxb. Maximum amount of β-sitosterol was found in stem extract, 0.158 µg and maximum amount of Betulinic acid was found in root, 0.101 µg.

The bands of β-sitosterol and Betulinic acid extracts ware identified and confirmed by comparing Rf value of extracts with the chromatogram of standard β-sitosterol solution and Betulinic acid. The solution with volume ranging from 1 µL-7 µL of stock solution A for β-sitosterol and 0.5 µL- 3.5 µL of Stock solution D for Betulinic acid yielded better results and therefore were used for the quantification (Fig.3). Mean reading of standard sample solution were used for the purpose of quantification of β-sitosterol (Table No.2) and Betulinic acid (Table No.3)

Linearity

Linearity of β-sitosterol and Betulinic acid was validated by linear polynomial regression equation and correlation coefficient (Figure 2 & 3). The linear correlation coefficient, 'r' = 0.9843 for β-sitosterol and 'r' = 0.9914 for Betulinic acid being close to '1' indicates a perfect positive correlation between the concentrations of the standards with their respective peak areas.

LOD/LOQ

Limit of detection LOD and the Limit for quantification LOQ with n=5 for β-sitosterol was found to be 0.3580 µg and 0.0038 µg, while for Betulinic acid was found to be 0.0352 µg and 0.1067 µg respectively. These values make it clear that the instrument shows an excellent sensitivity of the mentioned standard compounds at very low concentrations.

Intra-day and inter-day precision

The peak areas of similar separate bands of β-sitosterol and Betulinic acid were recorded for the same day as well as on different days as per ICH guidelines. Intra-day and inter-day precision and accuracy of the assay for β-sitosterol and Betulinic acid showed a good precision of the formulated method. The mean of % R.S.D value (n = 6) in intra-day and inter-day precisions studies for β-sitosterol were found to be 1.186 and 1.902 (Table 4 and 5). In the case of Betulinic acid were found to be 2.117 and 2.980 respectively (Table 6 and 7). The peak areas of β-sitosterol for all concentrations is below 2% which shows an excellent reliability while for Betulinic acid below 3% also shows a good reliability.

Recovery

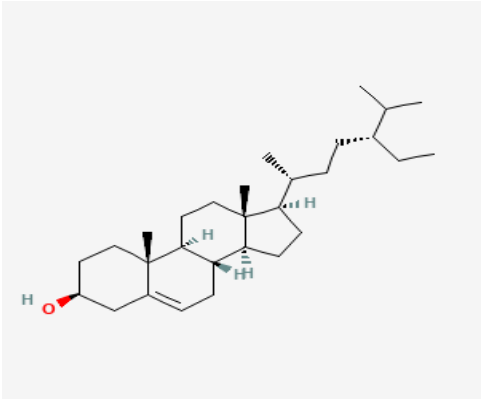
The peak area responded well when root plant extract (zero value) was spiked by 80%, 100% and 120% β-sitosterol and Betulinic acid separately. The recovery of β-sitosterol and Betulinic Acid was seen to be 95.187 % and 80.844 % respectively in roots of the plant, which signifies a good accuracy level for the

method as shown in Table 8, for β-sitosterol and Table 9 for Betulinic acid. This indicates the system suitability and adequate reproducibility of the equipment. (Fig.5)

Specificity

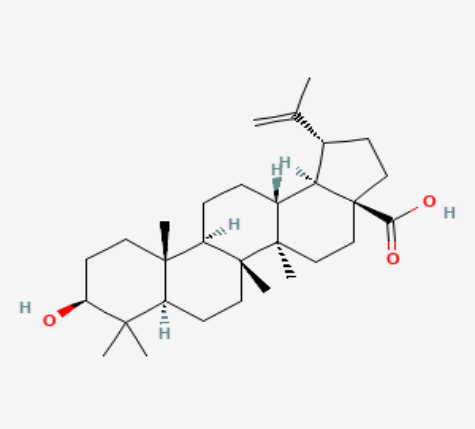
There was only the positive response of the presence of β-sitosterol and Betulinic acid and the standard solutions while a complete negative response was observed by the diluent Methanol and the mobile phase, n-hexane: Ethyl Acetate (8:2) (v/v) (Fig.6). Thus, indicating excellent specificity level of the method.

