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Estimation of Pterostilbene in *Pterocarpus marsupium* Heart wood and Extracts by HPLC and HPTLC

Katta Vijaykumar[1], Papolu B. Murthy[1], Sukala Kannababu[1], B. Syamasundar[2] & Gottumukkala V. Subbaraju*[1]

[1] *Laila Research Centre, Unit I, Phase III, Jawahar Autonagar, Vijayawada–520 007, India*
[2] *Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar–522 510,India*

ABSTRACT: Simple and reproducible methods for estimation of pterostilbene, an anti-diabetic agent found in *Pterocarpus marsupium* by high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC), were developed and validated. The methods showed satisfactory linearity and precision, good recovery and appropriate limits of detection (LOD) and quantification (LOQ).The content of pterostilbene was determined and the results obtained by HPLC and HPTLC methods were in good agreement. The methods developed are suitable for the quality control applications in *Pterocarpus marsupium* heart wood and extracts.

Key words: Pterostilbene, *Pterocarpus, marsupium*, HPLC, HPTLC.

Introduction

Pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) is a phytoalexin found in grapes and blue berries (1-3). It was first isolated from *Pterocarpus santalinus* (red sandal wood) (4) and also found to be one of the active constituents of the heart wood of *Pterocarpus marsupium*, a tree used in the Indian system of medicine for the treatment of diabetes (5, 6). *Pterocarpus marsupium* is a moderate to large deciduous tree commonly known as Asanahm bijkah (Sanskrit) and Red kino tree (English) and used extensively in the treatment of diarrhoea, toothache, fever, urinary tract and skin infections (7) Extracts from this plant have been also reported to possess anti-inflammatory activity (8) and wound healing property (9). The alcoholic extracts of the stem bark of *Pterocarpus marsupium* exhibited significant hepatoprotective activity (10) and anticataract activity (11). Pterostilbene exhibits a wide range of biological activities, namely antioxidant activity (12, 13), hypolipidemic activity (14), cancer chemoprevention activity (15-17). Pterostilbene is also useful in the treatment of arthritis, fibro-myalgia, pain (18) and inflammation (19).

* *Corresponding author Telephone:+91 866 2541303; Fax +91 866 2546216; e-mail: subbarajugottumukkala@hotmail.com*

Herbal products have gained increasing importance, recently, in view of their safety and disease prevention properties. With the development of advanced analytical techniques, the modernization of traditional medicine has become an important area in the recent years.

The therapeutic and health protection effects of pterostilbene led to the development of analytical methods for the determination of pterostilbene in plant materials. High performance liquid chromatography (HPLC) (20, 21) methods reported for pterostilbene estimation in grapevine, berries and wines includes separation of pterostilbene using initial solid phase extraction (SPE), gradient elution and coupling diode array detection and fluorometry. High performance thin layer chromatographic (HPTLC) method was reported earlier for the estimation of pterostilbene in plant materials (22).

In the present study, accurate, simple, specific and reproducible HPLC and HPTLC methods have been developed and validated (23) for the determination of pterostilbene in *Pterocarpus marsupium* heart wood and extracts.

Experimental

Reagents and Apparatus

Standard pterostilbene was purchased from M/s

ChromaDex USA. Methanol and acetonitrile were of HPLC grade, phosphoric acid, hexane, ethyl acetate and formic acid were purchased from M/s Qualigens (Mumbai, India). Ultra pure water generated by the Barnstead Nano pure system (Model 3750, USA) was used. Methanol was used as a solvent for the preparation of standards and samples. Acetonitrile and 0.1% (v/v) phosphoric acid in water (55:45) and hexane-ethyl acetate-formic acid (8.0:1.9:0.1) were used as mobile phase for HPLC and HPTLC analyses respectively. All solutions were filtered through 0.45 μm pore size membrane filter using a Millipore Swinnex type filtration unit. *Pterocarpus marsupium* heart wood and extracts were provided by M/s Laila Impex, Vijayawada, India.

