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Original Article

HPTLC Method Development and Validation for Simultaneous Quantification of Purpurin and Alizarin in *Rubia cordifolia* L. Roots and their Marketed Preparations

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ABSTRACT

Background: *Rubia cordifolia* L. a spiky perennial climber of Rubiaceae family, is a rich reservoir of anthraquinone containing compounds purpurin and alizarin. It emerges as a versatile powerhouse possessing diverse pharmacological properties which includes antibacterial, antitumor, antioxidant, antiplatelet, potent blood purifier, diuretic, anti-stress, neuroprotective, anti-mutagenic, anticancer, anti-inflammatory, antidepressant, antidiabetic and vasodilation. Thereby it offers a holistic approach for disease prevention through its pharmacological properties. **Objectives:** However due to lack of standardization for *R. cordifolia* roots and its derived marketed herbal products, its quality control remains a daunting task..The present study aims to develop a simple, novel, reliable and specific HPTLC method for quantifying purpurin and alizarin in *R. cordifolia* roots as well as in its marketed preparations. **Materials and Methods:** The separation and quantification of purpurin and alizarin has been carried out on HPTLC plates pre-coated with Silica gel 60 F_{254} as stationary phase and toluene, ethyl acetate, glacial acetic acid (6:3.5:0.5; *v/v/v)* as mobile phase. Determination and quantification were performed by densitometric scanning at 254 nm. **Results:** The developed method gave compact spots at R_f 0.57±0.007 for purpurin and 0.73±0.008 for alizarin. The method was validated as per International Council for Harmonization Q2(R1) guidelines for specificity, precision, robustness, accuracy and recovery. Linearity range for purpurin and alizarin was 100-2000 ng/spot, which showed good regression coefficient for purpurin and alizarin; R^2=0.9967 and R^2=0.9941 at 254 nm respectively. The LOD and LOQ for validated marker compounds purpurin and alizarin at 254 nm were found as 23.87±0.52, 71.61±0.84 ng/spot and 18.36±0.72, 55.08±0.26 ng/spot respectively. The content of purpurin and alizarin in *R. cordifolia* plant extract was found as 17.960±0.658, 34.645±1.153 µg/mg, marketed preparation A, contains 21.741±0.746 and 6.824±0.485 µg/mg while marketed preparation B, contains 18.289±1.014 and 3.031±0.234 µg/mg of the sample. **Conclusion:** The developed method was found to be simple, precise, accurate, economical and convenient for rapid screening of bioactive marker purpurin and alizarin present in methanolic extracts of *R. cordifolia* roots and its marketed preparations.

Keywords: *Rubia cordifolia* L., Method validation, HPTLC, Purpurin, Alizarin, Densitometry.

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INTRODUCTION

In recent decade there has been a serge in utilizing plant based product for alleviation of diseased condition.[1,2] This has led to the increase demand of alternative medicine for various health concerns,thereby leading to a rise in growth of herbal product markets and interest in traditional systems of medicine worldwide.[3] Preparation which are commercially available have to be standardized for authentic analytical methods and procedures which can be realied upon for phytochemical composition. However this is a challenging task for the scientists.[4] Moreover, the ever increasing demand of commercialized preparation has resulted in decline of their quality, purity, authenticity due to lack of adequate regulations.[5]

Phytochemical complexity in herbal medicines makes them fight in a multidimensional way. Although, serving a plant medicine

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to treat the disease is challenging taskto identify, that which phytochemical are responsible to treat the disease as plants or herbal medicine are the complex mixture of phytochemicals.

Moreover, phytochemical-based evaluation and their pharmacology provide an expertise platform to identify the targeted phytochemicals responsible for the biological activity using modern analytical tools directly or indirectly in biological evaluation.[6] The modern techniques such as HPTLC, HPLC, NMR, LCMS, GCMS and many more provide an effective and robust research platform for qualitative and quantitative evaluation of herbal medicines.[7,8]

HPTLC is one of the more specific, robust and economic approaches which are used in qualitative and quantitative evaluation of the phytochemicals based on their molecular integrity or molecular nature. It has been exponentially used in the scientific validation of herbal or traditionally derived medicines.[9] HPTLC is highly sophisticated, automated, increasingly popular tool for the analysis of complex natural products. It is flexible and cost-efficient separation technique take less time and ideally suited for the analysis of constituents in botanicals and herbal drugs as many samples can be run parallelly.[10-13] The advantage of full automation, scanning at selective detection wavelength, less amount of sample preparations, many samples can be run simultaneously have enabled HPTLC to be a powerful analytical tool for quantitative estimation of compounds in complex mixtures of pharmaceuticals, natural products and food samples.[14]

Anthraquinone (AQ), also known as anthracenedione or dioxoanthracene, is an organic aromatic compound.[15] Anthraquinones (9,10-dioxoanthracenes) is an important class of natural compounds with a wide range of therapeutic applications.[16] The literature reports seventy nine naturally occurring anthraquinones which include emodin, aloe-emodin, physcion, alizarin, purpurin, damnacanthal, merindone, aloin, barbaloin, rhein and Chrysophanol.[17] *R. cordifolia* (Indian Madder) is a perennial, prickly or scabrous, climbing herb belongs to family rubiaceae was found to mainly grow near streams and rivers by the side of upper Ghats in evergreen forests which is up to three thousand seven hundred and fifty two meter above sea level.[18]

