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A VALIDATED QUANTITATIVE DETERMINATION OF ALIZARIN IN *RUBIA CORDIFOLIA* LINN. BY ISOCRATIC RP-HPLC

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ABSTRACT

A rapid, simple and specific reversed phase High Performance Liquid Chromatographic (RP-HPLC) method has been developed for the quantitative determination of alizarin in the methanolic extracts of roots and aerial parts of *Rubia cordifolia* Linn, an important medicinal plant used in Ayurveda system of medicine. Chromatographic analysis was performed by using a C_{18} column (250 × 4.6 mm) alizarin was isocratically eluted with a solvent system composed of methanol: water (80:20, v/v), pH 3.5 adjusted with formic acid, at a flow rate of 1 mL/min, 20 µL of the sample was injected, UV detection was performed at 254 nm using a photodiode array detector and retention time was found to be 4.782 min. Parameters such as linearity, limit of quantification (LOQ) and detection (LOD), precision and accuracy are studied. The method is linear for alizarin concentration in the range of 10-200 µg/mL with a correlation of 0.99903.The LOD and LOQ were found to be 52.54 ng/mL and 158 ng/mL respectively. The percent recoveries were found to be in the range of 98.50-102 %. Percent content of alizarin in roots and aerial parts of the plant were found to be 0.52 and 0.14 % respectively. Higher concentration of alizarin was found in roots in comparison with aerial parts. Since *Rubia cordifolia* Linn. is used in many ayurvedic formulations this method can be employed in quality control of the formulations containing *Rubia cordifolia* Linn.

KEYWORDS : ***Rubia coordifolia*, Alizarin, RP-HPLC, anthraquinones and Manjistha**

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INTRODUCTION

Rubia cordifolia Linn. (Rubiaceae), commonly known as Manjishta in Ayurveda, is a climbing plant grows in the North-West Himalayas, Nilgiris and other hilly districts of India[1]**.** This herb is used as one of the major ingredient of many commercially available products[2]**.** Roots of this plant possess many medicinal properties, extracts of *Rubia Cordifolia* have shown hepatoprotective, antineoplastic properties and is also useful in disintegration and elimination of urinary stones[3,4],roots are also used to cure jaundice, paralytic affections, urinary troubles, amenorrhea and to shrink and clean mother's uterus after child birth[5.] Roots of this plant have been used to dye silk and wool red since ancient times[6.] The major compounds of this plant are anthraquinones alizarin and purpurin and their derivatives, ruberythric acid (alizarin-primeyeroside), pseudopurpurin and lucidin primeveroside, rubiadin, munjistin, quinizarin, lucidin and 1,8-dihydroxyanthraquinone[7]. Alizarin (1, 2 dihydroxy anthraquinone) (Fig. 1) found as a major component in extracts of the roots of various members species of Rubiaceae[8]. Alizarin is an orange red powder principally used for dyeing textile fabrics; it has been also reported to posses modulatory role against genotoxicity of mutagens[9]. Although, HPLC method for the analysis of alizarin in *Rubia tinctorum* L. hairy root cultures have been reported in literature. Quantitative determination of alizarin in roots and aerial parts of *Rubia cordifolia* Linn. has not been reported yet. HPLC is one of the several chromatographic techniques that are being profusely utilized in laboratories all over the world for estimation of specific compounds in a sample[10]. Present study deals with the quantitative determination of alizarin by RP-HPLC in methanolic extracts of root & aerial parts of the plant.

