RESEARCH ARTICLE

HPTLC Densitometric Analysis for Quantification of Sennocide-B and Rhein in Leaf Extracts of *Senna alexandrina Mill.* and Their Commercial Formulations

Rizwan Ahmad[1,2], Zeeshan Fatima[1*], Sadath Ali[3], Suneela Dhaneshwar[4]

[1]*Amity Institute of Pharmacy, Lucknow, Amity University, Noida, Uttar Pradesh, India.*
[2]*Seth Vishambhar Nath Institute of Pharmacy, Barabanki, Uttar Pradesh, India.*
[3]*M.A.M College of Pharmacy, Gulbarga, Karnataka, India.*
[4]*Amity Institute of Pharmacy, Amity University, Mumbai, Maharashtra, India.*

Received: 05th March, 2024; Revised: 26th May, 2024; Accepted: 03rd July, 2024; Available Online: 31st August, 2024

ABSTRACT

The leaf extract of *Senna alexandrina* Mill., a plant from the Fabaceae family, contains bioactive anthraquinones sennoside-B and rhein, traditionally used in Indian medicine for various health issues. However, standardizing the quality of *S. alexandrina* and its commercial products is challenging. To address this, we established two high-performance thin-layer chromatography (HPTLC) methods for accurate analysis of these biomarkers in *S. alexandrina* leaf extract and its commercial formulations. For sennoside-B, a mobile phase combination of solvent for example, butanol, water, and glacial acetic acid (6:3.5:0.5, v/v/v) was used, with detection at 254 nm, producing spots at an Rf value of 0.37 ± 0.006. The method showed linearity between 100 to 2000 ng/spot (R^2 = 0.9983 ± 0.0017), with a limit of detection (LoD) of 22.84 ± 0.554 ng/band and a limit of quantitation (LoQ) of 69.22 ± 0.859 ng/band. For rhein, a mobile phase combination of solvent toluene, ethyl acetate, and glacial acetic acid in the ratio (7:2.5:0.5, v/v/v) was utilized and was also detected at 254 nm. The obtained spots had Rf value of 0.67 ± 0.004. The linearity was confirmed within the same range (R^2 = 0.9985 ± 0.0005), with an LoD of 15.49 ± 0.645 ng/spot and an LoQ of 46.94 ± 1.172 ng/spot. Herein, the methods have followed the guidelines of ICH (Q2) R1. These developed HPTLC methods are selective, simple, sensitive, accurate, and economical, making them suitable for everyday assessment of these bioactive markers in *S. alexandrina* and its commercial products. This study supports the quality control of *S. alexandrina*, ensuring product consistency, efficacy, and safety.

Keywords: Sennocide-B, HPTLC, Anthraquinone, *S. alexandrina Mill*., Rhein, HPTLC, Densitometry.

International Journal of Pharmaceutical Quality Assurance (2024); DOI: 10.25258/ijpqa.15.3.13

How to cite this article: Authors. Article Title. International Journal of Pharmaceutical Quality Assurance. 2024;15(3):1173-1183.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Herbal medications have recently become an important feature of many country's basic health care systems.[1] Plants have a wide range of chemicals with potential therapeutic applications against human diseases, such as bacteria, fungi, and viruses.[2] Plants are the primary focus of contemporary research on natural molecules and products,as they are more easily obtainable and can be chosen based on their ethno-medical applications.[3] Recently, several plant formulations have picked the interest of researchers in their search for phytochemicals for treatments of human ailments.[4] Herbal remedies employed to treat and cure a variety of diseases and physiological problems are described in ancient system of medicine such as Ayurveda, Unani [5], and Siddha.[6] Many ethnic groups employ numerous plant species to heal a variety of diseases.[7]

Herbal drugs needs to be standardized for their worldwide acceptance as it ensures the therapeutic efficacy and safety of the drug. As there is a complexity in plant-based medicines, the separation of phytochemicals through chromatographic techniques are employed to develop a fingerprinting profile, which identifies and detects impurities present in commercial formulations.[8] The available detection techniques include high-pressure liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC), etc. Since HPLC utilizes more solvents comparatively, hence is supposed to be costlier.[9] GC may possibly be utilized for essential oils in herbal preparations.[10] The easily available TLC is more cramped to the qualitative estimation of herbal medicines. Therefore TLC has been renovated into more advanced instrumentation known as high-performance thin layer chromatography (HPTLC)[11],

**Author for Correspondence: zfatima@amity.edu*

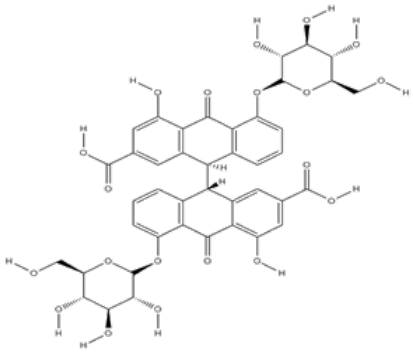
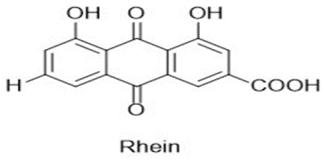


Figure 1: Sennocide-B[14]



Rhein

Figure 2: Rhein[14]

which can be used for botwh qualitative and quantitative analysis.[7,12]

Anthraquinones are important members of the quinone family that belong to the polyketide groups and are sometimes known as anthracenediones or dioxoanthracenes. They are aromatic in nature.[13] Sennoside-B and rhein are the two important anthraquinones that possess a wide range of pharmacological activities.

