ABSTRACT
A simple and reproducible HPTLC method for the determination of Alizarin in *Rubia cordifolia* and its formulations was developed and is described. The HPTLC method involve separation of components by TLC on stationary phase i.e. precoated silica gel GF60254 (20x10 cm, aluminium sheet) with a solvent system of toluene: ethyl acetate: formic acid (9.8:0.2:0.1) and detection was carried out by scanning and quantifying the peak at 254 nm for Alizarin. The sensitivity of HPTLC method was found to be 50 ng and the linearity was observed in the range of 0.1 µg to 1µg. The method was validated in terms of linearity and specificity. The proposed method being precise and sensitive can be used for detection, monitoring and quantification of Alizarin in *Rubia cordifolia* and its formulations.

KEYWORDS: *Rubia cordifolia*, Formulations, HPTLC, Alizarin, Standardization.

INTRODUCTION
Standardisation of Ayurvedic drugs, herbal formulations and plant materials is the need of the day. Many of them do not have standard identification tests or analytical procedures to maintain their consistent quality. Several Pharmacopoeias and books containing monographs on plant materials describes only the physio-chemical parameters and are lacking in identification and quantification of active compounds. Hence modern methods describing the
identification and quantification of in plants may be useful for proper standardisation of herbs and their formulations. *Rubia cordifolia*, Rubiaceae commonly known as Indian Maddar and Manjistha in Sanskrit is perennial herbaceous climber with long and cylindrical root with a thin red bark. The roots and stem are well known source of Anthraquinones. The roots has proven as antioxidant, anti-inflammatory, anticancer, immunomodulator and hepatoprotective and are extensively used against blood, urinary and skin diseases.[1,2,3,4] Rubia cordifolia is widely used in indigenous system of medicine, it was found to contain anthraquinones of was found to be the major active principle having biological activity. The predominant anthraquinones are alizarin (1,2-dihydroanthraquinone), purpurin (tri hydroxy anthraquinone) and manjistin (xantopurpurin-2-carboxylic acid).[5,6] Quality control of synthetic drugs offers no problem with very well defined parameters of analysis as compared to herbal formulations of plant origin, which are prone to deterioration and variation. The quality control of herbal medicines is therefore highly desired to ensure their authenticity, stability and consistency. HPTLC is becoming a routine analytical technique because of its advantages of low operating cost, high sample throughput, simplicity, and speed, the need for minimum sample clean up, reproducibility, accuracy, reliability, and robustness.[7,8] In the present study a suitable, sensitive, and reliable quantitative HPTLC method has been developed for quality control determination of Alizarin from *R.cordifolia* plant and its formulations.

**MATERIAL AND METHODS**

**Procurement and Authentication**

The root and stem of *Rubia cordifolia* were collected from the Pydhonie, Mumbai Market and authenticated at the Agharkar Institute (No: Auth. 07-40) Poona. Standard Alizarin was purchased from Sigma Aldrich Laboratories Mumbai.

**Extraction**

Around 50 g of air dried whole plant sample was ground to pass through No.60 mesh sieve and extracted with methanol by Soxhlet extraction method. The extract was filtered and concentrated. The final solution for spotting was prepared by dissolving 1g of concentrated extract in 100 ml of methanol and further diluting 10 ml of this solution to 100 ml with methanol. This solution was used further for HPTLC analysis as per the procedure mentioned below.

For *R. Cordifolia* formulation (10, 20, 40 mg/ml concentration gels) 500mg gel of each concentration were transferred to a test tube and extracted with methanol. The extracts were
filtered through whatman filter paper and the residue was washed with 10 ml of methanol. Both extract and washings were transferred to a 10 ml of volumetric flask and volume was made up to 10 ml with methanol.

**Chromatographic conditions**[^9][^10]

Instrument: A camag HPTLC system equipped with a sample applicator Linomat IV, twin through plate development chamber, TLC Scanner III and an Integration software CATS.

Adsorbent: TLC aluminium plate Precoated with silica gel GF254 (E.Merk)

Solvent system: Toluene: Ethyl acetate: Formic acid: (9.8:0.2:0.1)

Solvent run upto: 80 mm.

Scanning wavelength: 254 nm.

Standard preparation: A 0.05 mg/ml solution of Alizarin reference standard was Prepared in methanol.

**Procedure**

Standard solution of pure alizarin (1 mg/ml) in methanol was prepared to yield a stock solution of 100 μg/ml. From this various volumes of 1,2,4,5,6 μl of standard Alizarin solution were applied on precoated TLC silica gel G60F254 plates, using a camag linomat IV automatic sample applicator from about 1 cm edge of TLC plate using a band width of 8 mm. The chromatogram was developed upto 80 mm under chamber saturation condition, with n-butanol: acetic acid: water (36:6:8) in a twin through chamber. After removal from the chamber, the plate was air dried for 15 minutes, derivatised and heated at 120°C for 15 minutes in hot air oven. The plate was then scanned and quantified at 254 nm using camag TLC scanner III. Linearity curve for standard Alizarin in the range of 0.1 to 1μg was developed by plotting the peak area against concentration of Alizarin. The amount of Alizarin was determined using the standard calibration curve. The linearity curve shows a correlation coefficient of 0.999.

**Method Validation**

The developed HPTLC method was validated for specificity and reproducibility.[^12] Developed method is found to be specific for Alizarin (Rf = 0.15) even in the presence of other excipients in case of formulations (fig. 1) and also the *R. cordifolia* extract (fig.2, 3,4).
Linearity range was found to be in the range of 0.1 to 0.5 µg with a correlation coefficient (r) of 0.999 indicating good linearity between concentration and peak area. Precision of the HPTLC instrument was checked by scanning the same spot (900 ng) of Alizarin five times. Reproducibility of the method was checked by analysing a standard solution of Alizarin 300 ng/spot after application (5 µl) on a TLC plate (n=5) and the % CV for peak area was found to be 4.96. Hence the developed HPTLC method is reliable for quantitative monitoring of Alizarin in the raw materials as well as in its formulations.

