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Journal of Chromatography B, 744 (2000) 257–262

JOURNAL OF CHROMATOGRAPHY B

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Sensitive high-performance thin-layer chromatographic method for the estimation of diospyrin, a tumour inhibitory agent from the stem bark of *Diospyros montana* Roxb.

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Received 10 January 2000; received in revised form 12 April 2000; accepted 14 April 2000

Abstract

Diospyrin, a tumour inhibitory agent from the stem bark of *Diospyros montana* was isolated and characterised. A sensitive high-performance thin-layer chromatographic (HPTLC) method was developed for the estimation of diospyrin. The method was validated for precision (intra- and inter-day), repeatability and accuracy. The method was found to be precise, with the RSDs for intra-day in the range of 0.72–1.85% and RSDs for inter-day in the range of 1.06–2.95%, for different concentrations. Instrumental precision and repeatability of the method were found to be 0.086 and 0.937 (% CV), respectively. Accuracy of the method was checked by performing the recovery study at two levels and average percentage recovery was found to be 97.87%. The developed HPTLC method was adopted for the estimation of diospyrin content of the stem bark of *D. montana* from different regions, which varied from 0.35 to 0.47% (w/w) in the samples.

Keywords: Diospyrin

1. Introduction

For the past few decades, anticancer compounds from natural sources have been gaining importance because of the vast chemical diversity that they offer. During the exploration of indigenous medicinal plants for anticancer activity, *Diospyros montana* Roxb. (family: Ebenaceae) was found to possess significant tumour inhibitory activity [1]. *D. montana* is a poisonous tree, widely found in the tropics, commonly known as *Tamala* in Sanskrit and *Bistendu* in Hindi. Traditionally the fruits are used to heal skin sores, while crushed leaves and fruits are used by tribal people to stupefy fish [2,3]. In Chinese medicine, the paste of the stem bark of *D. montana* was used to treat tumours [4]. Diospyrin, a binaphthoquinonoid from the stem bark, was found to be the active principle responsible for the anti-tumour activity [1,5].

To the best of our knowledge, so far no method has been reported for the estimation of diospyrin content in plant material. High-performance thin-layer chromatography (HPTLC) is emerging as an

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00277-2

important tool for evaluation of herbal drugs on a phytochemical basis [6–8]. As a part of our work on standardisation of indigenous medicinal plants, we report a simple, specific and sensitive method for the estimation of diospyrin, from the stem bark of *D. montana* using HPTLC.

2. Materials and methods

2.1. Materials and reagents

Samples of stem bark of *D. montana* were collected from four different parts of Karnataka, India, viz., Shimoga, Sringeri, Sagar and Soraba and were authenticated at the Botany Department, D.V.S. Senior college, Shimoga, Karnataka (voucher specimens were preserved in our Pharmacognosy and Phytochemistry Department). All the four samples were air dried at source. Later they were dried at 55°C in a hot air oven, powdered to 40 mesh and stored in air-tight containers. All the reagents used for analysis were of analytical grade.

2.2. Isolation and characterisation of diospyrin

Diospyrin was isolated and purified from stem bark of *D. montana* by the reported method of Kapil and Dhar [9]. Briefly the method is as follows: powdered stem bark of *D. montana* (100 g) was defatted by refluxing with light petroleum (60–80°C). The marc obtained after filtration was further extracted with carbon tetrachloride. The extract was concentrated and cooled in a refrigerator. Amorphous powder of diospyrin separated was washed repeatedly with ethanol and was recrystallised from chloroform to get pure diospyrin (0.225%, w/w, yield).