Figure 1.β-Sitosterol



Synonyms: β-Sitosterol[17]
Sitosterol
Cupreol
Azuprostat
22,23-Dihydrostigmasterol
Quebrachol
Triastonal
(-)-beta-Sitosterol
Mol. weight: 414.71[17]
Group of Compound: Terpenes-Triterpenes

Figure 2. Betulinic Acid



[17]Synonyms: Betulinic acid
Betulic acid
Mairin
Lupatic Acid
C30H48O3
3beta-Hydroxy-20(29)-lupaene-28-oic acid
3-Hydroxylup-20(29)-en-28-oic acid
.beta.-betulinic acid
Betulinicacid
Gratiolone
Formula: $C_{30}H_{48}O_3$
Mol. weight: 456.7
Group of Compound: Terpenes-Pentacyclic Triterpenoi

Fig.3 Polynomial Dynamic range of β-sitosterol

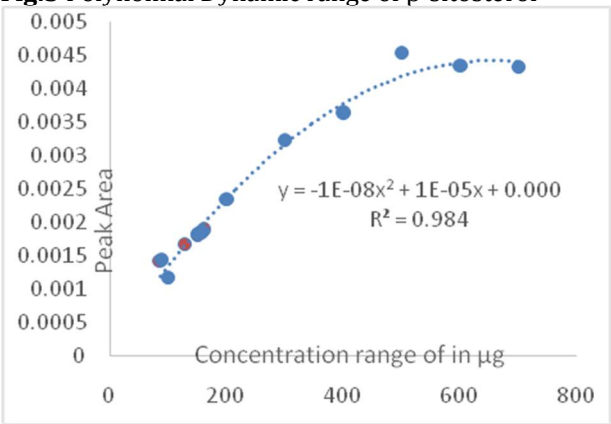


Fig.4 Linear Dynamic range of Betulinic Acid

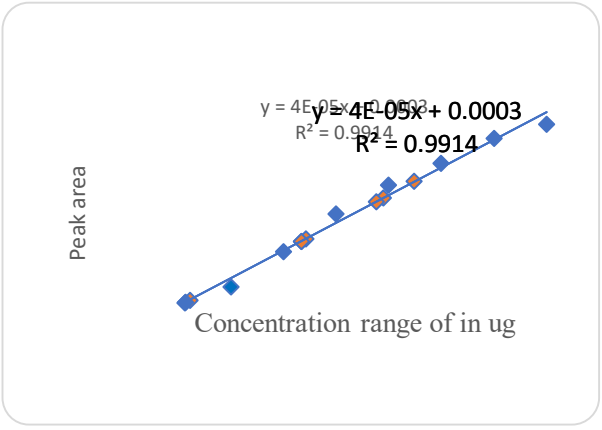
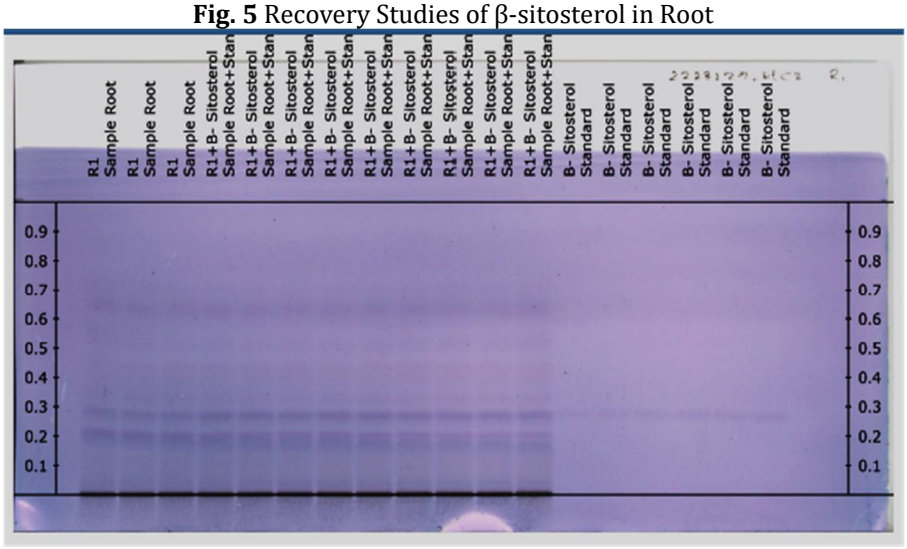


