# Research Journal of
# Phytochemistry

ISSN 1819-3471

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Phytochemistry 4 (3): 207-212, 2010
ISSN 1819-3471 / DOI: 10.3923/rjphyto.2007.46.51


# HPTLC Method for the Quantification of Plumbagin in Three *Plumbago* Species*

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**Abstract:** Plumbagin is a naturally occurring naphthoquinone, found in abundance in the genus *Plumbago*. A simple, sensitive, selective, precise and robust HPTLC quantitative method of analysis of plumbagin has been developed and validated. The quantity of plumbagin in the crude extracts, obtained from the three common species of *Plumbago* namely, *Plumbago zeylanica*, *Plumbago rosea* and *Plumbago capensis* has been determined. Total Plumbagin content of *P. rosea*, *P. capensis* and *P. zeylanica* was found to be 0.569, 0.429 and 0.247 mg%, respectively. Densitometric analysis of Plumbagin was carried out in the absorbance mode at 265 um. This method gave compact spots at Rf = 0.78 corresponding to plumbagin. The linear regression analysis data for the calibration plots for Plumbagin had shown good linear relationship with $R^2 = 0.998 \pm 0.0035$ in the concentration range of 20-80 ng/spot. The method was validated for robustness and precision. The limit of detection is noticed to be of 2 ng and is statistically tested for repeatability by inter day and intra day precision tests as per ICH guidelines and its updated international convention. Since this method resolves and quantifies Plumbagin effectively, it can be used to quantify the concentration of Plumbagin in the herbal preparations.

**Key words:** *Plumbago*, robustness, precision, plumbagin, HPTLC

## INTRODUCTION

*P. zeylanica* is a semi climbing subshrub that grows throughout Asia and Africa. This plant is a perennial, sub-scanted shrub found wild in peninsular India and in low elevations in Taiwan (Anonymous, 1969). The whole plant and its roots have been used as folk medicine in the treatment of rheumatic pain, dysmenorrhea, carbuncles, contusion of the extremities, ulcers and elimination of intestinal parasites (Li, 1998). *P. capensis* Thunb, a small scandent shrub, indigenous to S.Africa is a lesser known species in ethnopharmacognosy. *P. rosea* also called *P. indica* L. a shrubby perennial found growing throughout India often as a cultivated plant or as an ornamental plant. It is reported to be wild or indigenous in Sikkim and Khasi hills (Chiu and Chang, 2003). The root bark of *P. rosea* are important indigenous medicine but less commonly used than those of *P. zeylanica*. Plumbagin is present in families of Plumbaginaceae and Droseraceae (Botanical Dermatology Database, 1991a,b). Pure plumbagin is used primarily to Botanical dermatology databas eexploit its properties as a superoxide generator, an antibiotic and an anti neoplastic agent. *P. zeylanica* is grown as a perennial herb in the plains of Bengal and Southern India. Extracts of the root have been reported to be a powerful poison when given internally or applied to the Ostium uteri leads to abortion (Premakumari *et al*., 1977). When administered to hyperlipidaemic rabbits, plumbagin reduced serum cholesterol and LDL cholesterol by 53 to 86% and 61 to 91% (Sharma and *et al*., 1999). Anti-helicobacter activity of *P. zeylanica* has also been reported (Wang and Huang, 2005). In the present

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**Originally Published in Research Journal of Phytochemistry, 2007*

=study, a HPTLC method has been developed for the determination of Plumbagin in the hydroalcoholic extracts of three *Plumbago* species. The proposed method has been validated as per ICH guidelines (ICH, 1994 and 1996) and its updated international convention (ICH, 2000).

## MATERIALS AND METHODS

### Plant Material

Air dried roots of *P. capensis*, *P. rosea* and *P. zeylanica*, collected from TAMPCOL farm, Chennai was identified by botanists at CSDMRI, Chennai and a voucher specimen was deposited at CARISM herbarium. The roots of the three species have been used for the study as Plumbagin was reported to be accumulated mostly in roots and widely used for medicinal purpose (Van der Vijver, 1972).

### Chemicals and Reagents

Standard Plumbagin was procured from Hi-media, India Pvt. Ltd. All other chemicals used were of analytical grade (Merck, India) and the HPTLC plates used were from E-Merck, Germany.