Sample preparation

Pterocarpus marsupium heart wood samples

Weighed about 1 g of *Pterocarpus marsupium* heart wood powder into a round bottom flask, added about 30 mL methanol and refluxed on a water bath for about 30 min. The same operation was repeated twice with methanol (2 X 30 mL) and combined all the alcoholic extracts and made up to 100 mL with methanol. The solution was filtered on 4.5 μm membrane filter.

Pterocarpus marsupium extracts

About 100 mg of dry sample was dissolved in 75 mL of methanol, sonicated for 10min, diluted to 100 mL with methanol and filtered through 0.45 μm membrane filter

Calibration curve of standard pterostilbene–HPLC method

1 mg mL^{-1} pterostilbene standard solution was prepared in methanol. Standard working solutions were prepared by diluting standard stock solutions with methanol in the concentration range 2.5-300.0 ng μL^{-1}. 20 μL from each working standard solution was injected in six replicates. Calibration curve was generated by linear regression based on the peak areas.

Calibration curve of standard pterostilbene–HPTLC method

1 mg mL^{-1} pterostilbene standard solution was prepared in methanol. Standard working solutions were prepared by diluting standard stock solution with methanol in the concentration range 20-120 ng mL^{-1}. 10 mL from each working standard solution was spotted on the TLC plate to obtain a final concentration range of 0.2-1.2 μg / spot. Each concentration was spotted six times on TLC plates. Calibration curve was generated by linear regression based on the peak areas.

HPLC instrumentation

The HPLC system supplied by Shimadzu comprising LC-10 ATVP pumps, SCL- 10 AVP system controller and SIL-10 ADVP auto injector was used. The column was Phenomenex Luna C_{18} 5μm, (250 X 4.6 mm) at ambient temperature. Isocratic elution was carried out with acetonitrile: 0.1 (v/v) phosphoric acid in water (55 : 45) at a flow rate of 1 mL min^{-1}, detection was at 313 nm using SPD-M 10 AVP photodiode array detector. Class VP software was used for integration and calibration. Evaluation was via peak areas with linear regression.

HPTLC instrumentation

The samples were spotted in the form of bands with a Camag microlitre syringe on a pre-coated silica gel aluminium plates 60_F-254 (20 cm X 10 cm with 250 mm thickness, E.Merck, Darmstadt, Germany) using a Camag Linomat IV (Muttenz, Switzerland) applicator. The plates were pre-washed with methanol and activated at 60°C for 5 min prior to chromatography. Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag Muttenz, Switzerland) using mobile phase consists of hexane : ethyl acetate : formic acid (8.0:1.9:0.1). The length of the chromatogram run was 8 cm. Subsequent to the scanning TLC plates were air dried and scanning was performed on a Camag TLC scanner in its absorbance mode at 313 nm and operated by Cats software 4.03. Evaluation was via peak areas with linear regression.

Estimation of pterostilbene

To estimate the content of pterostilbene in the *Pterocarpus marsupium* heart wood and extract samples, aliquots of 10 μL were subjected to HPTLC and aliquots of 20 μL were injected into HPLC. The HPTLC plates were developed to a distance of 8 cm from the point of application, dried and scanned. HPLC analysis was continued for 20 min, since the retention time of pterostilbene was 10.4 ± 0.5 min. The content of pterostilbene was calculated by linear regression, and mean percentages were calculated from six replicate experiments.

Results and Discussion

Consistent quality for herbal products can only be assured by the use of validated analytical methods for

identification and quantification of the active ingredients. The HPLC and HPTLC methods for quantitative estimation of pterostilbene were validated with regard to their specificity, precision, accuracy and linearity.

HPLC method validation

The composition of the HPLC mobile phase was optimized to achieve good resolution. The best resolution and peak shape was obtained by acetonitrile : 0.1 (v/v) phosphoric acid in water (55 : 45) as mobile phase. The compound with a retention time 10.4 ± 0.5 min was identified as pterostilbene (Figure 1). Specificity can be ascertained by comparing the standard and samples peak purity. The peak corresponding the pterostilbene in the sample was confirmed by comparing the spectrum obtained by using photodiode array detector, which was completely in agreement with the standard (Figure 2).