Sennoside-B (Figure 1) is a member of the class of sennocides and an oxo dicarboxylic[14] a type of naturally occurring anthraquinone found in *S. alexandrina* and *Rheum Officinale Baill* (Rhubarb). The principal purgative components sennoside A and B (SA, SB), were initially separated and identified from Senna leaves and were subsequently ascribed to the anthraquinone family by Stoll.[15] *Senna* plants was found to possess many pharmacological properties such as antioxidant, antimalarial, relaxant, and antimicrobial.[16] It also act as an immunity booster [17]. The phytoconstituent SB from Senna was found to prevent cell growth in human osteosarcoma [18]. It was observed that sennoside B was effective in the treatment of epilepsy without having any undesirable effects.[19] Sennoside B was also found to be useful in treating proliferative diseases in which PDGF (platelet-derived growth factor) signaling plays a central role.[20] Rhein (Figure 2) is chemically 4, 5-dihydroxyanthraquinone-2-carboxylic acid.[14] Rhein is used as a neuro-protective, antimicrobial, antitumor and hypoglycemic agent and it also increases uric acid excretion and reduces inflammatory responses.[21] It can cure ulcerative colitis by proper regulation of gut microbes.[22] It displays extensive antifibrotic effects and alleviates lipid accumulation and fibrosis development.[23]

The role of two commercial formulations A and B taken for the quantitative estimations of biomarkers sennocide-B and rhein are mainly used as a laxative, purgative, antioxidant, antimalarial, relaxant and antimicrobial immunity booster, hepatoprotective, nephroprotective, anti-inflammatory and uncancerous. Earlier researchers have established the importance of correlating *S. alexandrina* leaves and their commercial formulations A and B using HPTLC analysis.[24,25] This correlation is essential for ensuring the consistency and quality of commercial formulations A and B derived from *S. alexandrina*. Due to the lack of phytochemical evaluation of *S. alexandrina* and its commercial formulations, the study is aimed to quantify some major phytochemicals using modern analytical techniques such as HPTLC densitometric analysis. The HPTLC method will enable us to quantify sennocide-B and rhein biomarkers in both the *S. alexandrina* leaves and their commercial formulations, offering insights into their composition and regulatory compliance. This research will contribute to the field by providing a novel and reliable analytical technique for the estimation of sennocide-B and rhein biomarkers in *S. alexandrina* leaf extracts and its two commercial formulations A and B. It will enhance the ability to assess the quality and authenticity of commercial formulations containing *S. alexandrina* leaves and ensure their pharmacological effectiveness.

MATERIALS AND METHODS

Procurement of Plant material and marketed preparations

S. alexandrina leaves were purchased from local market in Lucknow, Uttar Pradesh, India. Authentication was carried out by Prof. R.B. Ram, Dean, School of Agricultural Sciences and Technology (SAST), Baba Sahab Bhim Rao Ambedkar University, Lucknow, through letter no: 01/Dean /SAST/ BBAU/LKO, and a voucher specimen was deposited for further reference, by correlating their morphological and microscopical characters with those given in the literature. The leaves were dried in shade, finely powdered, and stored temperature (30±2°C). Two commercial formulations A and B, from Lupin Limited, India and Vidisa SBS Biotech, India, respectively, were purchased from the local market, which contains dried powdered leaves of *S. alexandrina.*

Procurement of Chemicals and reagents

The thin layer chromatography plates pre-coated with silica gel (thickness: 0.25 mm) 60 F_{254} were purchased from Merck. The reference standards of sennocide-B and rhein were obtained from Sigma Aldrich and Selleck Chemicals, respectively. During the chromatographic investigations, Merck (Germany) provided all of the HPLC-grade chemicals. In 99.5% pure methanol was utilized in the extraction procedure.

Sample Preparation

Accurately weighed 10g each of dried and powdered leaves of *S. alexandrina* and powder of its two commercial formulations A and B were refluxed at 50ºC for 2 hours, using 100 mL methanol as an extracting solvent. The obtained extract was filtered, concentrated and stored in a dried and cool place for further use.[16,25,26]

HPTLC Analysis

HPTLC analysis of each prepared sample was done for their chemical profiling and quantification of marker compounds.

Preparation of sample for HPTLC analysis

About 30 mg of each prepared sample of *S. alexandrina* leaf and its two commercial formulations A and B were dissolved in methanol, vortexed, and centrifuged for 10 minutes at 3000 rotations per minute. The resulting clear supernatant was then stored for further use.

Preparation of standard solution

1mg of standard sennocide-B and rhein was separately dissolved in methanol, vortexed, and centrifuged for 10 minutes at 3000 rpm. The clear liquid that was obtained was then stored as standards in separate vials, each containing a standard concentration of 1-mg/mL.

Chromatographic conditions for method developments for individual markers

From the stock solutions, 4 µL of *S. alexandrina* leaf extract and two commercial formulations (A and B) were prepared. For standards, sennoside-B and rhein, volumes of 0.2, 0.5, 1, 2, and 4 µL (concentrations of 100, 250, 500, 1000, and 2000 ng/spot) were applied. Using a Camag 100 µL sample syringe (Hamilton) and Linomat V applicator (Camag), 4 mm wide bands were created on Silica Gel 60 F254 HPTLC plates (10x10 cm; Merck), one for each standard. Consistent application parameters, including a rate of 150 nL/s with nitrogen gas flow, ensured reproducibility. Several solvent systems were tested, resulting in the selection of butanol: water: glacial acetic acid (But-OH:H_2O:GAA) (6:3.5:0.5; v/v/v) for sennoside-B and Toluene: ethyl acetate: glacial acetic acid (Tol:EA:GAA) (7:2.5:0.5; v/v/v) for rhein. Development occurred in a twin trough glass chamber (20 × 10 cm) pre-saturated with the mobile phase vapors for 15 minutes, ensuring optimal separation. The plates were developed to 80 mm and analyzed under visible, short, and long-wavelength UV light. Scanning at 254 nm with a speed of 100 mm/s and slit dimensions of 6 × 0.3 mm using a Camag HPTLC system (including a sample applicator, twin trough development chamber, and TLC Scanner III: software Wincats) allowed precise detection and quantification [27]. This thorough analysis ensured accurate visualization and measurement of the marker compounds. Chromatographic conditions for method developments for particular markers have been shown in Table 1.

Table 1: Chromatographic conditions for method developments for individual markers

Parameters	*Markers*	
	Sennocide-B	*Rhein*
Solvent system	But-OH:H_2O:GAA (6:3.5:0.5; v/v/v)	Tol:EA:GAA (7:2.5:0.5)
Sample preparation (Plant extract & Formulations)	30 mg in 1-mL of MeOH followed by filtration through a 0.45 µm filter	30 mg in 1-mL of MeOH followed by filtration through a 0.45 µm filter
Application volume of Plant extract & Formulations (µL)	4	4
Standard preparation	1-mg of sennoside-B in 1-mL of methanol(1-mg/mL)	1-mg of rheinin 1-mL of methanol (1-mg/mL)
HPTLC application volume of standard sol on plate	0.2–4 µL(0.2, 0.5, 1.0, 2.0 and 4.0 µL)	0.2–4 µL(0.2, 0.5, 1.0, 2.0 and 4.0 µL)
HPTLC concentration of applied volume of standard sol on plate (ng/spot)	100, 250, 500, 1000 & 2000	100, 250, 500, 1000 & 2000
Use of saturation pad	Used	Used
Saturation time (minutes)	25	25
Detection wavelength (nm)	254	254
Distance between spots (mm)	4	4
Drying time (minutes)	10	10
Drying temperature (ºC)	45	45
Development time (minutes)	15	15
Band width (µm)	0.3	0.3
Slit dimension (mm)	6	6
Temperature	RT (25 ± 2ºC)	RT (25 ± 2ºC)