Purpurin, 1,2,4-trihydroxyanthraquinone (Figure 1)[19] and Alizarin, 1,2-dihydroxyanthraquinone (Figure 2)[20] natural anthraquinone compounds from *R. cordifolia* were found to possess a wide range of pharmacological effect that includes antibacterial, antitumor, antioxidant, antiplatelet, potent blood purifier, diuretic, anti-stress, neuroprotective, anti-mutagenic, anticancer, anti-inflammatory, antidepressant, antidiabetic and vasodilation in cardiac health.[21]

The free anthraquinones and combined anthraquinones compounds present in *R. cordifolia* potentiate significant antioxidant properties.[22] The role of two marketed preparations A and B, taken for the quantitative estimations of biomarkers compounds are mainly used as antibacterial, antioxidant, blood purifier, diuretic, anti-stress, anti-inflammatory. We establish the importance of correlating *R. cordifolia* plants and their marketed preparations using High-Performance Thin-Layer Chromatography (HPTLC) analysis. This correlation is essential for ensuring the consistency and quality of herbal products derived from *R. cordifolia*, such as two marketed preparations A and B, taken for the study. The HPTLC method enables us to quantify purpurin and alizarin in both the *R. cordifolia* plant and its marketed preparations, offering insights into their composition and regulatory compliance. Due to the lack of phytochemicals evaluation of *R. cordifolia* and their derived existing marketed products, the study is aimed to determine and quantify some major phytochemicals using modern analytical techniques such as HPTLC densitometric analysis. This research contributes to the field by providing a novel and reliable analytical method for the quantification of purpurin and alizarin in *R. cordifolia* and its marketed preparations. It enhances the ability to assess the quality and authenticity of products containing R. *cordifolia* and ensures their pharmacological effectiveness.

MATERIALS AND METHODS

Procurement of Plant material and marketed preparations

The roots of *R. cordifolia* were purchased from local market, 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. The roots were dried in shade, finely powdered and stored in airtight container at room temperature (30±2°C). Two marketed preparations A and B, were purchased from the local market, which contain purpurin and alizarin as the major components along with other constituents.

Procurement of Chemicals and reagents

Pre-coated TLC plates, Silica gel 60 F_{254}. Thickness 0.25 mm were purchased from Merck. The reference standard of Purpurin (229148) and Alizarin (122777) were procured from Sigma-Aldrich company with percentage purity 90% and 97% respectively. All the solvents and reagents used during the chromatographic studies were of HPLC grade, supplied by Merck (Germany). The methanol used for extraction process was of 99.5% purity.

Sample Preparation

Accurately weighed 10g each, dried and powdered roots of *R. cordifolia* and powder of its marketed preparations A and B, were

refluxed at 50°C for 2 hr, using 100 mL methanol as an extracting solvent. The obtained extract was filtered using a muslin cloth followed by Whatman's filter paper, concentrated on water bath and stored in a dried and cool place for further use.

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

30 mg of each prepared sample (plant and marketed preparations extract) was dissolved in methanol, vortexed and centrifuged at 3000 rpm for 10 min and then clear supernatant obtained was stored.

Preparation of Standard Solution

1 mg of each standard (Purpurin and Alizarin) was dissolved in 1 mL of methanol, vortexed and centrifuged at 3000 rpm for 10 min and then clear supernatant obtained was stored as standards in vials. Thereafter, half of the volume from each vial of the standard solution was mixed in another vial to obtain the mixed standard of 0.5 mg/mL concentration each.

Chromatographic Conditions

From the stock solution of samples and standards, 4 µL from each sample and 0.2-4 µL (0.2, 0.5, 1.0, 2.0 and 4.0 µL) of mixed standard with concentration of 100, 250, 500, 1000 and 2000 ng/spot were applied to pre-washed and activated Silica gel 60 F_{254} pre-coated HPTLC plates (20x10 cm; Merck, Germany) using a Camag 100 µL sample syringe (Hamilton, Switzerland) having a Camag Linomat V applicator (Camag, Switzerland), The spot were having 4 mm wide band length. The application parameters involved during the entire length of validation analysis were similar. 150 nL/s was selected as application rate for flow of nitrogen gas. Trial and error method was involved to select the appropriate solvent system. The solvent system comprising of toluene, ethyl acetate, glacial acetic acid (6:3.5:0.5; v/v/v) under laboratory conditions (25°C±2°C and 40-50% relative humidity) was finalized for quantification of purpurin and alizarin markers. It provided good resolution. The TLC plate was developed with linear ascending development using the finalized solvent system as a mobile phase. 15 min prior to the development of TLC the twin trough glass chamber (10×10 cm) was saturated with mobile phase vapors. The TLC plates were run in duplicate up to a length 80 mm. The developed plates were further visualized under visible, short UV and long UV light. The slit dimensions were 6×0.3 mm and scanning speed was 100 mm/s. The quantification of purpurin and alizarin was carried out at 254 nm using Camag HPTLC system (Muttenz, Switzerland) equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC scanner III using Wincats 1.2.3 software.[23]

Method Validation

The method was validated in term of assessing precision, accuracy and robustness, Limit of Detection (LOD), Limit of Quantitation (LOQ), as per ICH guidelines.[24]