Precision is a measure of either reproducibility or repeatability of the analytical method in normal operating conditions. Intermediate precisions express the laboratory variations, by intra-day and inter-day variation. Six determinations of three concentrations standard pterostilbene on the same day (intra-day) and on different days (inter-day) were carried out and expressed as percent relative standard deviation (RSD%) or co-efficient of variation (CV). The results depicted in Table 1A, show that no significant intra-day and inter-day variations were observed in the analysis of pterostilbene. The RSD% for intra-and inter-day analysis was found to be in the range 0.11-0.69 %, which are less than 2%.

The accuracy of the method was determined from recovery studies. The pre analyzed samples were spiked with three concentrations of standard pterostilbene and the mixtures were analyzed by the proposed method. The recoveries are in the range of 98.28 -101.25% reported in the Table 2A. The average recovery percentage value was found to be 99.56 ± 1.25%.

The linearity of standard curve was evaluated by determining six standard working solutions containing 2.5–300.0 ng μL^{-1} pterostilbene. Peak area and concentrations were subjected to least square linear regression analysis to calculate this calibration equation and correlation coefficient. Linearity was obtained over a concentration range of 0.05-6.00 µg per injection with a correlation coefficient of 0.9999±0.0001. The linearity of calibration graph and adherence of the system to Beer's

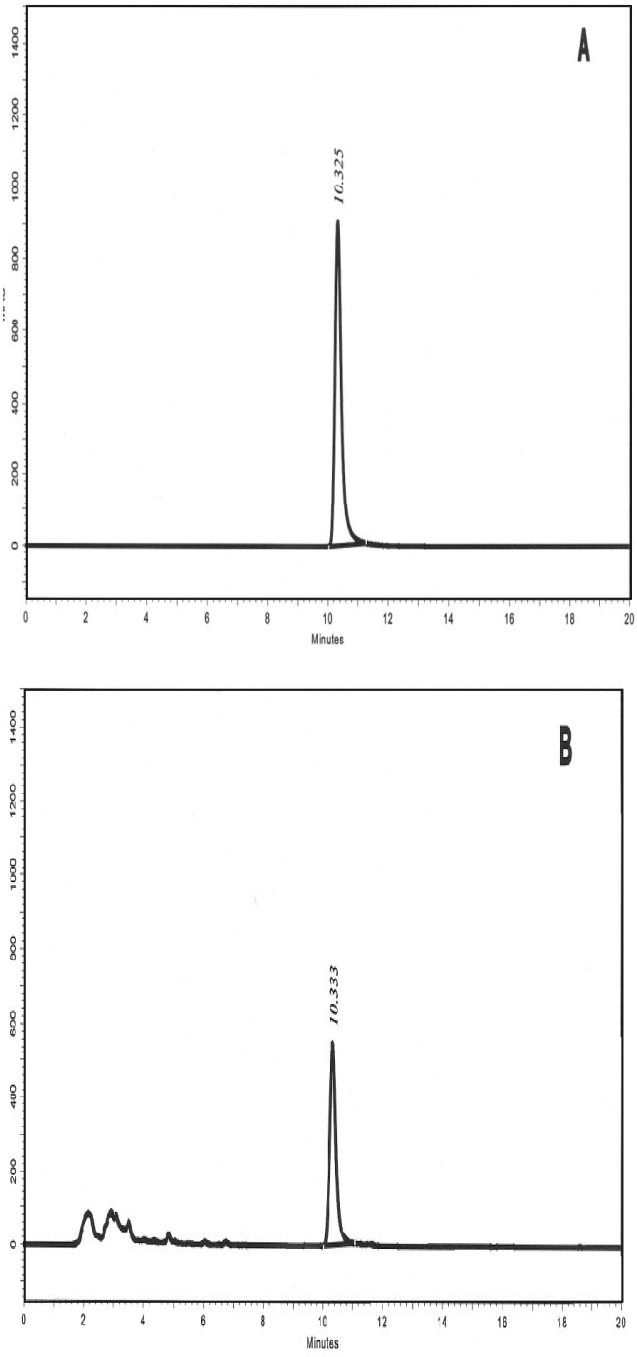

Figure 1: HPLC Chromatograms of (A) Pterostilbene standard (B) Pterocarpus marsupium heart wood extract.

