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Screening of *Plumbago* Species for the Bio-active Marker Plumbagin

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Abstract

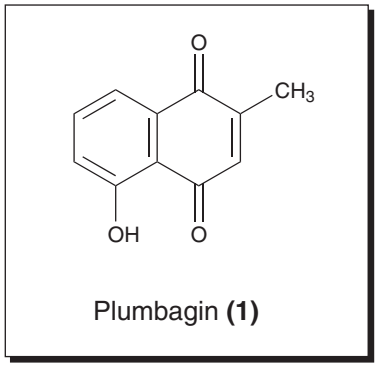
Three locally available *Plumbago* species (*Plumbago auriculata*, *Plumbago rosea* and *Plumbago zeylanica*) have been quantitatively screened for the bioactive marker plumbagin by high performance thin layer chromatography. In general, the root parts were found to be the rich sources for plumbagin. Most significantly, *P. rosea* was found to accumulate maximum plumbagin in the roots. In contrast, *P. auriculata* and *P. zeylanica* are high yielding species for the leaf and stem parts, respectively. Studies have also been extended to evaluate some *P. rosea* samples collected from southern India and a traditional medicine, Chitrakadi vati, which contains *P. zeylanica* as the major constituents.

Keywords: Plumbaginaceae, *Plumbago auriculata*, *Plumbago rosea*, *Plumbago zeylanica*, plumbagin, HPTLC.

Introduction

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a highly desirable natural product derived from the species of *Plumbago*, *Drosera* and *Diospyros* for biological and chemical research (Veluri et al., 1999). Plumbagin was found to exhibit a variety of interesting pharmacological activities like antitumor (Uma Devi et al., 1999), antifertility (Kini et al., 1997), antimalarial (Nakornchai et al., 1995), hyperglycemic (Olagunju et al., 1999), hypolipidemic (Sharma et al., 1991) and antimicrobial (Krishnaswamy & Purushothaman, 1980). Interesting research activity is going on in the chemical front in making a number of plumbagin analogues by employing Schmid reaction, Thiel-Winter addition, allylic bromination and oxy-mercuration reactions (Dinda et al., 1999).

In continuation of our search for potential species of some well-known herbal drugs (Mallavadani et al., 2002), we herein report the work done on the medicinally highly reputed *Plumbago* species. The genus *Plumbago* (Plumbaginaceae) consisting of shrubs, is found wild in southern and eastern parts of India and occasionally cultivated as ornamental plants (Baniprasad et al., 1995). There are three species found in India, *P. auriculata* Lam. (Syn. *P. capensis Thumb.*), *P. rosea* Linn. (Syn. *P. indica* Linn.) and *P. zeylanica* Linn. All these three species are locally available. In Indian traditional medicine, *P. zeylanica* (diarrhoea, dyspepsia, skin-diseases including leprosy) and *P. rosea* (leukoderma, abortifacient) have been extensively used (Kirtikar et al., 1935; Varier et al., 1995). Pharmacological screening of various extracts of *Plumbago* species revealed that plumbagin **(1)** is the bio-active marker and is responsible for their varied therapeutic activities.



Plumbagin **(1)**

In view of the growing demand for plumbagin, we have done extensive HPTLC based quantification studies on root, stem and leaf parts of *P. auriculata*, *P. rosea* and *P. zeylanica*. A literature search revealed that only two research groups

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have worked on plumbagin quantification. However, the studies are totally different as the species studied by the first group is *P. europaea* (Al-Nuri et al., 1994; Honnoun et al., 1999) and the other on the root part of *P. zeylanica* (Gupta et al., 1993, 1999).

Experimental

General

M.P. Buchi capillary melting point apparatus and uncorrected; IR: JASCO FTIR; UV: Beckman DU-64 spectrophotometer; ^{1}H– and ^{13}C–NMR: Jeol DPX at 300 MHz and 75 MHz, respectively. HPTLC: TLC plates precoated (0.2 mm) silica gel F_{254} aluminium sheets of E-Merck grade, Scanner: CAMAG TLC Scanner-III with CATS 4 software.

Plant material

The whole plant material of *P. auriculata*, *P. rosea* and *P. zeylanica* were collected locally during October–December 2000 and the authenticity of these three species was confirmed by a taxonomist. Voucher specimens (000201–03) are available at the Forest and Marine Products Division, Regional Research Laboratory, Bhubaneswar, India. Two samples of *P. rosea* roots were also collected from southern India i.e., Thiruvanantapuram and Madras through traders. The authenticity of these samples was also confirmed by a taxonomist. One traditional medicine, namely Chitrakadi vati, which contains *P. zeylanica* as the major constituent, was purchased from a local market.