RESULTS AND DISCUSSION

Development of Analytical Method

Analytical method development and validation is a process which ensures that the analytical procedure employed is suitable for its applicability. It the mandatory process for any good analytical practices. High Performance Thin Layer Chromatography is largely used in analytical technique for standardization and quality monitoring because of its simplicity, high efficiency, cost effectiveness, less time consumption automation and scanning at selective wavelength.[25] Chromatographic analysis is a rational approach to meet the requirement for more effective, result oriented and powerful quality control of Indian traditional and herbal medicines.[26] The optimized chromatographic analysis not only identifies, authenticate and quantify but it is an approach to express the various patterns of chemical ingredients distributed in the herbal drugs and their commercial formulations.[27] For method development and validation to analyze purpurin and alizarin in marketed preparations, we explored different detection wavelengths, solvent systems and compositions of mobile phase. According to preliminary results, detection was done on wavelength of 254 nm.The finalized mobile system as toluene, ethyl acetate, glacial acetic acid (6:3.5:0.5; *v/v/v*) gave good resolution, dense compact and well separated spots at (R_f) 0.57 for purpurin and 0.73 for alizarin (Figures 3(a) and 3(b)).

HPTLC Analysis for Simultaneous Development of Purpurin and Alizarin

HPTLC analysis and validation for simultaneous development of purpurin and alizarin was performed successively. The resulted outcomes revealed several major and minor metabolites at wavelength 254 nm while validation analysis revealed the developed method was linear, robust and accurate. The developed method was validated to determine the precision, accuracy and robustness of the obtained outcomes. The content of purpurin and alizarin in plant samples and its marketed preparations was found to be as one of the major constituents which was expressed in percentage (w/w) sample or extract. The HPTLC chromatograms are represented in Figure 3(c) to 3(g) respectively. The previous literatures on *R. cordifolia* have reported the same outcomes as it was found in our study.[28,29]

Method Validation

The validation profiling of the developed method was performed as per the ICH guidelines: Q2(R1). The parameters such as linearity, specificity, LOD, LOQ, accuracy, precision and robustness were determined successively.

Linearity

A good linear relationalship for both purpurin and alizarin was obtained with the development method over the ranges as shown in Table 1 and is represented graphically in Figures 4 and 5. The calibration curve was plotted against peak area and concentration of applied sample from 100-2000 ng at 254 nm which showed regression equation and regression coefficient for purpurin and alizarin y=9.6798x+203.04, R^2=0.9967 and y=14.114x+876.78, R^2=0.9941 at 254 nm, respectively.

Specificity

The specificity of the method was confirmed by analyzing the standard compounds and the extracts. The band for purpurin and alizarin in the sample were confirmed by comparing the R_f values and UV spectra of the band with those of the standard. The peak purity of purpurin and alizarin was assessed by comparing the spectra at three different levels. Viz, peak Start (S), peak apex (M) and peak End (E) positions of the bands. The experimental observations reveals that the developed method was found to be specific for chromatographic analysis. Since there was no peak tailing, altered retention factor and peak area.

LOD and LOQ

The LOD is the lowermost limit of the marker compound detected in an analyte while LOQ is the lowermost limit of the marker compound quantified in an analyte. The analytes can be detected and quantified with adequate accuracy, precision and variability through the standard deviation of the response and the slope of the calibration curve. Equation 1 and 2 were used to compute the LOD and LOQ values respectively.

$$LOD=3.3\sigma/ S \text{ ----------(1)}$$

$$LOQ= 10\ \sigma/ S \text{ ----------(2)}$$

Where σ denotes standard deviation of the response and S corresponds to calibration curve slope.

The LOD and LOQ for validated marker compounds purpurin and alizarin at 254 nm were found as 23.87±0.52, 71.61±0.84 ng/spot and 18.36±0.72, 55.08±0.26 ng/spot respectively. The experimental observations reveal good sensitivity of the system with respect to the developed and validated method. Calibration data with LOD and LOQ of analyzed analyte is summarized in Table 2.

Table 1: Calibration parameters for purpurin and alizarin.

Sl. No.	Concentration of purpurin /alizarin[ng/mL]	Purpurin Average Area (*n*=3)	Alizarin Average Area (*n*=3)
1	100	985.63	1441.84
2	250	2621.87	4738.76
3	500	4829.4	7840.97
4	1000	10642.25	16271.31
5	2000	19272.55	28543.63

Table 2: Linearity parameters of the developed method for purpurin and alizarin.

Parameters	Biomarkers	
	Purpurin	Alizarin
R_f value	0.57±0.007	0.73±0.008
Linearity range (ng/spot)	100-2000 ng/spot	100-2000 ng/spot
Scanning wavelength	254 nm	254 nm
Regression equation	y=9.6799x+216.8	y=14.124x+892.01
Regression coefficient	0.9967±0.0003	0.9941±0.0005
Slop±SD	9.6799±0.0323	14.124±0.0231
LOD±SD (ng/spot)	23.87±0.52	18.36±0.72
LOQ±SD (ng/spot)	71.61±0.84	55.08±0.26

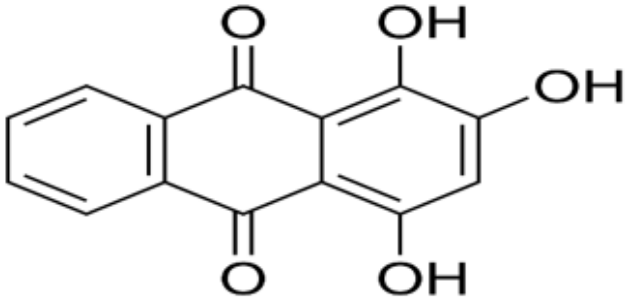


Figure 1: Structure of Purpurin.