*Methanol: Me-OH, RT-Room Temparature

Validation of HPTLC Method

The validation of the developed analytical method was performed as per the guidelines of ICH Q2(R1).[28]

RESULTS AND DISCUSSION

Development of analytical method

The application of high-performance thin-layer chromatography is widespread in the standardization and quality monitoring of herbal drugs due to its high efficiency, low cost, low time consumption, automation, and selective wavelength scanning.[29] The chromatographic analysis is more result-oriented and efficacious quality control analytical method for herbal medicines.[30] Chromatographic techniques optimization authenticates and quantifies chemical patterns distributed in the herbal and their commercial formulations.[31] For method development and validation to analyze sennocide-B and rhein in commercial formulations, we explored detection wavelength and solvent systems. The finalized mobile system as But-OH:H2O:GAA (6:3.5:0.5; v/v/v) and Tol:EA:GAA (7:2.5:0.5) using densitometry at 254 nm gave good band separation at (Rf) 0.37 for sennocide-B and and 0.67 for rhein**.** The developed HPTLC plate for quantification of Sennocide-B has been shown in (Figure 3) , Chromatogram of Standard

marker sennocide-B at R_f 0.37 (Figure 4), Chromatogram of *S. alexandrina* leaf extracts showing sennocide-B biomarker at Rf 0.37 (Figure 5), Chromatogram of commercial formulation A extracts showing sennocide-B biomarker at Rf 0.37 (Figure 6), Chromatogram of commercial formulation B extracts showing sennocide-B biomarker at Rf 0.37 at (Figure 7), 3D Chromatogram of standard sennocide-B at Rf 0.37 observed in *S. alexandrina* leaf extracts and commercial formulations A and B extracts (Figure 8). Calibration curve of sennocide-B as standard at 254 nm has been shown in (Figure 9).

Similarly the developed HPTLC plate for quantification of rhein at 254 nm has been shown in (Figure 10), Chromatograms of Standard marker rhein at R_f 0.67 (Figure 11), Chromatogram of *S. alexandrina* leaf extracts showing rhein biomarker at Rf 0.67 (Figure 12), Chromatogram of commercial formulation A extracts showing rhein biomarker at Rf 0.67 (Figure 13), Chromatogram of commercial formulation B extracts showing rhein biomarker at Rf 0.67 at (Figure 14), 3D Chromatogram of standard rhein at Rf 0.67 observed in *S. alexandrina* leaf extracts and commercial formulations A and B extracts (Figure 15). The calibration curve of rhein as standard at 254 nm has been shown in Figure16.

HPTLC Chromatograms of developed plate for quantification of sennocide-B in plant & formulation extracts

Results are depicted in Figures 3-8.

Method validation in plant and formulation extracts for Sennocide-B

Linearity

The regression equation and coefficient for sennoside-B were determined by plotting the calibration curve peak area (AUC) *versus* concentration (ng/band) ranging from 100 to 2000 ng at 254 nm.[28] The resulting equation was y = 2.8589x + 29.075, with a regression coefficient (R^2) of 0.9983 ± 0.0017. The calibration parameters and calibration curve of sennocide-B has been represented in Table 2 and Figure 9.

Specificity

Herein, the specificity of the method was executed and the experimental explanations determined that the method was highly specific for chromatographic estimation. The absence of peak tailing supported this conclusion, consistent retention factors, and stable peak areas, indicating that the method accurately and reliably identified sennoside-B without interference from other components in the samples.

Table 2: Calibration parameters for Sennocide-B

S. No.	*Concentration of sennocide-B [ng/mL]*	*Sennocide-B average area (n = 3)*
1	100	280.14
2	250	862.34
3	500	1447.48
4	1000	2763.32
5	2000	5798.95

Table 3: Linearity parameters for sennocide-B

Parameters	*Biomarkers*
	Sennocide-B
Rf value	0.37 ± 0.006
Linearity range (ng/spot)	100–2000 ng/spot
Scanning wavelength	254 nm
Regression equation	y = 2.8589x + 29.075
Regression coefficient	0.9983 ± 0.0017
Slop ± SD	2.8589 ± 0.0105
LoD ± SD (ng/spot)	22.84 ± 0.554
LoQ ± SD (ng/spot)	69.22 ± 0.859

LoD and LoQ

The limit of detection (LoD) value was and determined to be 22.84 ± 0.554 ng/spot and the limit of quantitation (LoQ) for the validated marker compound sennoside-B at 254 nm was obtained as 69.22 ± 0.859 ng/spot. This determination was made using the following equations.

LoD = (3.3 × (**σ**)/ (S) ----------(1), σ- representing the standard deviation of the response, and S- slope of the calibration curve

LoQ = (10 × (**σ**) / (S)----------(2)

Linearity parameters with LoD and LoQ of analyzed analyte is summarized in Table 3.

Precision

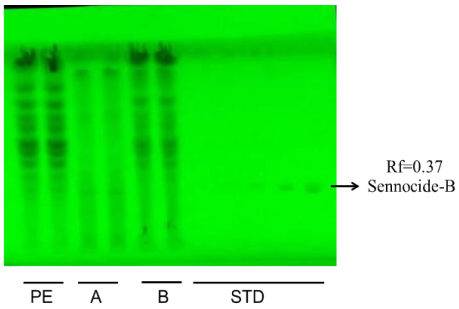
Herein, three concentrations of the marker compound (250, 500, and 1000 ng/ band) were analyzed in triplicate for intra-day and inter-day analyses. The standard deviation (SD), mean peak area, and percentage relative standard deviation (%RSD) were determined for each concentration. These precision metrics demonstrate the method's reliability and repeatability within a single day and across multiple days. Detailed precision data are presented in Table 4.

Accuracy

Pre-analyzed samples of sennoside-B at a concentration of 500 ng were spiked with additional amounts of the standard sennoside-B at 50, 100, and 150% levels. These spiked samples were prepared in triplicate and re-analyzed. The accuracy was computed as the percentage (%) of analyte retrieved. The recovery study was conducted at 254 nm for sennoside-B, and the results demonstrated excellent recovery percentages, ranging from 97.85 to 99.20%, in the spiked samples. This high recovery rate confirms the accuracy of the developed method. Detailed accuracy data for the analyzed marker compound are summarized in Table 5.