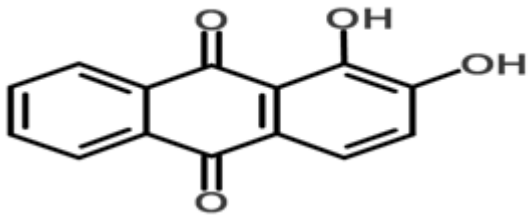


Figure 1
Structure of alizarin

MATERIALS & METHODS

Roots and aerial parts of *Rubia cordifolia* Linn. were collected from Chopta forest (Himalayas) from an altitude of 1800 m and were identified by Prof. Javed Ahmad, Head, Dept. of Botany, Jamia Hamdard. Voucher specimen is deposited at the herbarium of Department of Botany, Jamia Hamdard. New Delhi. HPLC grade standard compound, alizarin was purchased from Sigma Aldrich (India). HPLC grade methanol and water were purchased from Merck, India. HPLC analysis was carried out on a Waters HPLC system (Binary Pump 600 controller), Waters PDA detector (996) and an autosampler (2707). Empower 2 software was used to control the system and for monitoring and analysis of results. For chromatographic separation R_pC_{18} column (250×4.6 mm, particle size 5 µm) was used. Further, a sonicator, rotary evaporator (R-200/205/V (Buchi), a pH meter (company name) a hot air oven were also used.

(i) HPLC Sample Preparation

Roots and aerial parts of *Rubia cordifolia* Linn. were dried under shade and grinded to make a fine powder in a grinder. One gram of each of finely powdered plant material was extracted with 50 ml of MeOH and was sonicated for 20 min. The solution was filtered using Whatman filter paper No 1. each solution was evaporated under vacuum in a rotary evaporator to make an extract and subsequently redissolved in 10 ml of methanol. Prior to injection all solutions were filtered through 0.22 µm nylon filters 20 µL of each sample solution was injected into the column in triplicate for HPLC analysis.

(ii) Preparation of mobile phase and stock solutions

An optimum mobile phase composition was achieved by using different compositions of Methanol–Water. The final composition was optimized as 80:20 (v/v), Solvent system was filtered before use by using 0.22 µm nylon filters. The other conditions were as follows: pH of the solvent system was adjusted at 3.5 by using formic acid and the compound was analyzed at a flow rate of 1 mL/min detection wavelength was set at 254 nm. Stock solution of 1 mg/mL of alizarin was prepared in methanol. A series of standard working solutions in the linear range of 10-200 µg/mL for alizarin were obtained by diluting the mixture of the stock standard solution with methanol and these were used for the preparation of calibration curve. All standard solutions were stored at 4°C and were stable for at least 20 days.

(iii) Calibration

Standard solutions of 10-200 µg/mL of alizarin were prepared in methanol from the stock solution of 1 mg/mL and were used for the preparation of calibration graph. 20 µL of each of the standard solution was injected by the autosampler with concentrations mentioned above and the linearity of response for alizarin was determined. Calibration curve was drawn by plotting the peak areas alizarin against the corresponding concentration.

(iv) Method Validation

The developed method is validated as per the ICH guidelines [11]. The method is validated by determining linearity, precision, specificity, accuracy, limit of detection (LOD), limit of quantification (LOQ) and recovery.

Standard solutions of 10-200 µg/mL of alizarin were analyzed to check the linearity of response.

The limit of detection (LOD) and limit of quantification (LOQ) were obtained by using the standard formula as per the ICH guidelines

$LOD = 3.3\ \sigma/S$ $LOD = 10\ \sigma/S$

Where σ is Standard deviation of the response and S is the slope of the calibration curve

The specificity of the method was ascertained by analyzing the standard and the samples.

(v) Precision & Accuracy

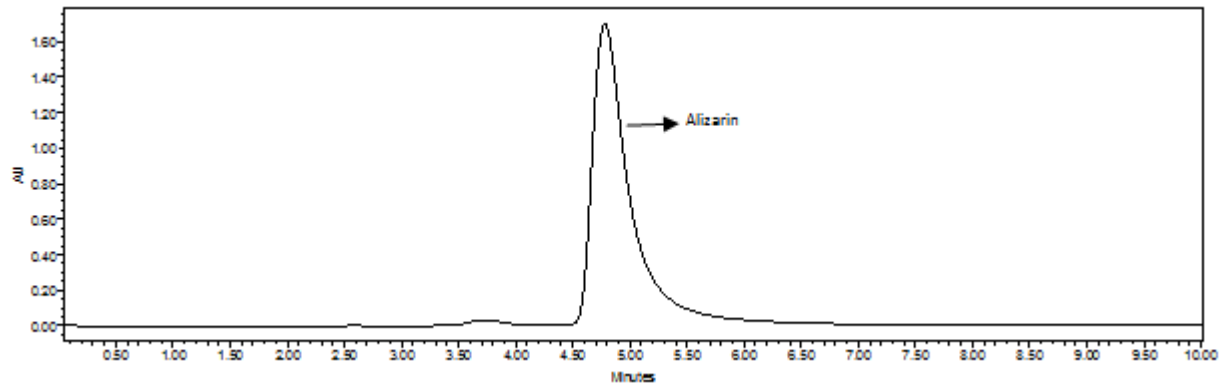
The accuracy of the method was determined by spiking a known amount of solutions of alizarin in triplicate at three different levels 80, 100 and 120 %. Percentage recovery was determined. Standard solutions were injected six times to evaluate inter and intra-day precision and % RSD value was calculated.