law was validated by high value correlation coefficient.

Limit of detection (LOD) and limit of quantification (LOQ) were studied to check the sensitivity of the method under the working conditions proposed. The LOD and LOQ were 0.28 and 0.85 ng per injection respectively.

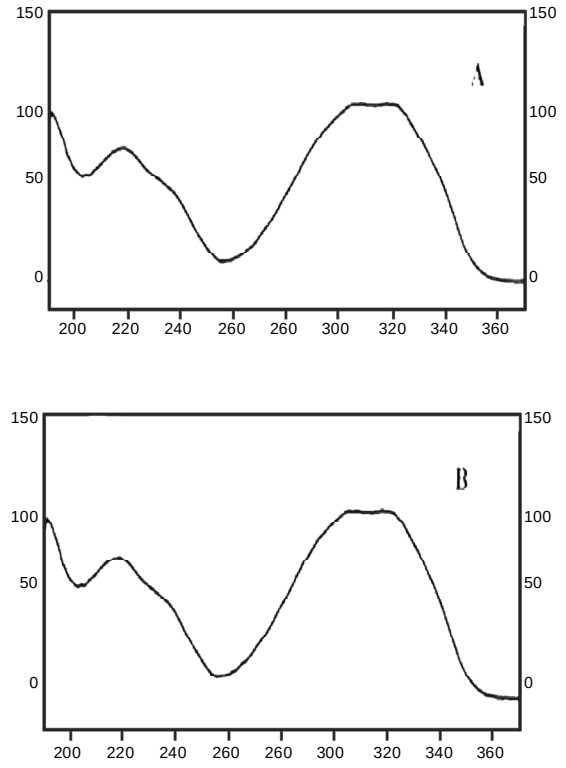

Figure 2: Spectra of pterostilbene obtained by diode array detector. (A) Pterostilbene standard (B) *Pterocarpus marsupium* heart wood extract.

Table 1
Intra-day and Inter-day precision of HPLC (A) and HPTLC (B) methods (n=6)

A. HPLC method

	Intra-day precision		*Inter-day precision*	
Pterostilbene µg	*Pterostilbene* µg	*RSD* %	*Pterostilbene* µg	*RSD* %
0.50	0.4973±0.0007	0.14	0.4960±0.0006	0.12
1.00	0.9996±0.0038	0.38	0.9994±0.0011	0.11
5.00	5.0663±0.0111	0.22	5.0166±0.0346	0.69

B.HPTLC method

	Intra-day precision		*Inter-day precision*	
Pterostilbene µg	*Pterostilbene* µg	*RSD* %	*Pterostilbene* µg	*RSD* %
0.20	0.1999±0.0032	1.60	0.1914±0.0028	1.46
0.70	0.6998±0.0118	1.69	0.6902±0.0119	1.72
1.20	1.2003±0.0179	1.49	1.1592±0.0115	0.99

Table 2
Recovery Study (n=6)

A. HPLC method

Amount Pterostilbene added µg	*Amount Pterostilbene recovered µg*	*% Recovery*
0.2970	0.2919	98.28
0.9900	0.9816	99.15
2.4750	2.5059	101.25

B. HPTLC method

Amount Pterostilbene added µg	*Amount Pterostilbene recovered µg*	*% Recover*
0.30	0.3017	100.56
0.45	0.4573	101.62
0.80	0.7873	98.41

HPTLC method validation

The composition of the mobile phase for TLC was optimized by testing different solvent mixtures of varying polarity. The best results were obtained using hexane-ethyl acetate-formic acid (8.0:1.9:0.1). The selected mobile phase produced highly symmetrical peaks showing good resolution (Figure 3). The compound with a R_f value of 0.25 ± 0.03 was identified as pterostilbene.