Robustness

The robustness of the method was evaluated by varying the mobile phase ratio and chamber saturation time. The quantitative impact was assessed by measuring the %RSD, as shown in Table 6. In this study, %RSD estimates remained below 2%, indicating the method's robustness and reliability, with consistent performance despite minor changes in experimental conditions.



PE: Plant extract of leaves of *senna alexandrina* ;
A : Commercial formulation A ; B: Commercial formulation B;
STD: Standard Sennocide-B

Figure 3: HPTLC plate of *Senna alexandrina* leaf extracts, commercial formulations A and B & Standard sennocide-B at 254 nm

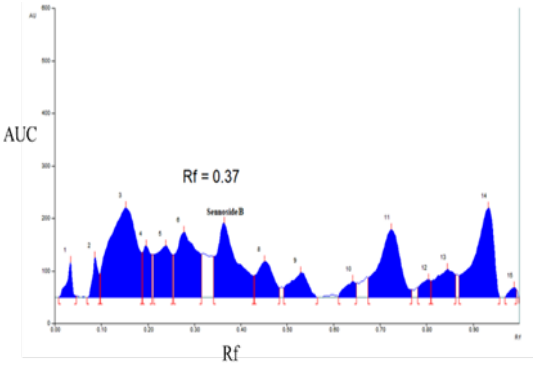


Figure 6: HPTLC chromatogram of commercial formulation A extracts showing sennocide-B biomarker at Rf 0.37 at 254 nm

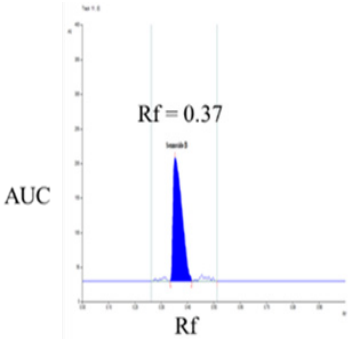


Figure 4: HPTLC Chromatograms of Standard marker sennocide-B at R_f 0.37 at 254 nm

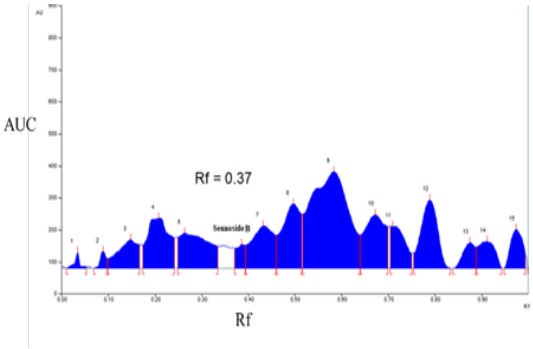


Figure 7: HPTLC chromatogram of commercial formulation B extracts showing sennocide-B biomarker at R_f 0.37 at 254 nm.

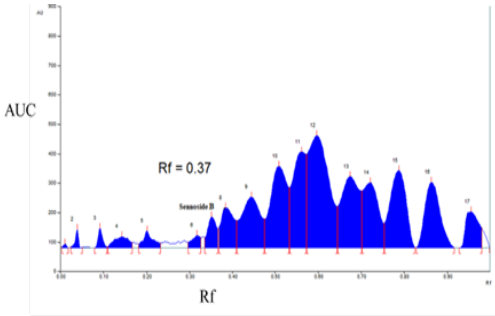


Figure 5: HPTLC chromatogram of *S. alexandrina* leaf extracts showing sennocide-B biomarker at Rf 0.37 at 254 nm

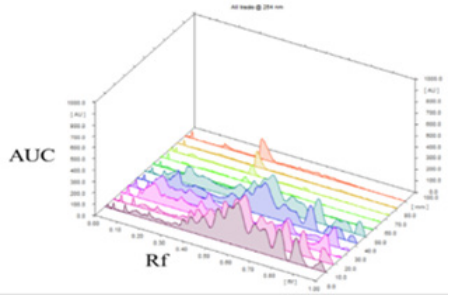


Figure 8: 3D chromatogram of standard sennocide-B at Rf 0.37 observed in *S. alexandrina* leaf extracts and commercial formulations A and B extracts at 254 nm

Table. 4: Intraday and Interday precision of developed and validated method at 254 nm for sennocide-B

Standard	*Intra–day*				*Inter–day*			
	Concentration	*Response (Area)*			*Concentration*	*Response (Area)*		
		Peak area (mean)	*± SD*	*% RSD*		*Peak area (mean)*	*± SD*	*% RSD*
Sennocide-B	250	862.34	13.36	1.54	250	871.62	14.72	1.68
	500	1447.48	25.78	1.78	500	1433.59	26.71	1.86
	1000	2763.32	46.23	1.67	1000	2778.95	41.92	1.50

**Concentration is expressed as ng/spot*

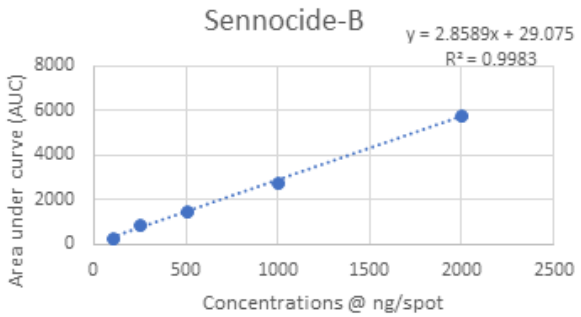


Figure 9: Calibration curves of sennocide-B as standard at 254 nm

Drug Content of Sennocide-B

The drug content of sennocide-B was quantified through the calibration equation as obtained from validation analysis. The drug content of the marker was expressed in µg/mg, *w/w*. The content of the marker compound has been described in Table 7.

HPTLC Chromatograms of developed plate for quantification of rhein in plant & formulation extracts

Results are depicted in Figures 10-15.

Method validation of rhein in plant and formulation extracts

This comprehensive validation process ensured the reliability and applicability of the method by evaluating several critical parameters. The validated method was established by taking into account various parameters, including linearity, specificity, LOD, LOQ, precision, accuracy, and robustness [28].