Table 3: Intraday and Interday precision of developed and validated method of purpurin and alizarin at 254 nm.

Standard	Intra-day				Inter-day			
	Conc (ng/spot)	Response (Area)			Conc (ng/spot)	Response (Area)		
		Mean peak area	±SD	% RSD		Mean peak area	±SD	% RSD
Purpurin	250	2621.87	28.79	1.09	250	2637.42	31.64	1.19
	500	4829.40	47.64	0.98	500	4841.80	53.61	1.10
	1000	10642.25	112.43	1.05	1000	10639.27	96.83	0.91
Alizarin	250	4738.76	56.53	1.19	250	4756.88	62.31	1.30
	500	7840.97	87.37	1.11	500	7842.73	83.67	1.06
	1000	16271.30	143.72	0.88	1000	16274.10	156.92	0.96

Table 4: Accuracy of developed and validated method at 254 nm.

Drug	Percentage of standard spiked to the sample	Amount added (ng/spot)	Amount recovered (ng/spot)	% recovery	Average recovery
Purpurin	50	750	736.67	98.22	97.96
		750	743.28	99.10	
		750	724.38	96.58	
	100	1000	991.42	99.14	99.15
		1000	996.73	99.67	
		1000	986.51	98.65	
	150	1250	1236.74	98.93	98.40
		1250	1223.92	97.91	
		1250	1229.63	98.37	
Alizarin	50	750	742.36	98.98	99.14
		750	745.82	99.44	
		750	742.51	99.00	
	100	1000	983.41	98.34	98.42
		1000	987.65	98.76	
		1000	981.72	98.17	
	150	1250	1221.82	97.74	98.49
		1250	1234.71	98.77	
		1250	1237.14	98.97	

Precision

The intra-day and inter-day precision for the method was measured by using 3 concentrations in triplicate (250, 500 and 1000 ng per band) followed by estimation of mean peak area, standard deviation and %RSD. The precision data of the analyzed analytes is summarized in Table 3.

Accuracy

The pre-analyzed samples of purpurin and alizarin at concentrations of 500 ng, were spiked with extra 50%, 100% and 150% of the standard purpurin and alizarin by the standard addition method in triplicate and the mixtures were re-analyzed by the proposed method and the accuracy was then calculated as percentage of analyte recovered from the assay. The recovery study was carried out at 254 nm for both the markers. The resulted data revealed good recovery percentage in samples spiked with purpurin and alizarin i,e 97.96-99.15% and 98.42-99.14 respectively. This confirmed the accuracy of the developed method. The accuracy data of the analyzed marker compounds is summarized in Table 4.

Robustness

The ability of an analytical procedure to with stand minor intentional variations in method parameters without being significantly affected is known as its robustness. For determination of method robustness, mobile phase ratio and chamber saturation time were varied within a realistic range and quantitative influence of the variables was determined in % RSD, as shown in

Table 5: Robustness study of the developed HPTLC method.

Change in mobile phase ratio; Toluene, Ethyl acetate, Glacial acetic acid (6:3.5:0.5; *v/v/v*)					
Drug	**Mobile phase ratio**	**R_f**	**Conc (ng/spopt)**	**Area±SD (ng/Spot)**	**% RSD**
Purpurin	6: 3: 1; *v/ v/v*	0.56	500	4829.40±81.53	1.68
		0.57	1000	10642.25±143.77	1.35
	6:3.5:0.5; *v/v/v*	0.57	500	4847.58±74.67	1.54
		0.57	1000	10712.71±149.76	1.39
	7: 2.5:0.5; *v/ v/v*	0.58	500	4861.93±84.45	1.73
		0.57	1000	10608.76±156.95	1.47
Alizarin	6: 3: 1; *v/ v/v*	0.74	500	7813.64±138.77	1.77
		0.73	1000	16346.73±224.52	1.37
	6:3.5:0.5; *v/v/v*	0.73	500	7840.97±153.26	1.95
		0.73	1000	16271.31±212.43	1.30
	7: 2.5:0.5; *v/ v/v*	0.72	500	7892.42±144.43	1.82
		0.71	1000	16198.32±236.72	1.46
Change in the chamber saturation time					
	Time in min	**R_f**	**Conc (ng/spopt)**	**Area±SD (ng/Spot)**	**% RSD**
Purpurin	25	0.58	500	4853.52±77.51	1.59
		0.57	1000	10648.32±137.64	1.29
	30	0.57	500	4867.82±82.77	1.70
		0.57	1000	10673.52±129.57	1.21
	35	0.56	500	4889.63±84.52	1.72
		0.57	1000	10684.42±133.12	1.24
Alizarin	25	0.71	500	7846.23±151.23	1.92
		0.72	1000	16323.11±187.97	1.15
	30	0.73	500	7857.95±139.66	1.77
		0.73	1000	16376.99±205.22	1.25
	35	0.74	500	7871.83±147.42	1.87
		0.75	1000	16394.21±198.63	1.21

Table 6: Drug content of marker compounds in roots of *Rubia cordifolia* L. extract and its marketed preparations.