RESULTS AND DISCUSSION

The separation of alizarin by RP-HPLC was carried out under optimized conditions. Optimization of mobile phase was carried out using various concentrations of methanol and water. Three different compositions of methanol and water were used, 75:25, 85: 15 and 80:20. The optimum mobile phase was found to be in the ratio of 80:20, pH of the mobile phase was maintained at 3.5 and gave a satisfactory separation of alizarin. Using this mobile phase retention time of alizarin was found to be 4.782 min. The peaks of alizarin in samples were confirmed by comparing the retention time (Fig 2) and UV spectrum (Fig 3) with the standard**.** A linear calibration curve was obtained in the given concentration range of alizarin. A linear relationship between peak areas and concentrations was obtained in the range of 10-200 µg/mL. This shows that the

method is linear**.** Calibration data is shown in Table 1. LOD and LOQ were calculated by the method as described previously, and were found to be 52.54 ng/mL and 158 ng/mL respectively. The percentage of recovery found to be satisfactory and was in between 98.5-102% validation summary is shown in Table 2. % RSD for inter-day precision was higher than that of intra-day precision.

a



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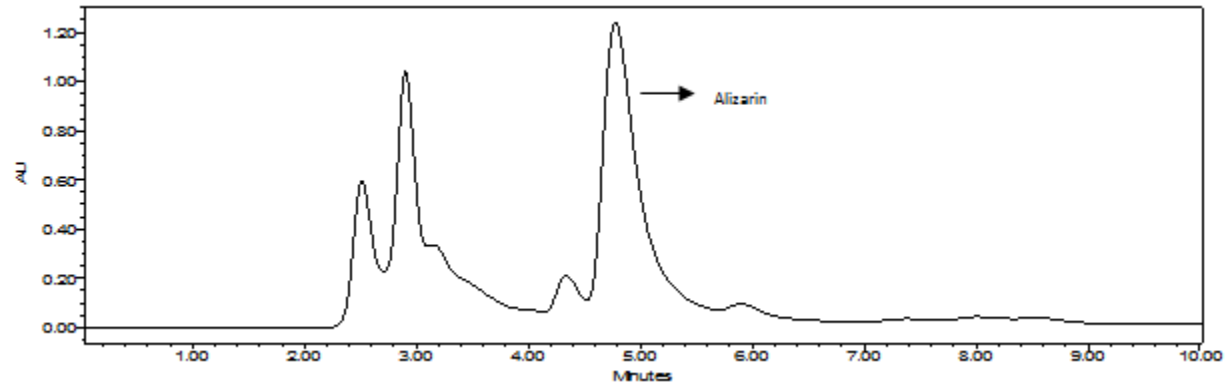

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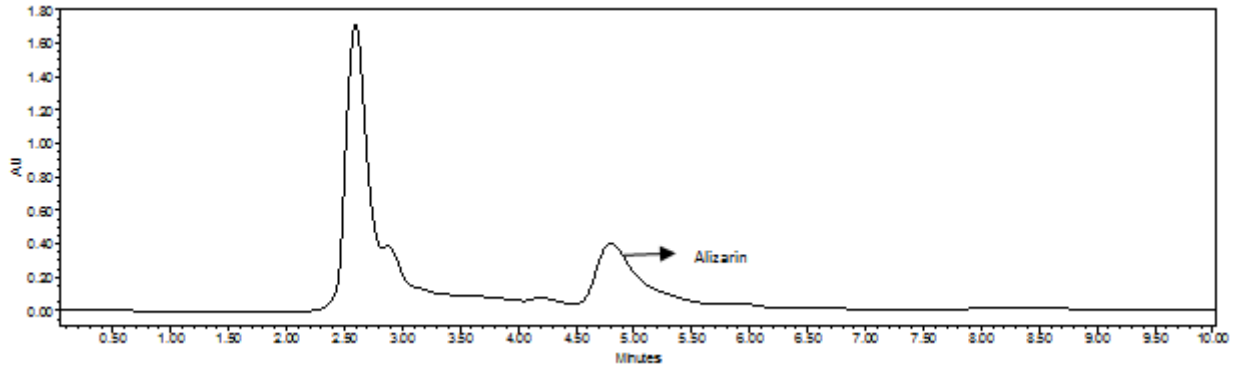