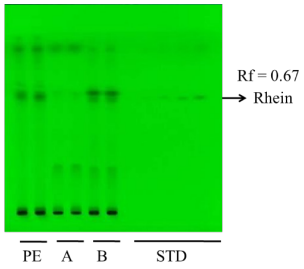
Table 5: Accuracy validated method for sennocide-B (at 254 nm wavelength)

Drug	*Percentage of standard spiked to the sample*	*Amount added*	*Amount recovered*	*Precent recovery*	*Average recovery*
Sennocide-B	50	750	738.56	98.47	98.90
		750	742.43	98.99	
		750	744.32	99.24	
	100	1000	993.13	99.31	99.20
		1000	995.73	99.57	
		1000	987.22	98.72	
	150	1250	1221.35	97.70	97.85
		1250	1224.81	97.98	
		1250	1223.67	97.89	

**Amount added and recovered expressed in concentration as (ng/spot)*

Table 6: HPTLC developed method for robustness for sennocide-B

Change in mobile phase ratio; But-OH:H_2O:GAA (6:3.5:0.5; v/v/v)					
Drug	*Mobile phase solvent ratio*	*Rf*	*Conc (ng/spot)*	*Area ± SD (ng/Spot)*	*% RSD*
Sennocide-B	6: 3: 1; *v/ v/v*	0.36	500	1421.68 ± 21.56	1.51
		0.37	1000	2733.53 ± 45.22	1.65
	6:3.5:0.5; *v/v/v*	0.37	500	1447.48 ± 24.71	1.70
		0.37	1000	2763.32 ± 44.76	1.61
	7: 2.5:0.5; *v/ v/v*	0.38	500	1462.77 ± 25.53	1.74
		0.37	1000	2777.43 ± 51.57	1.85
Change in the chamber saturation time					
	Time in minutes	*Rf*	*Conc (ng/spot)*	*Area ± SD (ng/Spot)*	*% RSD*
Sennocide-B	20	0.38	500	1413.63 ± 19.45	1.37
		0.37	1000	2725.63 ± 43.77	1.60
	25	0.37	500	1452.33 ± 22.47	1.54
		0.37	1000	2755.93 ± 38.83	1.40
	30	0.36	500	1467.54 ± 27.32	1.86
		0.37	1000	2765.72 ± 41.22	1.49



PE: Plant extract of leaves of *senna alexandrina* ;
A : Commercial formulation A ;
B: Commercial formulation B; STD: Standard Rhein

Figure 10: HPTLC plate of *S. alexandrina* leaf extracts, commercial formulations A and B & Standard rhein at 254 nm

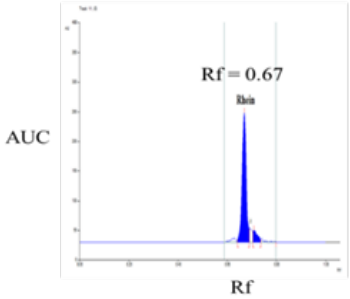


Figure 11: HPTLC chromatograms of standard marker rhein at R_f 0.67 at 254 nm

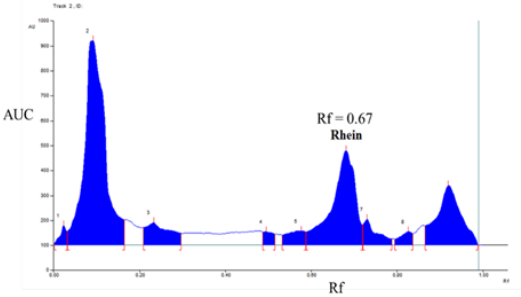


Figure 12: HPTLC chromatogram of *S. alexandrina* leaf extracts showing rhein biomarker at R_f 0.67 at 254 nm.

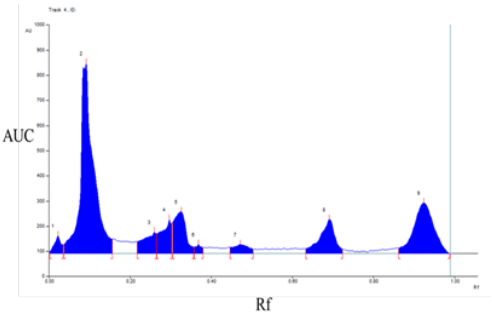


Figure 13: HPTLC chromatogram of commercial formulation A extracts in which biomarker rhein was not detected at 254 nm

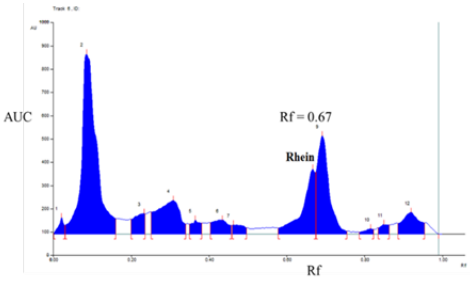


Figure14: HPTLC chromatogram of commercial formulation B extracts showing rhein biomarker at Rf 0.67 at 254 nm

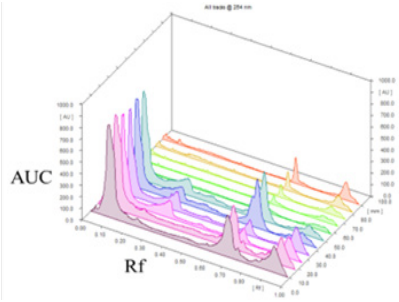


Figure15: 3D chromatogram of standard rhein at Rf 0.67 observed in *S. alexandrina* leaf extracts , commercial formulations A and B at 254 nm

Linearity

The regression equation and coefficient for rhein were determined by plotting peak area *versus* concentration (ng/band) ranging from 100 to 2000 ng at 254 nm. The resulting regression equation was y = 1.2528x + 339.3, with a regression coefficient (R^2) of 0.9985 ± 0.0005. These calibration parameters, along with the calibration curve for rhein, are presented in Table 8 and illustrated in Figure 16. This linearity ensures precise and consistent results for the analysis of rhein in various samples.

Table 7: Drug content of marker compounds in leaf of *S. alexandrina* plant extract and its commercial formulations

Samples	*Content of drug in the sample (µg/mg)*
	Sennocide-B
	254 nm
S. alexandrina plant extract	8.168 ± 0.247
Commercial formulation A	16.698 ± 0.894
Commercial formulation B	2.250 ± 0.016

Specificity

For specificity, the spectra were examined at three different points along the chromatographic band and the experimental observations reveal that the developed method was found to be specific for chromatographic estimation. Since there was no peak tailing, it altered the retention factor and peak area.