Processing, extraction and isolation

The root, stem and leaf parts of the three *Plumbago* species were shade-dried and powdered in a grinder separately. To isolate authentic plumbagin, the powdered root material (500 g) of *P. rosea* was packed in a Soxhlet extractor and extracted with hot acetone. The acetone solubles on concentration under vacuum gave a dark reddish-brown residue (25 g), which on column chromatography (hexane-ethyl acetate, 94 : 6) furnished plumbagin as orange yellow coloured shining crystals (800 mg), m.p. 78 °C [lit. (Pollock & Stevens, 1965) m.p. 78–9 °C]; R_f 0.44 [Pet. ether (60–80 °): ethyl acetate – 94 : 6]. Its identity was further confirmed by spectral (UV, IR, ^{1}H and ^{13}C NMR and mass) data.

Quantitative estimation of marker compound by HPTLC

HPTLC conditions

The HPTLC system consists of a Linomate applicator and CAMAG TLC SCANNER – III with CATS 4 software; stationary phase: pre-coated silica gel F_{254} aluminium plate (E-Merck grade); mobile phase: Pet. ether (60–80 °): ethyl acetate – 94 : 6; development: vertical in saturated chamber; detection: i) UV light – 254 nm – yellow fluorescence, ii) 5% alcoholic KOH – pink colour.

Test sample solution

In view of the sublimating nature of plumbagin, a simple and very efficient procedure has been employed to prepare the test sample solutions. The powdered plant sample (2 g) was weighed accurately and extracted exhaustively with acetone in a 6 ml Soxhlet extractor. The acetone solution was directly filtered into a graduated test tube (10 ml) through a Hirsh funnel and made up to volume with acetone.

Standard solution

The standard solution was prepared by dissolving 1 mg of the pure plumbagin in 1 ml of acetone.

Procedure of sample application

Test sample solutions [each 10 µl (2000 µg) for root parts and 15 µl (3000 µg) for leaf and stem parts] and different concentrations of the standard solution [1 µl (1 µg), 2 µl (2 µg) and 3 µl (3 µg)] were applied in different tracks as bands by a LINOMATE applicator. Sample solutions of various parts of the three *Plumbago* species have been applied separately and developed the chromatograms.

Scanning

The plate was kept in the above mentioned solvent system and allowed to run up to a distance of 9 cm. After drying, it was scanned densitometrically at 265 nm.

Method development and validation

Repeatability: The plant material was extracted by the above mentioned procedure and analysed in triplicate.
Reproducibility: Identical volumes of the standard solution (1 µg in 1 µl) were applied five times on HPTLC plates and analysed by densitometry.
Linearity range: Calibration graphs for HPTLC were recorded with sample amounts ranging from 1–10 µg (correlation coefficient, r = 0.9997).

Result and discussion

As plumbagin, the bio-active marker of *Plumbago* species, is highly desired by the herbal drug industry for quality evaluation and also for making new chemical entities, a HPTLC based search has been undertaken. The authentic sample of the marker compound was obtained by isolation from the roots of *Plumbago rosea* and was throughly characterised by

comparing its physical, chemical and spectroscopic data with reported values (Tezuka et al., 1973). Due to the sublimating nature of plumbagin, extreme precautions have been taken to avoid sample loss and we developed a simple and efficient procedure to generate test sample solutions as mentioned above. Preliminary TLC studies revealed that the solvent system petroleum ether (60–80 °): ethyl acetate – 94 : 6 was ideal and gave a single spot with a R_f 0.44 for the marker and well resolved spots for the test samples. The spots of the chromatogram were visualised both in UV at 254 nm and by spraying with 5% alcoholic KOH. Initially the HPTLC fingerprinting was done on the marker compound and parameters were optimised. Under identical parameters, the fingerprinting pattern of the test samples were recorded. The marker compound was found to be present at R_f 0.44. The finger printing pattern of the high yielding *P. rosea* roots collected locally is given in Fig. 1. The three dimensional patterns of the test sample and standard revealed that the peak corresponding to R_f 0.44 is superimposable in all the samples. The spectrum characteristics corresponding to this peak were also found exactly matching, indicating the compounds corresponding to R_f 0.44 of the standard and test samples are identical. The peak purity test was done by comparing the spectra of the standard and test samples. Linearity of the calibration curve was achieved between 1–10 µg. The correlation coefficient for a calibration curve between 1–10 µg was found to be 0.9997. The percentage of the bioactive marker was determined with the help of calculation mode by using the peak area parameter and the data are presented in Table 1.