Sample's	**Content of drug in the sample (µg/mg)**	
	Purpurin	**Alizarin**
	254 nm	**254 nm**
Rubia cordifolia root extract	17.960±0.658	34.645±1.153
Marketed preparation A	21.741±0.746	6.824±0.485
Marketed preparation B	18.289±1.014	3.031±0.234

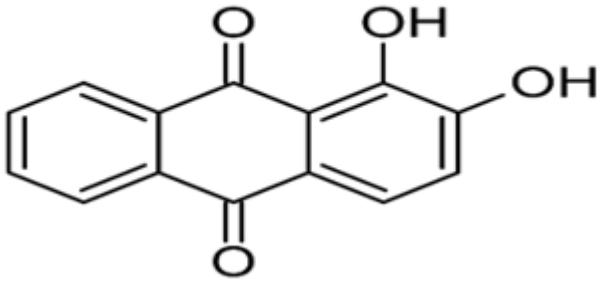
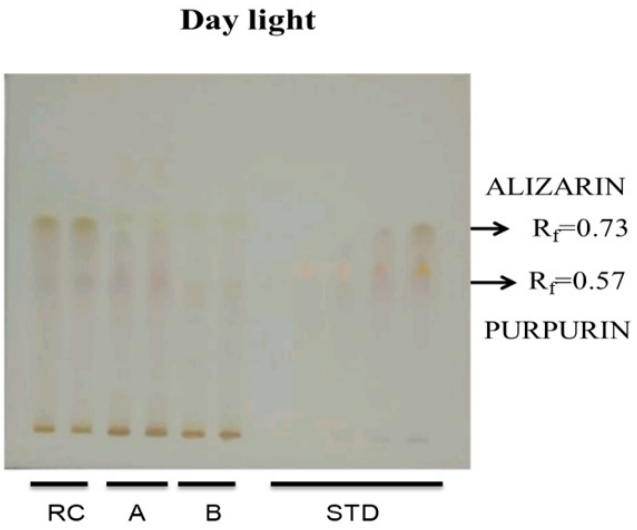
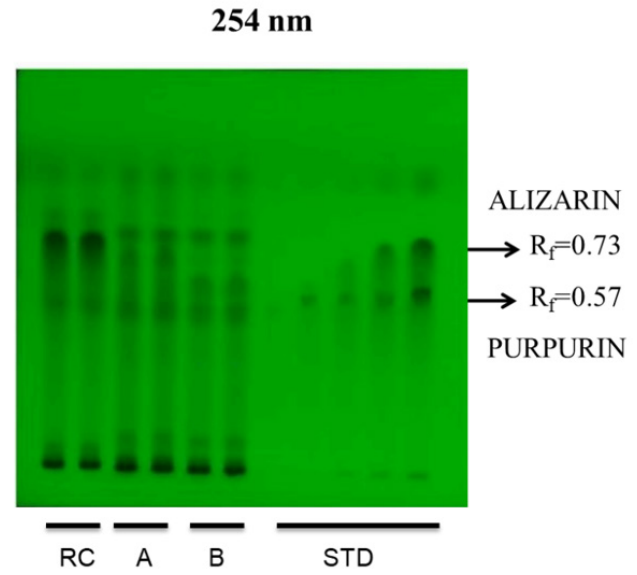


Figure 2: Structure of Alizarin.



RC=*Rubia cordifolia* root extract; A=Marketed preparation A; B=Marketed preparation B; STD= Mixed Standards: Alizarin+Purpurin.

Figure 3(a): HPTLC plate of plant sample and different marketed preparations at day light.



RC=*Rubia cordifolia* root extract; A=Marketed preparation A; B=Marketed preparation B; STD=Mixed Standards: Alizarin+Purpurin.

Figure 3(b): HPTLC plate of plant sample and different marketed preparations at 254 nm.

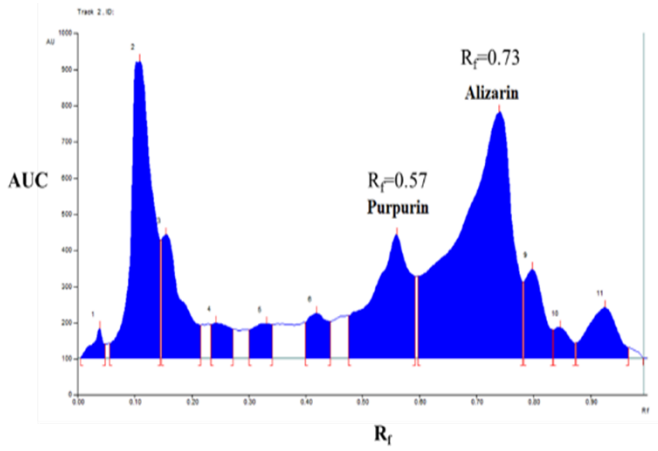


Figure 3(c): HPTLC chromatogram of *R. cordifolia* root extract showing purpurin and alizarin biomarkers at R_f 0.57 and 0.73 respectively at 254 nm.

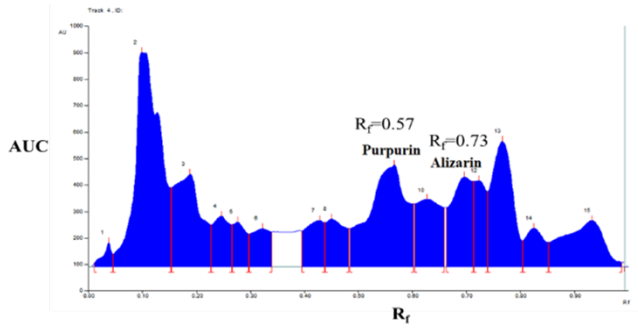


Figure 3(d): HPTLC chromatogram of Marketed preparation A, showing purpurin and alizarin at R_f 0.57 and 0.73 respectively at 254 nm.