Table 8: Calibration parameters for rhein

S. No.	*Concentration of rhein [ng/mL]*	*rhein Average Area (n = 3)*
1	100	463.50
2	250	606.24
3	500	988.24
4	1000	1641.30
5	2000	2820.50

Table 9: Linearity study for rhein

Parameters	*Biomarkers*
	Rhein
R_f value	0.67 ± 0.004
Linearity range (ng/spot)	100-2000 ng/spot
Scanning wavelength	254 nm
Regression equation	y = 1.2528x + 339.3
Regression coefficient	0.9985 ± 0.0005
Slop ± S.D.	1.2528 ± 0.0012
LoD ± S.D.	16.19 ± 0.645
LoQ ± S.D.	51.88 ± 1.172

Sensitivity (LoD and LoQ)

The limit of detection (LoD) was obtained as and 16.19 ± 0.645 ng/spot and the limit of quantitation (LoQ) was determined as 51.88 ± 1.172 ng/spot, for marker compound rhein at 254 nm. This concludes that the method can reliably measure the quantity of rhein present in a sample, ensuring precise and accurate results. In this study, similar equations used as per the above validated method.

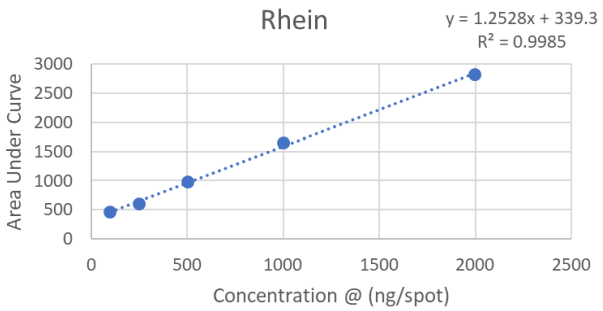


Figure 16: Calibration curve diagram at 254 nm (for rhein standard)

Calibration data with LoD and LoQ of analyzed analyte is summarized in Table 9.

Precision

To evaluate the precision of the method, a rigorous testing protocol was conducted. Specifically, three concentration levels of the analyte (250, 500 ng, and 1000 ng per band) were prepared in triplicate, and their peak areas were measured. The calculations of this precision analysis are summarized in Table 10, which provides a comprehensive overview of the method's performance.

Accuracy

The accuracy of the established method was assessed through a standard addition experiment. A pre-analyzed

Table 10: Precision study at 254 nm for rhein

Standard	*Intra–day*				*Inter–day*			
	Concentration	*Response (Area)*			*Concentration*	*Response (Area)*		
		Peak area (Mean)	*± S.D.*	*%RSD*		*Peak area (Mean)*	*± S.D.*	*%RSD*
Rhein	250	606.24	11.53	1.90	250	618.74	12.13	1.96
	500	988.24	17.58	1.77	500	979.63	18.72	1.91
	1000	1641.30	27.32	1.66	1000	1657.59	24.31	1.46

**Concentration expressed as (ng/spot), S.D. -Standard deviations, %RSD-Percent Relatiove Standard deviations*

Table 11: Accuracy of developed and validated method at 254 nm for rhein

Drug	*Percentage of standard spiked to the sample*	*Amount added*	*Amount recovered*	*% recovery*	*Average recovery*
	50	750	744.42	99.25	99.01
		750	740.31	98.70	
		750	743.11	99.08	
	100	1000	985.45	98.54	98.63
Rhein		1000	989.96	98.99	
		1000	983.67	98.36	
	150	1250	1219.14	97.53	97.77
		1250	1221.63	97.73	
		1250	1225.94	98.07	

**Amount added and recovered expressed in concentration* (ng/spot)

Table 12: Robustness study for rhein

Change in mobile phase ratio; Tol: EA: GAA (7:2.5:0.5; v/v/v)					
Drug	*Mobile phase composition*	*Rf*	*Conc (ng/spot)*	*Area ± SD (ng/Spot)*	*% RSD*
Rhein	6:3.5:0.5; *v/v/v*	0.66	500	983.43 ± 16.67	1.69
		0.67	1000	1637.30 ± 26.11	1.59
	7: 2.5:0.5; *v/ v/v*	0.67	500	988.24 ± 17.58	1.77
		0.67	1000	1641.30 ± 27.32	1.66
	6: 3: 1; *v/ v/v*	0.65	500	993.24 ± 16.89	1.70
		0.67	1000	1645.30 ± 28.35	1.72
Change in the chamber saturation time					
	Time in minutes	*Rf*	*Conc (ng/spot)*	*Area ± SD (ng/Spot)*	*% RSD*
Rhein	20	0.68	500	978.66 ± 15.58	1.59
		0.67	1000	1632.30 ± 28.64	1.75
	25	0.67	500	990.12 ± 18.21	1.83
		0.67	1000	1643.32 ± 26.37	1.60
	30	0.66	500	985.77 ± 16.95	1.71
		0.67	1000	1649.88 ± 31.21	1.89

Table 13: Drug content of marker compounds in *S. alexandrina* L. leaf extract and its commercial formulations

Sample's	*Content of drug in the sample (µg/mg); w/w*
	Rhein
	254 nm
S. alexandrina Plant extract	120.692 ± 0.895
Commercial formulation A	NA
Commercial formulation B	51.873 ± 0.217

sample containing 500 ng of rhein was spiked with additional standard rhein amounts at (50, 100, and 150%) of the initial concentration. These mixtures were re-analyzed in triplicate at 254 nm. The recovery percentages, ranging from 97.77 to 99.01%, demonstrate the method's high accuracy, precision, and reliability in detecting rhein. Detailed recovery data are presented in Table 11, validating the method's suitability for quantitative analysis of rhein in various samples.

Robustness

In this study, method parameters were deliberately varied within a realistic range to assess robustness, with results

Table 14: Comparative results of marker compounds sennocide-B and rhein in leaf of *S. alexandrina* L. extract and its commercial products A & B

Parameters	*Biomarkers*	
	Sennocide-B	*Rhein*
Rf-Value	0.37 ± 0.006	0.67 ± 0.004
Scanning wavelength (nm)	254	254
Linearity	100–2000	100–2000
Regression equation	y = 2.8589x + 29.075	y = 1.2528x + 339.3
Regression coefficient (r^2) ± SD	0.9983 ± 0.0017	= 0.9985 ± 0.0005
Slope ± SD	2.8589 ± 0.0105	1.2528 ± 0.0012
LoD ± SD	22.84 ± 0.554	16.19 ± 0.645
LoQ ± SD	69.22 ± 0.859	51.88 ± 1.172
Precision (%RSD range)		
Intra day	1.54–1.78	1.66–1.90
Inter day	1.50–1.86	1.46–1.96
Accuracy (%drug recovered)	97.85–99.20	97.99–99.01
Content of drug in the sample (µg/mg); w/w		
S. alexandrina plant extract	8.168 ± 0.247	120.692 ± 0.895
Commercial formulation A	16.698 ± 0.894	NA
Commercial formulation B	2.250 ± 0.016	51.873 ± 0.217

*Linearity is expressed in concentration as (ng/spot)

evaluated using percentage relative standard deviation (%RSD). As shown in Table 12, the %RSD values remained under 2%, indicating that the method tolerates minor variations without compromising performance. This robustness ensures the reliability and consistency of analytical results, confirming the method's suitability for routine analysis.