Figure 2
Chromatograms of alizarin (RT=4.782) (a) standard, (b) root sample (c) aerial parts

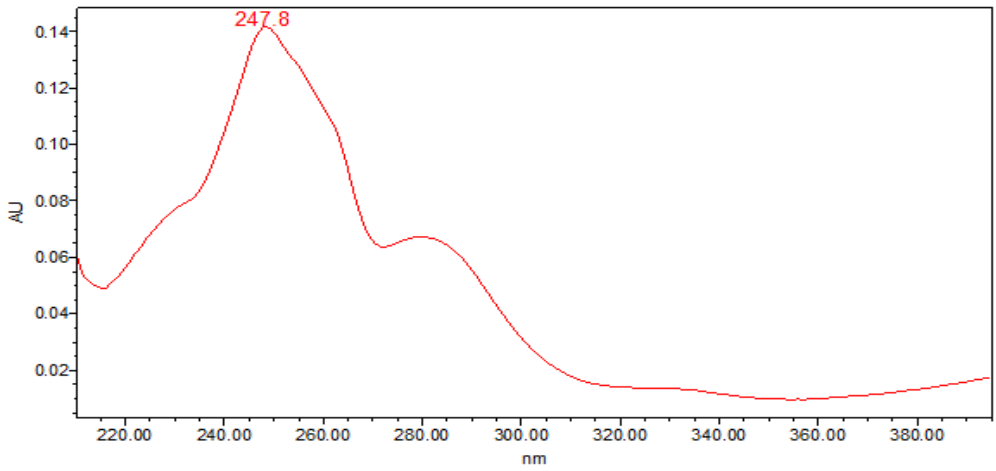

Figure 3
Spectrum of alizarin

Table 1
Calibration data

Parameter (Units)	Alizarin
Regression equation	Y=8680X + 27491
Linearity range (µg/mL)	10-200
r^2±SD	0.99903±0.0005
LOD (ng/mL)	52.54
LOQ (ng/mL)	158

Table 2
Validation Summary of Alizarin

Validation Parameter	Alizarin
Accuracy	98.50-102 %
Intra-day precision (%RSD)	1.25
Inter-day precision (%RSD)	1.92
Selectivity	Selective

Quantification of alizarin in hairy roots of *Rubia tinctorum* and *Rubia Cordifolia* has been reported in the previous studies [7, 12]. This analysis showed that alizarin is present in both roots and aerial parts of this plant although results show (Table 3) higher concentration of alizarin in roots of *Rubia cordifolia* Linn. Quantification of alizarin in *Rubia cordifolia* depicts that roots are the primary source of alizarin. Therefore, this study supports the previous studies and revealed that roots of *Rubia cordifolia* Linn. are good source of alizarin further it also revealed that a significant amount of this compound is present in aerial parts as well. Thus the whole plant should be utilized to extract alizarin.

Table 3
Quantitative estimation of Alizarin in methanolic extracts of Rubia cordifolia Linn.

Plant Part	Content of alizarin (% w/w) of sample Mean ± SD
Root	0.52±0.092
Ariel parts	0.14±0.073

CONCLUSION

Proposed RP-HPLC method provides a better and easy analysis of alizarin using a very common solvent system, methanol and water. Thus, this method is suitable for analysis and quantification of alizarin and for quality control and standardization of drugs derived from this *Rubia cordifolia* Linn.

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