The specificity of the method was ascertained by analyzing standards and samples. The spot for pterostilbene in the sample was confirmed by comparing the R_f value and the spectrum of the spot with that of the standard. Peak purity of the sample was fully in conformity with the standard as show in Figure 4.

The reproducibility of the method was studied by applying six replicates of the three different concentrations of the standard .The results in the Table 1B showed that the RSD% for intra- and inter-day analysis was found to be in the range of 0.99 - 1.72%, that is less than 2%.

The accuracy of the method was determined from recovery studies. A known but varying amount of standards of pterostilbene was added to the pre analyzed sample and analyzed according to the procedure. The results are reported in Table 2B. The average recovery percentage value was found to be 100.26% ± 1.40%.

Linearity was evaluated by determining six standard working concentrations containing 20-120 ng μL^{-1} of pterostilbene. Peak area and concentrations were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficient. Linearity was found over the concentration range of 0.2 – 1.2 µg / spot with a correlation coefficient 0.9974 ± 0.0020. The linearity of the calibration graph and adherence of the system to Beer's law was validated by a high value correlation coefficient.

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), methanol (blank) was

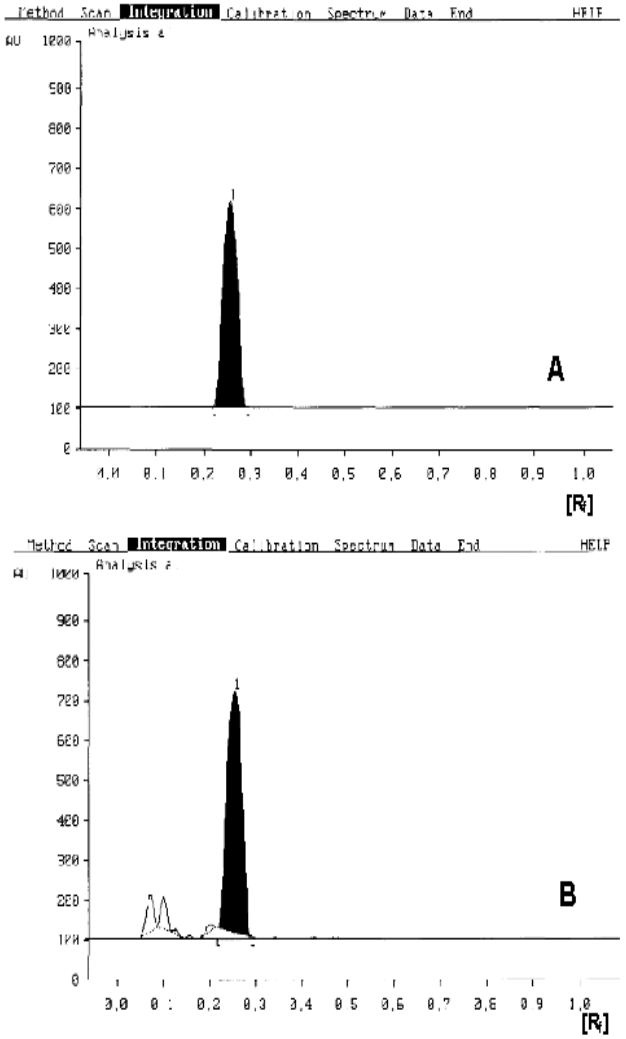

Figure 3: HPTLC Chromatograms of (A) Pterostilbene standard (B) *Pterocarpus marsupium* heart wood extract. Key to peak identity: 1, Pterostilbene.