### Preparation of Extracts

Air dried roots (100 g) of three species was extracted with 50% methanol (Hydroalcohol) by static cold maceration process. The extraction yield (%w/w) was calculated as ratio of the weight of the concentrated extract to the weight of the dried root.

### Analytical Procedure

### Chromatographic Conditions

Chromatography was performed on a 10×10 cm preactivated HPTLC Silica gel 60F254 plate. Samples and standard were applied to the plate as 6 mm wide band with an automatic TLC applicator Linomat V with $N_2$ flow (CAMAG, Switzerland), 8 mm from the bottom. Densitometric scanning was performed on CAMAG scanner III at 265 um. The plates were prewashed by methanol and activated at 60°C for 5 min prior to chromatography. The slit dimension was kept at 5×0.45 and 40 mm $sec^{-1}$ scanning speed was employed. The mobile phase consisted of toluenev: ethyl acetate: methanol (8:1:1) and 10 mL of mobile phase was used per chromatography. Linear ascending development was carried out in 10×10 cm twin glass chamber saturated with the mobile phase.

### Method Validation

### Precision

System repeatability was determined by six replicate applications and six times measurement of a sample solution at the analytical concentration of 10 ng/spot of Plumbagin. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of relative standard deviation (RSD%) and standard error was found to be less than 2%. Method repeatability was obtained from RSD value by repeating the assay six times in the same day for intra-day precision. Inter-day precision was assessed by the assay of six sample sets on different days. Inter-and intra-day variation for determination of Plumbagin in three different species was carried out at three different concentration levels of 20, 40 and 80 ng/spot.

### Robustness of the Method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like toluene:methanol (8:2, v/v) have been tried and chromatograms have been run. The plates are prewashed by methanol and activated at 60±5°C for 10 min prior to development. Time from spotting to chromatography and from chromatography to scanning is varied from 0,15, 30 and 60 min. Robustness of the method was done at three different concentration level 20, 40 and 80 ng/spot.

**Limit of Detection and Limit of Quantitation**

In order to estimate the Limit of Detection (LOD) and Limit of Quantitation (LOQ), blank chloroform was spotted six times. The signal to noise ratio was determined. LOD was considered as 2:1 and LOQ as 8:1. LOD and LOQ were experimentally verified by diluting known concentrations of Plumbagin until the average responses were approximately 2 or 8 times the standard deviation of the responses for six replicate experiments.

**Specificity**

The specificity of the method was ascertained by analyzing standard Plumbagin and extracts. The spots for plumbagin in sample was confirmed by comparing the Rf and spectra of the spot with that of sample. The peak purity of Plumbagin was assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot.

**Estimation of Plumbagin in Herbal Extract**

To determine the content of plumbagin in the herbal extract, 500 mg of hydroalcoholic extract is made upto 25 mL with chloroform. As naphthoquinones are reported to be easily soluble in chloroform, chloroform has been used as the solvent (Zhong *et al*., 1984; Siddhu and San karram, 1971). The resulting solution is centrifuged at 3000 rpm for 15 min and the supernatant is analyzed for plumbagin content. Forty microliter of the filtered solution is applied on the TLC plate followed by development and scanning. The analysis is repeated for six times and the possibility of interference from other components of extract in the analysis is studied.

## RESULTS AND DISCUSSION

**Development of the Optimum Mobile Phase**

The TLC procedure was optimized with a view to develop a stability indicating assay method. Both the standard and the sample were run in different solvent systems. The mobile phase consisting of toluene: ethyl acetate: methanol (8:1:1) gave better resolution. The spot at Rf 0.78 was identified as Plumbagin with the help of chromatogram of the standard compound. To improve the resolution of the spots, the plate was run in the same mobile phase twice.

**Calibration Graph**

Linearity was found over the concentration range of 20-80 ng/spot with $R^2 \pm SD = 0.998 \pm 0.0035$ (Table 1). Peak area and concentration were subjected to linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data has shown a good linear relationship over the concentration range of 20-80 ng/spot. The linearity of calibration graphs and adherance of the system to Beer's law are validated by high value of correlation coefficient and the SD for intercept value is noticed to be less than 2%. No significant difference is observed in the slopes of standard curves (ANOVA; $p<0.05$).