Drug content of rhein

The drug content of rhein was determined through the calibration equation as obtained from validation analysis. The drug content of the marker was expressed in µg/mg, *w/w*. The content of the marker compound has been described in Table 13.

CONCLUSION

Herein, Sennoside-B and rhein levels in *S. alexandrina* leaf extract and its commercial formulations may now be measured with a new, sensitive, repeatable, and dependable HPTLC method. This technique ensures precision and dependability by successfully separating these biomarkers from other plant elements. The final mobile systems for sennoside-B and rhein were optimized and validated as per guidelines. These systems produced dense and compact spots with Rf values of 0.67 for rhein and 0.37 for sennoside-B, indicating good separation. The quantitation of these markers was performed using the calibration equations mentioned in Tables 3 and 9. The content of each marker in the leaf extract and commercial formulations is described in Tables 7 and 13. The overlay of the 3D chromatogram of the leaf extracts, formulations, and individual markers of Sennocide-B and rhein is depicted in Figures 8 and 15, highlighting the excellent resolution and separation of the markers from other phytoconstituents. Recovery studies revealed percentages of 97.85 to 99.20% for Sennocide-B and 97.77 to 99.01% for rhein, demonstrating that there was no interference from other phytoconstituents present in the plant material. The HPTLC plates shown in Figures 3 and 10 illustrate that all sample constituents were clearly resolved without any tailing, further validating the method's effectiveness. Overall, this HPTLC method offers a reliable and efficient tool for the quantitative determination of Sennocide-B and rhein in *S. alexandrina* leaf extracts and commercial formulations. Furthermore, a comparative study of method development and validation of these two biomarkers sennocide-B and rhein present in *S. alexandrina* and its commercial formulations, has been summarized in Table 1. Sennocide-B and rhein exhibited significant differences in the leaf extract of *S. alexandrina* and its commercial formulations A and B . In summary, the developed HPTLC methods for the analysis of sennocide-B and rhein from the leaf extract of *S. alexandrina* and its commercial formulations reported here is very reliable, sensitive, and economic for rapid routine quality control analysis and quantification of Sennocide-B and rhein from leaf extract of *S. alexandrina* and its commercial products may also be used for standardization purposes. The generated scientific evidence will be of help for the regulatory purpose of *S. alexandrina* and their commercially derived products.

ACKNOWLEDGMENT

The authors would like to acknowledge Amity Institute of Pharmacy, Amity University Uttar Pradesh, Lucknow campus, Lucknow, U.P, India and Bioactive Natural Product Laboratory, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi-110062, India, for providing the research facilities.

REFERENCES

1. Khalilzadeh B, Shadjou N, Kanberoglu GS, et al. Advances in nanomaterial based optical biosensing and bioimaging of apoptosis via caspase-3 activity: a review. Microchimica Acta. 2018;185(9): 1-19. doi:10.1007/s00604-018-2980-6
2. Yeung KS, Hernandez M, Mao JJ, Haviland I, Gubili J. Herbal medicine for depression and anxiety: A systematic review with assessment of potential psycho-oncologic relevance. Phytotherapy Research. 2018;32(5):865-891. doi:10.1002/ptr.6033
3. Li FS, Weng JK. Demystifying traditional herbal medicine with modern approach. Nature Plants. 2017;3(8). doi:10.1038/nplants.2017.109
4. Wagner L, Cramer H, Klose P, Lauche R, Gass F, Dobos G, Langhorst J. Herbal medicine for cough: a systematic review and meta-analysis. Forschende Komplementärmedizin/ Research in Complementary Medicine. 2015 Dec 1;22(6):359-68. doi:10.1159/000442111
5. S PS, R KS, C SC, N K. Novel UV Spectrophotometric Method for the Quantitative Analysis of CefpodoximeProxetil in Pharmaceutical Formulations by First Derivative Technique. International Journal of Pharmaceutical Quality Assurance. 2017;8(03). doi:10.25258/ijpqa.v8i03.9577
6. Dhar, M. L., and M. M. Dhar. Screening of Indian plants for biological activity. Part I. 1968; 232-247. doi:10.5555/19680601779
7. Khan T, Ali M, Khan A, et al. Anticancer Plants: A Review of the Active Phytochemicals, Applications in Animal Models, and Regulatory Aspects. Biomolecules. 2019;10(1):47. doi:10.3390/biom10010047
8. Agatonovic-Kustrin S, Loescher CM. Qualitative and quantitative high performance thin layer chromatography analysis of Calendula officinalis using high resolution plate imaging and artificial neural network data modelling. Analytica Chimica Acta. 2013;798:103-108. doi:10.1016/j.aca.2013.08.048
9. Abood MA, Kadhim EJ. Phytochemical investigation of some active components in Iraqi Conyza canadensis (Syn. Erigeron canadensis). International Journal of Drug Delivery Technology. 2021;11:669-75. doi: 10.25258/ijddt.11.3.4
10. Agatonovic-Kustrin S, Hettiarachchi CG, Morton DW, Razic S. Analysis of phenolics in wine by high performance thin-layer chromatography with gradient elution and high resolution plate imaging. Journal of Pharmaceutical and Biomedical Analysis. 2015;102:93-99. doi:10.1016/j.jpba.2014.08.031
11. Ahmad S, Mujawar T, Batewal B, More P, Gaikwad A, Chumbhale D, Tare H. RP-UHPLC and HPTLC method development and validation for analysis of andrographolide from herbal hepatoprotective formulation. International Journal of Pharmaceutical Quality Assurance. 2023;14:96-104. Doi: 10.25258/ijpqa.14.1.17
12. Loescher CM, Morton DW, Razic S, Agatonovic-Kustrin S. High performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC) for the qualitative and quantitative analysis of Calendula officinalis—Advantages