Fig. 5 Recovery Studies of β-sitosterol in Root



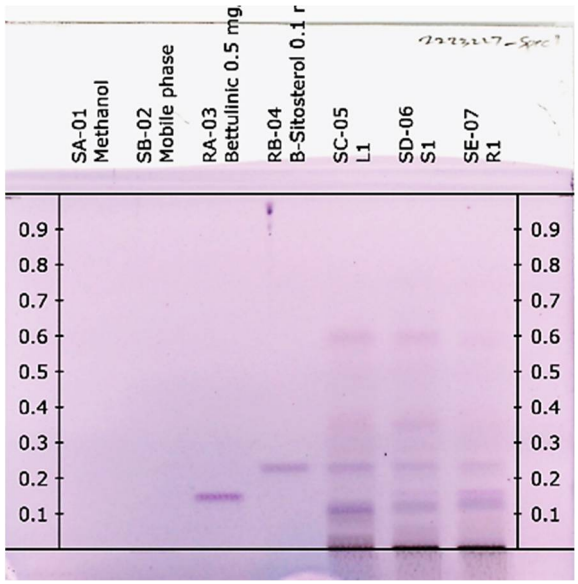


Fig 6. Specificity of β-sitosterol and Betulinic acid.

Table.No.1

Chromatographic parameters	Description
Stationary Phase	Merck Silica gel 60F254 pre-coated on Aluminium sheet.
Mobile Phase	n-hexane: Ethyl Acetate (8:2) (v/v)
Chamber saturation time	20 mins
Band Length	80 mm
Developing Distance	70 mm
Derivatizing reagent	ASA-Anisaldehyde Sulphuric Acid
Plate drying	2-4 min (after development) 5-7 min (after derivatization) Heating at 110 ºC for 1-3 min
Scanning Wavelenth	540 nm

Table No.2 The Rf values and peak areas of the serial dilutions of β-sitosterol and fixed amount of leaf, stem and root extract of *Spermadictyon suaveolens* Roxb.

Sr. No	Sample	Application Volume	Peak Area	End Rf	Amount of β-sitosterol per spot µg
1	β-sitosterol	1 µL	0.001171	0.227419	0.1
2	β-sitosterol	2 µL	0.002338	0.216129	0.2
3	β-sitosterol	3 µL	0.003231	0.214516	0.3
4	β-sitosterol	4 µL	0.003642	0.209677	0.4
5	β-sitosterol	5 µL	0.004530	0.214345	0.5
6	β-sitosterol	6 µL	0.004341	0.214516	0.6
7	β-sitosterol	7 µL	0.004327	0.214516	0.7
8	Leaf	20 µL	0.001841	0.208065	0.154661
9	Leaf	20 µL	0.001814	0.209677	0.150324
10	Leaf	20 µL	0.001816	0.209667	0.150646
11	Stem	20 µL	0.001877	0.21129	0.160443
12	Stem	20 µL	0.001850	0.212903	0.156106
13	Stem	20 µL	0.001863	0.213040	0.158194
14	Root	20 µL	0.001678	0.212903	0.128482
15	Root	20 µL	0.001421	0.209677	0.087205
16	Root	20 µL	0.001431	0.21023	0.088812
17	Mean β-sitosterol Leaf Amount				0.151877
18	Mean β-sitosterol Stem Amount				0.158247
19	Mean β-sitosterol Root Amount				0.101499

Table No.3 The Rf values and peak areas of the serial dilutions of Betulinic Acid and fixed amount of leaf, stem and root extract of *Spermadictyon suaveolens* Roxb.

Sr. No	Sample	Application Volume	Peak Area	End Rf	Amount of Betulinic Acid per spot µg
1	Betulinic acid	0.5µL	0.00092	0.146774	25
2	Betulinic acid	1µL	0.002093	0.143548	50
3	Betulinic acid	1.5µL	0.003346	0.141935	75
4	Betulinic acid	2µL	0.0043	0.135484	100
5	Betulinic acid	2.5µL	0.00504	0.134566	125
6	Betulinic acid	3µL	0.005859	0.137097	150
7	Betulinic acid	3.5µL	0.00633	0.140323	175
8	Leaf	20 µL	0.000399	0.129032	0.00334
9	Leaf	20 µL	0.000491	0.133871	0.00582
10	Leaf	20 µL	0.000412	0.132452	0.00369
11	Stem	20 µL	0.002443	0.143548	0.05840
12	Stem	20 µL	0.002525	0.145161	0.06066
13	Stem	20 µL	0.002444	0.143542	0.05848
14	Root	20 µL	0.004433	0.15	0.11210
15	Root	20 µL	0.003887	0.151613	0.09738
16	Root	20 µL	0.003765	0.151232	0.09409
17	Mean Leaf Amount				0.00429
18	Mean Stem Amount				0.05920
19	Mean Root Amount				0.10119