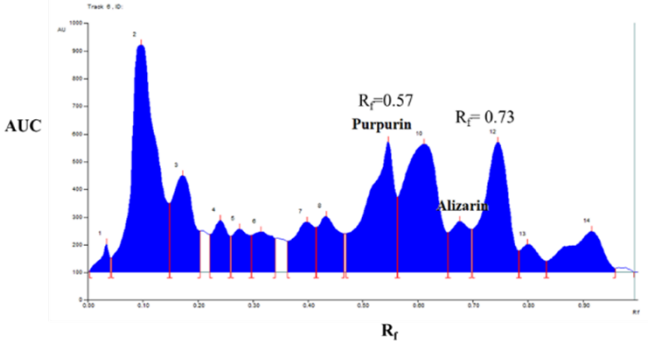


Figure 3(e): HPTLC chromatogram of Marketed preparation B, showing purpurin and alizarin at R_f 0.57 and 0.73 respectively at 254 nm.

Table 5. The % RSD of parameters after variations was found to be in acceptable range i.e less than 2%.

Drug Content of Purpurin and Alizarin

The drug content of purpurin and alizarin was determined through the calibration equation as obtained from validation analysis. The drug content of each marker was expressed in microgram per milligram of the sample extract (µg/mg, w/w). The content of each marker compound has been described in Table 6.

DISCUSSION

The aim of this study was to develop and validate a HPTLC method for simultaneously estimating the concentration of 2 marker compounds namely purpurin and alizarin in *R. cordifolia* plant extract and two marketed preparations A and B. The

mobile phase consisting of toluene, ethyl acetate, glacial acetic acid (6:3.5:0.5; *v/v/v*) was able to provide excellent separation and resolution of purpurin and alizarin peaks without any disturbance from the other compounds present in extract or marketed preparations (Figures 3(c), 3(d) and 3(e)). The quantification of purpurin and alizarin was determined using the calibration equations mentioned in Table 2. The drug content of each marker was expressed in µg/mg, w/w of the root extract and marketed preparations. The content of each marker compound has been described in Table 6. Overlay of 3D chromatograms of root extract, two marketed preparations and mixed standards of purpurin and alizarin are depicted in Figure 3(f).

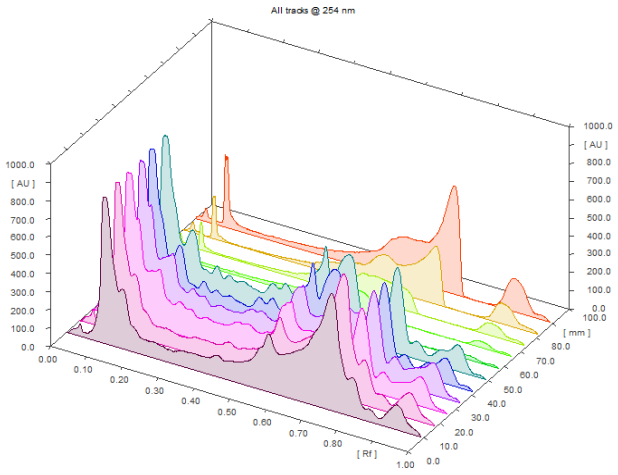


Figure 3(f): 3D chromatogram of standard purpurin and alizarin at R_f 0.57 and 0.73 observed in *R. cordifolia* root extract and Marketed preparation A and Marketed preparation B at 254 nm.

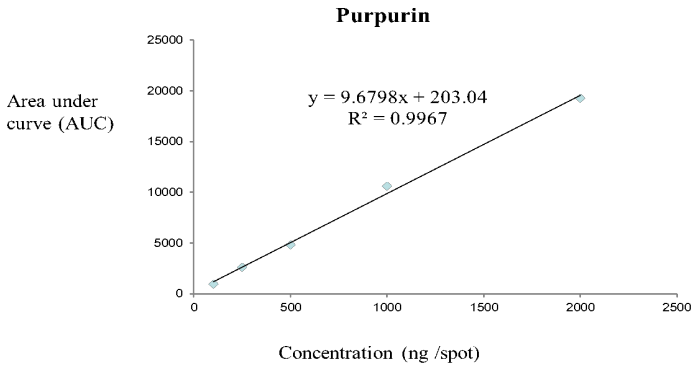


Figure 4: Calibration curves of Purpurin as standard at 254 nm.

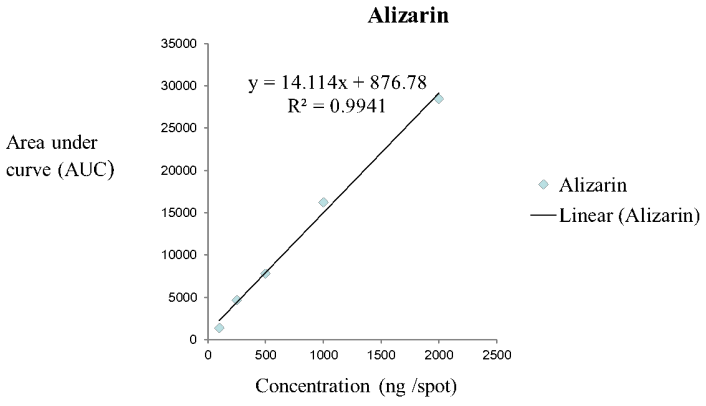


Figure 5: Calibration curves of Alizarin as standard at 254 nm.