and limitations. Journal of Pharmaceutical and Biomedical Analysis. 2014 Sep 1;98:52-9. doi:10.1016/j.jpba.2014.04.023
13. Fouillaud M, Venkatachalam M, Girard-Valenciennes E, Caro Y, Dufossé L. Anthraquinones and derivatives from marine-derived fungi: Structural diversity and selected biological activities. Marine drugs. 2016 Mar 25;14(4):64. doi:10.3390/md14040064
14. Gorkom BV, Vries ED, KARRENBELD, KLEIBEUKER. Anthranoid laxatives and their potential carcinogenic effects. Alimentary pharmacology & therapeutics. 1999 Apr;13(4):443-52. doi:10.1046/j.1365-2036.1999.00468
15. Stoll A, Becker B, Kussmaul WJHCA. Die Isolierung der Anthraglykoside aus Sennadrogen. 3. Mitteilung über Anthraglykoside. 1949;32(6):1892-1903. Doi: 10.1002/hlca.19490320613
16. Alshehri MM, Quispe C, Herrera-Bravo J, Sharifi-Rad J, Tutuncu S, Aydar EF, Topkaya C, Mertdinc Z, Ozcelik B, Aital M, Kumar NA. A Review of Recent Studies on the Antioxidant and Anti-Infectious Properties of Senna Plants. Oxidative Medicine and Cellular Longevity. 2022;2022(1):6025900. doi:10.1155/2022/6025900
17. Ikram A, Khalid W, Saeed F, Arshad MS, Afzaal M, Arshad MU. Senna: As immunity boosting herb against Covid-19 and several other diseases. Journal of Herbal Medicine. 2023 Feb 1;37:100626. doi:10.1016/j.hermed.2023.100626
18. Miyamoto M, Imai S, Shinohara M, Fujioka S, Goto M. Studies on the purgative substances. I. Isolation of sennoiside A, one of the most active principles from Rhubarb. Yakugaku zasshi: Journal of the Pharmaceutical Society of Japan. 1967 Sep;87(9):1040-3. Doi: 10.1248/yakushi1947.87.9_1040
19. Sahin H, Erbaş O. Beneficial effects of sennoside B on pentylenetetrazole-induced seizures in rats. Human & Experimental Toxicology. 2023 Mar 30;42: doi:10.1177/09603271231168764
20. Chen YC, Chang CN, Hsu HC, Chiou SJ, Lee LT, Hseu TH. Sennoside B inhibits PDGF receptor signaling and cell proliferation induced by PDGF-BB in human osteosarcoma cells. Life sciences. 2009 Jun 19;84(25-26):915-922. doi:10.1016/j.lfs.2009.04.003
21. Li GM, Chen JR, Zhang HQ, Cao XY, Sun C, Peng F, Yin YP, Lin Z, Yu L, Chen Y, Tang YL. Update on pharmacological activities, security, and pharmacokinetics of rhein. Evidence-Based Complementary and Alternative Medicine. 2021;2021(1):4582412. doi:10.1155/2021/4582412
22. Liu S, Yin R, Yang Z, Wei F, Hu J. The effects of rhein on D-GalN/LPS-induced acute liver injury in mice: Results from gut microbiome-metabolomics and host transcriptome analysis. Front Immunol. 2022;13:971409. doi:10.3389/fimmu.2022.971409
23. Xiao Q, Yu X, Yu X, et al. An integrated network pharmacology and cell metabolomics approach to reveal the role of rhein, a novel PPARα agonist, against renal fibrosis by activating the PPARα-CPT1A axis. Phytomedicine. Jul 20 2022;102:154147. doi:10.1016/j.phymed.2022.154147
24. Alam MN, Kaushik R, Singh L, Khan NA. Quantitative estimation of polyphenolic biomarker quercetin and chlorogenic acid in moringa oleifera leaves by hyphenated high-performance thin-layer chromatography (HPTLC) techniques. International Journal of Drug Delivery Technology. 2021;11:1123-1129. DOI: 10.25258/ijddt.11.4.2
25. Dhanani T, Singh R, Reddy N, Trivedi A, Kumar S. Comparison on extraction yield of sennoside A and sennoside B from senna (Cassia angustifolia) using conventional and non conventional extraction techniques and their quantification using a validated HPLC-PDA detection method. Natural Product Research. May 2017;31(9):1097-1101. doi:10.1080/14786419.2016.1258562
26. Epifano F, Fiorito S, Locatelli M, Taddeo VA, Genovese S. Screening for novel plant sources of prenyloxyanthraquinones: *Senna alexandrina* Mill. and Aloe vera (L.) Burm. F. Nat Prod Res. 2015;29(2):180-4. doi:10.1080/14786419.2014.971792
27. Gaurav, Zahiruddin S, Parveen B, Ibrahim M, Sharma I, Sharma S, Sharma AK, Parveen R, Ahmad S. TLC-MS bioautography-based identification of free-radical scavenging, α-amylase, and α-glucosidase inhibitor compounds of antidiabetic tablet BGR-34. ACS omega. 2020 Nov 12;5(46):29688-97. Doi: 10.1021/acsomega.0c02995
28. ICH I. Q2 (R1): Validation of analytical procedures: text and methodology. InInternational conference on harmonization, Geneva 2005 Nov 6 (Vol. 2005). Doi: 10.1002/9781118971147.ch5
29. Zahiruddin S, Parveen A, Khan W, Parveen R, Ahmad S. TLC-Based Metabolite Profiling and Bioactivity-Based Scientific Validation for Use of Water Extracts in AYUSH Formulations. Evid Based Complement Alternat Med. 2021;2021:2847440. doi:10.1155/2021/2847440
30. Chester K, Paliwal S, Khan W, Ahmad S. UPLC-ESI-MS/MS and HPTLC Method for Quantitative Estimation of Cytotoxic Glycosides and Aglycone in Bioactivity Guided Fractions of Solanum nigrum L. Frontiers in Pharmacology. 2017 Jul 6;8:434. doi: 10.3389/fphar.2017.00434
31. Khan W, Chester K, Anjum V, Ahmad W, Ahmad S, Narwaria A, Kumar DP, Katiyar CK. Chromatographic profiling of Pancharishta at different stages of its development using HPTLC, HPLC, GC–MS and UPLC–MS. Phytochemistry letters. 2017 Jun 1;20:391-400. Doi: 10.1016/j.phytol.2017.04.034