**Limit of Detection**
The lowest amount of analyte that could be detected is to be determined on the basis of signal to noise ratio. It was determined by spotting on plate different concentrations of Alizarin from 10 ng to 1000 ng. LOD was found to be 24 ng.

**Limit of Quantitation**
The lowest amount of Alizarin that could be quantitatively determined with definite precision and accuracy was calculated on the basis of signal to noise ratio. LOQ was found to be 80 ng.

**Linearity Range**
The linearity of the method was performed using standard Alizarin. The method was found to be linear in the range 100 ng to 500 ng. The correlation coefficient was found to be 0.999.

![Fig.1: HPTLC Chromatogram of Standard Alizarin showing all tracks at 580 nm.](image)
Fig. 2: HPTLC Chromatogram of Standard Alizarin – 100 ng

Fig. 3: HPTLC Chromatogram of Standard Alizarin – 200 ng
Fig. 4: HPTLC Chromatogram of Standard Alizarin – 300 ng

Fig. 5: HPTLC Chromatogram of Standard Alizarin – 400 ng
RESULT

The HPTLC chromatograms of *Rubia cordifolia* extract and formulations are given in figures.

Fig.6: HPTLC Chromatogram of Standard Alizarin – 500 ng

Fig.7: HPTLC Chromatogram of methanolic extract of *Rubia cordifolia*
Fig. 8: HPTLC Chromatogram Gel A (1% w/w) of *Rubia cordifolia*

<table>
<thead>
<tr>
<th>Peak</th>
<th>Start Rf</th>
<th>Start Height</th>
<th>Max Rf</th>
<th>Max Height</th>
<th>Max %</th>
<th>End Rf</th>
<th>End Height</th>
<th>Area</th>
<th>%</th>
<th>Assigned substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1m</td>
<td>0.11</td>
<td>0.2</td>
<td>0.15</td>
<td>99.4</td>
<td>100.0</td>
<td>0.21</td>
<td>0.7</td>
<td>2573.9</td>
<td>100.0</td>
<td>alizarin</td>
</tr>
</tbody>
</table>

Fig. 9: HPTLC Chromatogram of Gel B (2% w/w) of *Rubia cordifolia*

<table>
<thead>
<tr>
<th>Peak</th>
<th>Start Rf</th>
<th>Start Height</th>
<th>Max Rf</th>
<th>Max Height</th>
<th>Max %</th>
<th>End Rf</th>
<th>End Height</th>
<th>Area</th>
<th>%</th>
<th>Assigned substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.12</td>
<td>5.4</td>
<td>0.15</td>
<td>126.2</td>
<td>100.0</td>
<td>0.20</td>
<td>2.9</td>
<td>2730.6</td>
<td>100.0</td>
<td>alizarin</td>
</tr>
</tbody>
</table>
Fig. 10: HPTLC Chromatogram of Gel C (4% w/w) of *Rubia cordifolia*

**Linearity**

\[
Y = 104.4 + 15.49 \times X - 0.003137 \times X^2 \\
\text{r} = 0.99966 \quad \text{sdv} = 1.72
\]

Fig. 11: Linearity curve for standard Alizarin.
Fig. 12: Video image photograph of HPTLC plate of *R. cordifolia* extract and standard Alizarin as seen under @ 254nm. UV lamp.

Fig. 13: Video image photograph of HPTLC plate of *R. cordifolia* extract and standard Alizarin as seen under @ visible

Fig. 14: Video image photograph of HPTLC plate of *R. cordifolia* extract and standard Alizarin as seen under @ 366 nm
Table 1: Content of Alizarin in *Rubia cordifolia* extracts and formulations

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Alizarin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rubia cordifolia</em> extracts</td>
<td>1.32</td>
</tr>
<tr>
<td>Gel A (1% W/W)</td>
<td>0.02</td>
</tr>
<tr>
<td>Gel B (2% W/W)</td>
<td>0.035</td>
</tr>
<tr>
<td>Gel C (4% W/W)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Sample preparation and development of suitable mobile phase or solvent system are two important stages in development of the analytical procedures, which becomes more significant for herbal drugs because of their complexity of the chemical compounds and their affinity towards different solvent systems\(^{[13]}\) By using various mobile phase compositions a better resolution of Alizarin with symmetrical and reproducible peaks was achieved with Toluene: Ethyl acetate: Formic acid: (9.8:0.2:0.1). With the developed HPTLC method the Rf value of Alizarin was found to be 0.15.

**CONCLUSION**

Standardization is a very important aspect of herbal drug and chemical standardization is useful for the determining the optimal level of marker component. Chromatographic fingerprinting was done for identification and quantification of alizarin, it helped to identify the quality of *R.cordifolia* and also useful to measure the quantity of marker alizarin in total *R.cordifolia* root and stem extract and different concentration gel formulations. On micro fingerprinting analysis of *Rubia cordifolia* root and stem extracts at 254 nm Alizarin was seen as the spot around an Rf of 0.15 same spot of Alizarin at an Rf of 0.15 has been observed in the in-house developed formulations of *Rubia cordifolia* extract. The phytoconstituent Alizarin was found to be highest in the whole extract of *Rubia cordifolia* root and stem as compared to developed formulations.

**ACKNOWLEDGEMENTS:** We wish to thank Anchrom laboratories, Mumbai for supporting to carry out HPTLC studies.

**REFERENCES**