CONCLUSION

A rapid, simple, precise, accurate and specific HPTLC method has been developed for quantifying purpurin and alizarin in *R. cordifolia* and its marketed preparations. This method ensures high-quality control standards and effectively separates these compounds from others. While *R. cordifolia* contains various metabolites, purpurin and alizarin are the major ones. Notably, purpurin remains consistent, while alizarin's concentration varies between *R. cordifolia* roots and its marketed preparations. This HPTLC method is simple, sensitive, cost-effective and suitable for routine quality control, making it valuable for standardization and regulatory compliance. The generated scientific data stands as a valuable tool for chemical profiling and standardization, facilitating the quality monitoring of *R. cordifolia* roots and its marketed preparations. This approach not only enhances the understanding of the plant's chemical profiling but also contributes to regulatory compliance in the production of these herbal products.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ICH: International Conference on Harmonization; **r^2:** Regression coefficient; **R_f:** Retardation factor; **HPTLC:** High performance thin layer chromatography; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **σ:** Standard deviation; **RSD:** Relative standard deviation; **µg/mg:** Microgram/ milligram; **µL:** Microlitre; **ng:** Nanogram; **S:** Slope; **y=mx+c:** Linearity equation.

SUMMARY

Standardization and quality control of medicinal plants and their commercial products are one of the critical needs for ensuring their quality, safety and efficacy which assists in validation as well as regulation of herbal medicines and their herbal products. The present study aimed at quantitative estimation of purpurin and alizarin in *Rubia cordifolia* L. roots and their marketed preparations by HPTLC densitometric analysis which has not been reported in the literature so far. The developed method can have great application for standardization of herbal formulations and commercial products containing *R. cordifolia* plant extract in the herbal industry. This HPTLC method is simple, sensitive, cost-effective and suitable for routine quality control, making it valuable for standardization and regulatory compliance. The generated scientific data stands as a valuable tool for chemical profiling and standardization, facilitating the quality monitoring of *R. cordifolia* roots and its marketed preparations. This approach not only enhances the understanding of the plant's chemical profiling but also contributes to regulatory compliance in the production of these herbal products.

REFERENCES

1. Falzon CC, Balabanova A. Phytotherapy: an introduction to herbal medicine. Prim Care. 2017;44(2):217-27. doi: 10.1016/j.pop.2017.02.001, PMID 28501226.
2. Gaurav MU, Khan MU, Basist P, Zahiruddin S, Ibrahim M, Parveen R, *et al.* Nephroprotective potential of Boerhaavia diffusa and Tinospora cordifolia herbal combination against diclofenac induced nephrotoxicity. S Afr J Bot. 2022;151:238-47. doi: 10.1016/j.sajb.2022.01.038.
3. Zhang X, Qiu H, Li C, Cai P, Qi F. The positive role of traditional Chinese medicine as an adjunctive therapy for cancer. BioSci Trends. 2021;15(5):283-98. doi: 10.5582/bst.2021.01318, PMID 34421064.
4. Ajebli M, Eddouks M. Phytotherapy of hypertension: an updated overview. Endocr Metab Immune Disord Drug Targets. 2020;20(6):812-39. doi: 10.2174/1871530320666191227104648, PMID 31880255.
5. Bidikar CM, Hurkadale PJ, Nandanwadkar SM, Hegde HV. A validated Spectro densitometric regulatory compliant USP-HP-TLC protocol for quantification of polyphenols and antioxidants from polyherbal formulations containing Terminalia species. J Chromatogr B Analyt Technol Biomed Life Sci. 2022; 1207:123379. doi: 10.1016/j.jchromb.2022.123379, PMID 35940042.
6. Basist P, Parveen B, Zahiruddin S, Gautam G, Parveen R, Khan MA, *et al.* Potential nephroprotective phytochemicals: mechanism and future prospects. J Ethnopharmacol. 2022;283:114743. doi: 10.1016/j.jep.2021.114743, PMID 34655670.
7. Khan A, *et al.* Thin layer chromatography-mass spectrometry bioautographic identification of free radical scavenging compounds and metabolomic profile of Carica papaya linn. fruit and seeds using high-performance thin-layer chromatography, gas chromatography-mass spectrometry and ultra-performance liquid chromatography-mass spectrometry. Pharmacogn Mag. 2021;17;Suppl 1:S21-8.
8. Jia J, Chen T, Wang P, Chen G, You J, Liu Y, *et al.* Preparative Separation of methylswertianin, swerchirin and decussatin from the Tibetan medicinal plant Swertia mussotii using high-speed counter-current chromatography. Phytochem Anal. 2012;23(4):332-6. doi: 10.1002/pca.1362, PMID 22052665.
9. Nikam PH, *et al.* Future trends in standardization of herbal drugs. J Appl Pharm Sci. 2012(Issue):38-44.
10. Patel KG, Patel VG, Patel KV, Gandhi TR. Validated HPTLC method for quantitative determination of gallic acid in stem bark of Myrica esculenta Buch. Ham. J AOAC Int. 2010;93(5):1422-7. doi: 10.1093/jaoac/93.5.1422, PMID 21140652. ex. D.
11. Wang J, Tang F, Yue Y, Guo X, Yao X. Development and validation of an HPTLC method for simultaneous quantitation of isoorientin, isovitexin, orientin and vitexin in bamboo-leaf flavonoids. J AOAC Int. 2010;93(5):1376-83. doi: 10.1093/jaoac/93.5.1376, PMID 21140646.
12. Sudberg S, Sudberg EM, Terrazas J, Sudberg S, Patel K, Pineda J, *et al.* Fingerprint analysis and the application of HPTLC to the determination of identity and quality of botanicals, from an industry perspective. J AOAC Int. 2010;93(5):1367-75. doi: 10.1093/jaoac/93.5.1367, PMID 21140645.
13. Jiang YM, Wei JC.-M. and J.-C. Wei. A new species of Everniastrum containing diffractaic acid. Lichenologist. 1993;25(1):57-60. doi: 10.1006/lich.1993.1013.
14. Campo P, Sorial GA, Suidan MT, Venosa AD. Statistical evaluation of an analytical GC/MS method for the determination of long chain fatty acids. Talanta. 2006;68(3):888-94. doi: 10.1016/j.talanta.2005.06.031, PMID 18970406.
15. Jain V. A scoping review on recent progress on purpurin and its derivatives. Lett Drug Des Discov. 2023;20.
16. Chien SC, Wu YC, Chen ZW, Yang WC. Naturally occurring anthraquinones: chemistry and therapeutic potential in autoimmune diabetes. Evid Based Complement Alternat Med. 2015; 2015:357357. doi: 10.1155/2015/357357, PMID 25866536.
17. Wen M, Chen Q, Chen W, Yang J, Zhou X, Zhang C, *et al.* A comprehensive review of Rubia cordifolia L.: traditional uses, phytochemistry, pharmacological activities and clinical applications. Front Pharmacol. 2022;13:965390. doi: 10.3389/fphar.2022.965390, PMID 36160419.
18. Shan M, Yu S, Yan H, Chen P, Zhang L, Ding A. A review of the botany, phytochemistry, pharmacology and toxicology of Rubiae Radix et rhizoma. Molecules. 2016;21(12). doi: 10.3390/molecules21121747.
19. Nam W, Kim SP, Nam SH, Friedman M. Structure-antioxidative and anti-inflammatory activity relationships of purpurin and related anthraquinones in chemical and cell assays. Molecules. 2017;22(2). doi: 10.3390/molecules22020265, PMID 28208613.
20. Derksen GC, Lelyveld GP, van Beek TA, Capelle A, de Groot AE. Two validated HPLC methods for the quantification of alizarin and other anthraquinones in Rubia