Table No.4 Intra-day precision for β-sitosterol

Sr. no	Conc. of β-sitosterol	Peak Area of β-sitosterol	RF
1	0.1 mg/ml	0.00437	0.202
2	0.1 mg/ml	0.00448	0.2
3	0.1 mg/ml	0.00444	0.202
4	0.1 mg/ml	0.00451	0.194
5	0.1 mg/ml	0.00456	0.197
6	0.1 mg/ml	0.00461	0.195
Mean		0.004495	0.198333333
S.D		8.54985E-05	
% R.S.D		1.902	

Table No.5 Inter-day precision for β-sitosterol

S. n	Conc. Of β-sitosterol	Peak Area of β-sitosterol			Mean	SD	%RSD	Mean % R.S.D
		Day 1	Day 2	Day 3				
1	0.5 mg/ml	0.00451	0.00448	0.0044	0.00446	5.68624E-05	1.27399	1.186
2	0.5 mg/ml	0.00448	0.00461	0.00456	0.00455	6.55744E-05	1.441195	
3	0.5 mg/ml	0.00444	0.00451	0.0045	0.00448	3.78594E-05	0.844447	

Table No.6 Intra-day precision for Betulinic Acid

Sr. no	Conc. of Betulinic Acid	Peak Area of Betulinic Acid	RF
1	0.5 mg/ml	0.00153	0.132
2	0.5 mg/ml	0.00148	0.127
3	0.5 mg/ml	0.00151	0.131
4	0.5 mg/ml	0.00147	0.131
5	0.5 mg/ml	0.00142	0.126
6	0.5 mg/ml	0.00154	0.129
Mean		0.001491667	
S.D		4.44597E-05	
% R.S.D		2.980	

Table No.7 Inter-day precision for Betulinic Acid

Sr. no	Conc. of Betulinic Acid	Peak Area of Betulinic Acid			Mean	SD	%RSD	Mean % R.S.D
		1	2	3				
1	0.1 mg/ml	0.0015	0.00147	0.00148	0.001483	1.52753E-05	1.029792	2.117
2	0.1 mg/ml	0.00153	0.00151	0.00148	0.001506	2.51661E-05	1.670317	
3	0.1 mg/ml	0.00162	0.00151	0.00154	0.001556	5.68624E-05	3.652831	

Table No.8 Recovery studies of β-sitosterol in Root of *Spermadictyon suaveolens* Roxb.

Marker β-sitosterol	% Marker added	Marker added in µL	Area Average of 3 reading	Expected %	% Recovery	Average Recovery % 95.187
	80	0.8	0.00281	0.00311	89.369	
	100	1	0.00332	0.003567	93.362	
	120	1.2	0.00376	0.00366	102.829	

Table No.9 Recovery studies of Betulinic Acid in Root of *Spermadictyon suaveolens* Roxb.

Marker Betulinic Acid	% Marker added	Marker added in µL	Area - Average of 3 reading	Expected Value%	% Recovery	Average Recovery % 80.844
	80	1.6	0.00501	0.00610	93.240	
	100	2	0.00447	0.00624	80.328	
	120	2.4	0.00890	0.00649	68.964	

CONCLUSION

A simple, precise and novel method has been validated for the quantification, and identification of β-sitosterol and Betulinic acid in the methanolic extract of shade dried powder of leaf, stem and root of

Spermadictyon suaveolens Roxb. This method may be further used for qualitative and quantitative determination the compounds β-sitosterol and Betulinic acid in the selected plant. A novel compound Betulinic acid has been here successfully identified and quantified for the very first time through HPTLC in the plant *Spermadictyon suaveolens* Roxb. The method for both β-sitosterol and Betulinic acid was validated in terms of accuracy, precision, linearity and specificity showing that this may be used for quality control purposes.

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Conflict of Interest: No conflict-of-Interest rests among the authors.