## VALIDATION OF THE METHOD

**Precision**

The repeatability of sample application and measurement of peak area have been expressed in terms of RSD% and was observed to be <2% for plumbagin. The results show that there was no

Table 1: Linear regression data for the calibration curves (n = 6)

| Linearity range (ng/spot) | $R^2 \pm SD$ | Slope±SD | Confidence limit of slope |
|---|---|---|---|
| Plumbagin 20-80 | 0.998±0.0035 | 0. 28±0.007 | 0.275-0.286 |

significant intra-and inter-day variation has been observed in the analysis of Plumbagin at three different concentrations level 20, 40 and 80 ng/spot. The RSD % for intra-and inter-day analysis is found to be <2% in all the cases (Table 2). The standard deviation of peak areas was calculated for each parameter and RSD% was less than 2% in all the cases. The low values indicate robustness of the method (Table 3).

**LOD and LOQ**

The LOD was found to be 2 ng/spot for Plumbagin with signal/noise ratio of 2:1 while the LOQ was seen to be 4 ng/spot for Plumbagin with signal/noise ratio of 8:1.

**Specificity**

The peak purity of Plumbagin was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e., r (start, middle) = 0.999825 and r (middle, end) = 0.996673. Good correlation was (R= 0.9995) also obtained between standard and sample overlay spectra of plumbagin (Fig. 1).

**Estimation of Plumbagin in Hydroalcoholic Extract**

The spot at Rf = 0.78 corresponding to plumbagin was observed in the chromatogram of the extracts along with other components. There was no interference from other components present in the chromatogram (Fig. 2-4). Total Plumbagin content was found to be 0.569% w/w and 0.429% w/w in *P. rosea* and *P. capensis* while *P. zeylanica* had 0.247% w/w of Plumbagin (Fig. 2-4).

Table 2: Intra and inter day precision of HPTLC method (n = 6)

| | Intra day precision | | | Inter day precision | | |
|---|---|---|---|---|---|---|
| Amount (ng/spot) | SD of areas | RSD (%) | SE | SD of areas | RSD (%) | SE |
| 20 | 1.12 | 0.87 | 0.35 | 2.02 | 1.54 | 0.87 |
| 40 | 1.54 | 0.76 | 0.32 | 1.92 | 1.52 | 0.76 |
| 80 | 1.23 | 0.64 | 0.34 | 1.56 | 1.43 | 0.52 |

Table 3: Robustness testing

| Parameter | SD of peak areas | RSD (%) |
|---|---|---|
| Mobile phase composition | 1.86 | 1.24 |
| Time from spotting to chromatography | 0.78 | 0.43 |
| Time from chromatography to scanning | 0.68 | 0.19 |

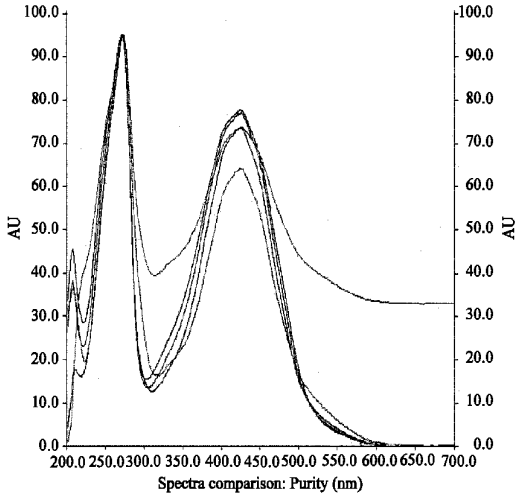


Fig. 1: Spectra of Plumbagin in *P. rosea* (Violet), *P. capensis* (Brown) and *P. zeylanica*