It is very significant to note from the data (see Table 1) that the bio-active marker was found more in roots, than in stem and leaf parts of all the three species. Among the root parts, the *P. rosea* of Bhubaneswar contains maximum plumbagin (19.13×10^{-2}%). For the plumbagin content in stem and leaf, the high yielding species are *P. zeylanica* (1.1×10^{-2}%) and *P. auriculata* (2.1×10^{-2}%), respectively. The low plumbagin concentration in two *P. rosea* samples collected from Thiruvanantapuram and Madras may be due to delay in processing of the material after collection. This seems to be likely as plumbagin is sublimating in nature and lost over a period of time. Evidence for this can also be seen from the orange yellow stains of the packing material through which the plant materials have been procured. The above observations suggest that fresh *Plumbago* species have to be used in the herbal drug formulations to get maximum bioactive marker concentration and, thereby, proper drug action. One of the popular traditional medicine, viz., Chitrakadi vati, has been successfully evaluated with the present method and found to contain plumbagin in 1.65×10^{-2}%.

In conclusion, *P. rosea* is high plumbagin yielding species among root parts, whereas in *P. zeylanica* and *P. auriculata*, higher amounts are found in the stem and leaf parts, respectively.

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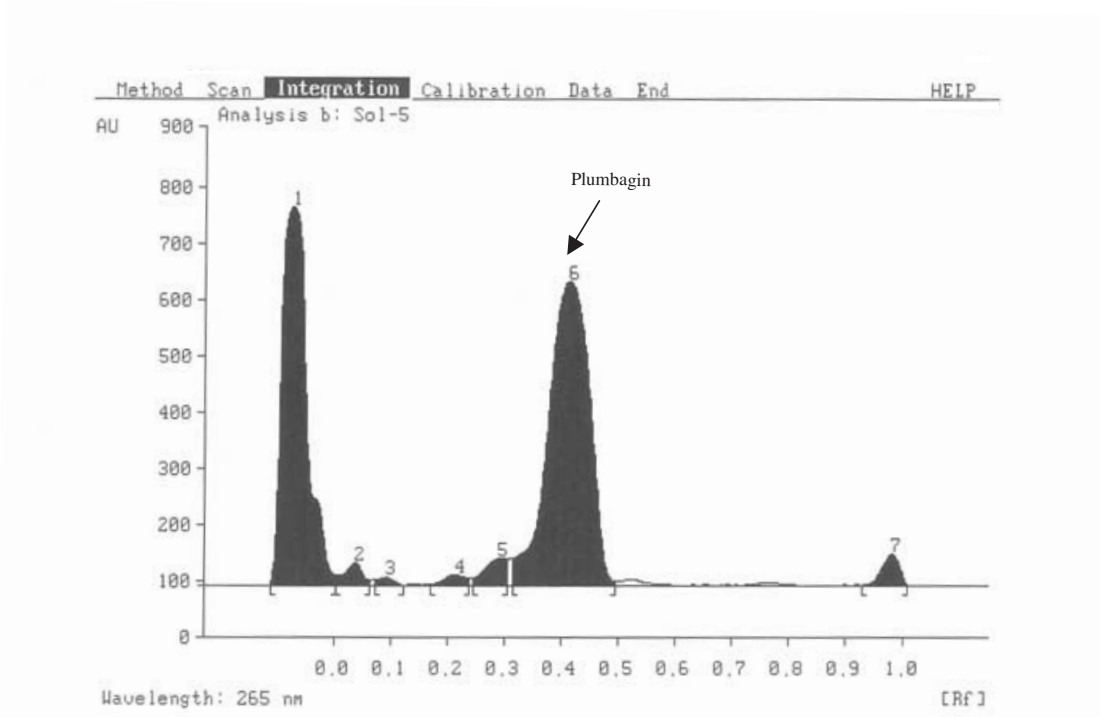

Figure 1. HPTLC fingerprinting of *P. rosea* root (Bhubaneswar).

Table 1. Accumulation of the bioactive marker plumbagin in various plumbago species.

Sl. No.	Species	Area of Collection	Part	Content (%)
1.	*P. auriculata*	Bhubaneswar	Root	15×10^{-2}
			Stem	0.38×10^{-2}
			Leaf	2.09×10^{-2}
2.	*P. rosea*	Bhubaneswar	Root	19.13×10^{-2}
			Stem	0.22×10^{-2}
			Leaf	0.32×10^{-2}
3.	*P. zeylanica*	Bhubaneswar	Root	14.31×10^{-2}
			Stem	1.1×10^{-2}
			Leaf	0.35×10^{-2}
4.	*P. rosea*	Thiruvanantapuram	Root	8.46×10^{-2}
5.	*P. rosea*	Madras	Root	9.4×10^{-2}
6.	*P. zeylanica* In Chitrakadi vati	Nrayan Pharm. Pvt. Ltd., Bhubaneswar	N.S.	1.65×10^{-2}

N.S. = Not Specified

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