tinctorum cultivars. Phytochem Anal. 2004;15(6):397-406. doi: 10.1002/pca.800, PMID 15599964.

21. Wang W, Zhang J, Qi W, Su R, He Z, Peng X. Alizarin and purpurin from Rubia tinctorum L. suppress insulin fibrillation and reduce the amyloid-induced cytotoxicity. ACS Chem Neurosci. 2021;12(12):2182-93. doi: 10.1021/acschemneuro.1c00177, PMID 34033711.
22. Di Pompo G, Poli F, Mandrone M, Lorenzi B, Roncuzzi L, Baldini N, *et al.* Comparative 'in vitro' evaluation of the antiresorptive activity residing in four Ayurvedic medicinal plants. Hemidesmus indicus emerges for its potential in the treatment of bone loss diseases. J Ethnopharmacol. 2014;154(2):462-70. doi: 10.1016/j.jep.2014.04.033, PMID 24786575.
23. Zahiruddin S, Parveen B, Ibrahim M, Sharma I, Sharma S, *et al.* TLC-MS bioautography-based identification of free-radical scavenging, α-amylase and α-glucosidase inhibitor compounds of antidiabetic tablet BGR-34. ACS Omega. 2020;5(46):29688-97. doi: 10.1021/acsomega.0c02995, PMID 33251404.
24. Hsi SL, Surman P, Al-Kassas R. Development of a stability-indicating UPLC method for determination of isotretinoin in bulk drug. Pharm Dev Technol. 2019;24(2):189-98. doi: 10.1080/10837450.2018.1454469, PMID 29558234.
25. 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, PMID 35003294.
26. 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. Front Pharmacol. 2017;8:434. doi: 10.3389/fphar.2017.00434, PMID 28729835.
27. Khan W, Chester K, Anjum V, Ahmad W, Ahmad S, Narwaria A, *et al.* Chromatographic profiling of Pancharishta at different stages of its development using HPTLC, HPLC, GC–MS and UPLC–MS. Phytochem Lett. 2017;20:391-400. doi: 10.1016/j.phytol.2017.04.034.
28. Thakur A, Hamrapurkar P. Quantitative densitometric HPTLC analysis of purpurin in the parts of Rubia cordifolia and in pharmaceutical dosage forms. JPC J Planar Chromatogr Mod TLC. 2009;22(2):109-13. doi: 10.1556/JPC.22.2009.2.6.
29. Natarajan S, Mishra P, Vadivel M, Basha MG, Kumar A, Velusamy S. ISSR characterization and quantification of purpurin and alizarin in Rubia cordifolia L. populations from India. Biochem Genet. 2019;57(1):56-72. doi: 10.1007/s10528-018-9875-4, PMID 30039443.

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