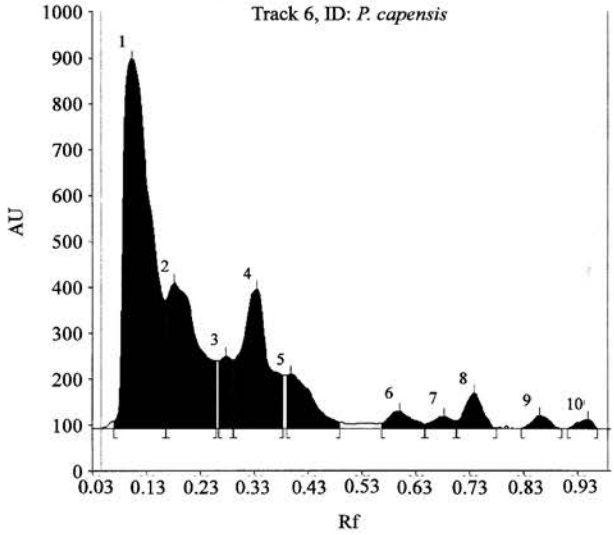


Fig. 2: HPTLC chromatogram of *Plumbago capensis*

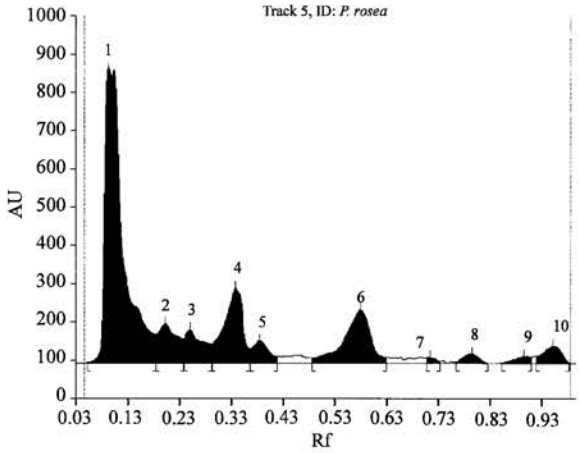


Fig. 3: HPTLC chromatogram of *P. rosea*

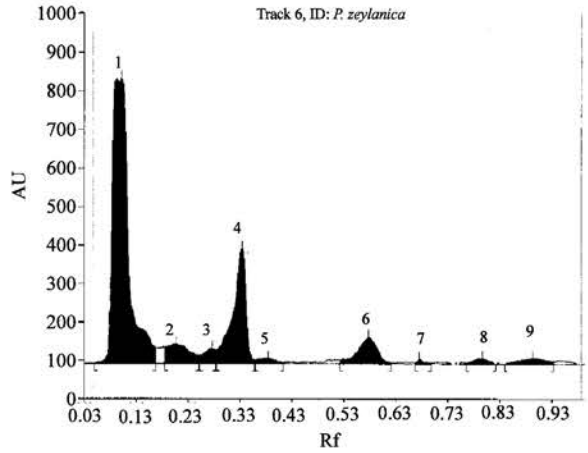


Fig. 4: HPTLC chromatogram of *P. zeylanica*

## CONCLUSIONS

The developed HPTLC method was precise, specific, accurate and robust for determination of Plumbagin in the three species of *Plumbago* namely *P. rosea*, *P. capensis* and *P. zeylanica*. A comparative study of this bio marker compound has shown that it is present in higher amount in the species *P. rosea* while the concentration was low in the hydroalcoholic extract of *P. zeylanica*. The activity of a plant extract is always influenced by the quantity of active principle present in the extract. Since Plumbagin is used in the variety of different chronic diseases, it is very essential to develop a standardization method from which one can optimize its quantity in the herbal formulations. Moreover, HPTLC being an easier and cheaper compared to its ally technique HPLC, was taken for this study to develop a chromatography method for Plumbagin quantification. In polyherbal formulations, hydroalcoholic extracts have been widely used. So, a method has been developed to ascertain the quantity of Plumbagin in the hydroalcoholic extract. So, it is necessary to choose or develop a right extraction procedure so that active ingredients are present in right quantity.

## ACKNOWLEDGMENT

We thank Our Vice-Chancellor, Prof. R. Sethuraman, SASTRA Deemed University, Thanjavur for his constant support to our Centre. Our sincere thanks are due to Department of Science and Technology, Govt. of India and Citadel Fine Pharmaceuticals, Cheunai for their financial assistance.

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