REFERENCES

1. Mokat D., Kavita M., and Rakshe A., (2016) Pharmacognostic studies of drug *Spermadictyon suaveolens Roxb.* Int. J. of Pharmacognosy., vol. 3, no. 5, pp. 234–239, doi: 10.13040/IJPSR.0975-8232.IJP.3(5).234-39.
2. Papitha R., Ravi L., and Selvaraj C. I., [2017] Phytochemical Studies and Gc-Ms Analysis of *Spermadictyon Suaveolens Roxb*. Int. J. of Pharmacy and Pharmaceutical Sciences, vol. 9, no. 3, p. 143, 2017, doi: 10.22159/ijpps.2017v9i3.16059.
3. Muhammad Ajaib U. H., Shazia Khalid U., (2014). *Spermadictyon suaveolens*: A potential natural anti-microbial and antioxidant source. Int. J. of Phytomedicine. vol. 6, pp. 256–267, 2014.
4. Rajni Ranjan and Deokule S. S., (2016). Research article an efficient protocol devised for rapid callus induction from leaf explants of *Spermadictyon suaveolens Roxb*. Int. J. Curr. Res., vol. Vol.8, no. 11, pp. 41583–41587.
5. Pradhan, B.K., Badola, H.K., (2008). Ethnomedicinal plant use by Lepcha tribe of Dzongu valley, bordering Khangchendzonga Biosphere Reserve, in North Sikkim, India. J. Ethnobiology Ethnomedicine 4, 22. https://doi.org/10.1186/1746-4269-4-22.
6. Saraf A. and Samant A., (2015). Development and Validation of HPTLC Method for Quantification of Biomarker β-Sitosterol in the Leaves of *Achyranthes aspera Linn*. Chem Sci Trans, vol. 4, no. 4, 2015, doi: 10.13140/RG.2.1.2783.6885.
7. Babu S. and Jayaraman S., (2020). An update on β-sitosterol: A potential herbal nutraceutical for diabetic management. Biomedicine and Pharmacotherapy, vol. 131, doi: 10.1016/j.biopha.2020.110702.
8. Pisha, E., Chai, H., Lee, IS. et al. [1995]. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat. Med. 1, 1046–1051 (1995). https://doi.org/10.1038/nm1095-1046.
9. Chaijan M. and Panpipat W., [2020]. Instability of β-sitosteryl oleate and β-sitosterol loaded in oil-in-water emulsion. NFS Journal, vol. 21, no. August, pp. 22–27, 2020, doi: 10.1016/j.nfs.2020.08.004.
10. Saraf A. A., Shinde P. S., [2018]. Chemical fingerprint profile of various secondary metabolites of medicinal plant *Derris heyneana* (Wight and Arn) Benth. Research Journal of Pharmacognosy and Phytochemistry, vol. 10, no. 2, p. 193, 2018, doi: 10.5958/0975-4385.2018.00031.6.
11. Song T. J., Park C et al. Antidiabetic effects of betulinic acid mediated by the activation of the AMP-activated protein kinase pathway. PLOS One, vol. 16, no. 4 April, pp. 1–16, 2021, doi: 10.1371/journal.pone.0249109.
12. Lou, H.; Li, H.; Zhang, S.;Lu, H.; Chen, Q. [2021] A Review on Preparation of Betulinic Acid and Its Biological Activities. Molecules, 26, 5583. https://doi.org/10.3390/molecules26185583
13. Saraf A. A., Shinde P. S., [2017] Development and Validation of HPTLC Method for Quantification of Biomarker β-Sitosterol in *Derris heyneana* (Wight and Arn) Benth. Chem Sci Trans. vol. 6, no. 1, Jan. 2017, doi: 10.7598/cst2017.1307
14. Debele T. A., Mekuria S. L., and Tsai H. C., [2017]. Synthesis and characterization of redox-sensitive heparin-β-sitosterol micelles: Their application as carriers for the pharmaceutical agent, doxorubicin, and investigation of their antimetastatic activities in vitro. Materials Science and Engineering C. vol. 75, pp. 1326–1338, doi: 10.1016/j.msec.2017.03.052.
15. Stefanini., Oresic L., [2022] Validation of analytical procedures: ICH guidelines Q2(R2). In: European Medicines Agency, 31 March 2022 EMA/CHMP/ICH/82072/2006 Committee for Medicinal Products for Human Use. pp. 1–34.
16. McNaught A. D., Wilkinson A., [1997] IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Blackwell Scientific Publications, Oxford. Online version (2019) created by S. J. Chalk. ISBN 0-9678550-9-8. https://doi.org/10.1351/goldbook.
17. PubChem Compound Summary for CID 64971. B. Acid. R. A. [2023] from https://pubchem. ncbi. nlm. nih. gov/compound/Betulinic-Acid. National Center for Biotechnology Information.