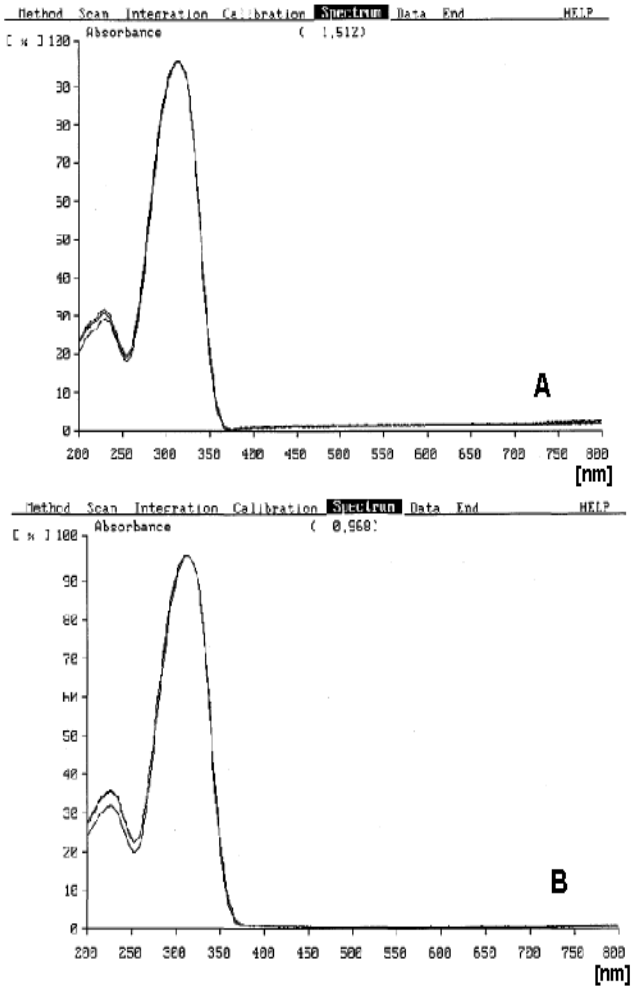

Figure 4: Overlay spectra of (A) Pterostilbene standard (B) Sample at Peak start, Peak maximum and Peak end in absorbance mode in the UV range, taken on the Camag TLC scanner III.

spotted six times. LOD and LOQ were determined based on the standard deviation of the response of the blank and slope estimated from the calibration curve of the pterostilbene. The LOD and LOQ was found to be 0.14 and 0.43 ng / spot for pterostilbene.

Application of HPLC and HPTLC methods

The methods developed here were applied for the estimation of pterostilbene in two different *Pterocarpus marsupium* heart wood samples and extracts. The results obtained are presented in Table 3. RSD% values were found to be less than 2% which shows that the precision of the methods is reasonably good. Interference studies (data not shown) revealed that the presence of commonly used excipients like starch, maltodextrin, yellow dextrin and colloidal silicon dioxide, does not interfere.

Table 3
Estimation of Pterostilbene by proposed HPLC and HPTLC methods (n=6)

Sample	*HPLC Method*		*HPTIC Method*	
	Estimated amount mg/100mg	*RSD %*	*Estimated amount mg/100mg*	*RSD %*
Pterocarpus marsupium heartwood Sample-1	0.3560	0.50	0.3490	1.34
Pterocarpus marsupium heartwood Sample-2	0.6180	1.06	0.6205	1.35
Pterocarpus marsupium heartwood extract Sample-1	5.8000	0.96	5.8560	1.29
Pterocarpus marsupium heartwood extact Sample-2	5.3230	1.98	5.1090	1.95

Conclusions

The HPLC and HPTLC methods developed are useful for quantitative estimation of pterostilbene in the range of 2.5 – 300.0 μg mL^{-1} and 20 – 120 μg mL^{-1} respectively, in plant materials and extracts. Due to non interferences with common excipients, the methods could be used for the determination of pterostilbene in dosage forms also. The methods are simple, sensitive and statistically validated for linearity, accuracy and precision.

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