The identity of the compound was checked by recording the melting point (Melting Point Apparatus, Toshniwal, India), UV spectra in ethanol (Jasco 7850 UV–Vis spectrophotometer), IR spectra (on Buck Scientific IR spectrophotometer-500), mass spectra [on a Perkin-Elmer, PE Sciex atmospheric pressure ionisation (API) 165 mass spectrometry (MS) system] and compared with the reported data [10]. The purity of the compound was established by analysis with a differential scanning calorimeter (Perkin-Elmer) and HPTLC (CAMAG). This diospyrin was used for developing the HPTLC method and as a standard for the analysis of diospyrin content in the stem bark samples.

2.3. Preparation of standard solutions

A stock solution of diospyrin (100 μg/ml) was prepared by dissolving an accurately weighed 10-mg amount of diospyrin in 100 ml of chloroform in a volumetric flask. Standard solutions of 10 μg/ml, 20 μg/ml, 30 μg/ml, 40 μg/ml, 50 μg/ml were prepared by transferring aliquots (1–5 ml) of stock solution to a 10-ml volumetric flask and adjusting the volume to 10 ml with chloroform.

2.4. Preparation of sample solution

An accurately weighed 1-g amount of powder of the stem bark of *D. montana* was extracted with chloroform (2×50 ml) under reflux for 2 h in a water bath. The flask was cooled and the extract filtered. The filtrates were pooled, transferred to a 100-ml volumetric flask and the volume was adjusted with chloroform, which constitutes the sample solution.

2.5. Calibration curve for diospyrin

A 10-μl volume of each of the standard solutions (100 ng, 200 ng, 300 ng, 400 ng, 500 ng per respective spot) was applied (band width: 5 mm, distance between the bands: 5 mm) on a precoated silica gel G60 HPTLC plate (E. Merck), 10 mm from the bottom edge, using a CAMAG Linomat IV automatic sample spotter. The plate was developed in a solvent system (8 ml) of toluene–ethyl acetate–cyclohexane–glacial acetic acid (6:1:1:0.1, v/v) in a CAMAG glass twin-trough chamber (10×10 cm) previously saturated with the solvent system for 30 min (temperature 25±2°C, relative humidity 40%). The development distance was 45 mm. After removing the plate from the chamber, it was dried in air and scanned and quantified at 445 nm using a CAMAG TLC scanner 3 and Cats 4 software. Data of peak area were recorded. A calibration curve was

obtained by plotting peak area vs. concentration of diospyrin applied.

2.6. Validation

The method was validated for precision, repeatability and accuracy. Precision of the instrument was checked by repeated scanning of the same spot of diospyrin (concentration: 300 ng) seven times and the coefficient of variation (CV) was calculated. The repeatability of the method was tested by analysing 300 ng/spot of standard solution of diospyrin after application on a TLC plate ($n=5$) and % CV was calculated. Variability of the method was studied by analysing aliquots of different concentrations of standard solutions of diospyrin (100 ng/spot, 300 ng/spot and 500 ng/spot) on the same day (intra-day precision) and on different days (inter-day precision) and relative standard deviation (RSD) was calculated.

Accuracy of the method was tested by performing the recovery studies at two levels by addition of 50% and 100% of diospyrin to one of the sample powders. To 1.4 g of stem bark powder (containing 5.05 mg of diospyrin), known amounts of standard diospyrin were added (2.5 mg and 5 mg) and extracted and estimated as described in Section 2.7. The percentage recovery as well as the average percentage recovery were calculated.

2.7. Estimation of diospyrin from D. montana stem bark

A 10-μl volume of sample solution was applied in triplicate on a precoated silica gel G60 HPTLC plate (E. Merck) with the CAMAG Linomat IV automatic sample spotter. The plate was developed and scanned. The peak areas were recorded. The amount of diospyrin present in the sample was calculated using the calibration curve for diospyrin.

3. Results and discussion

One of the major therapeutic areas where natural products have made a major impact on longevity and quality of life is in the chemotherapy of cancer. In fact, most of the important anticancer drugs are natural products, from plants or microorganisms [11]. In a series of explorations of indigenous medicinal plants for compounds with anticancer activity, diospyrin from *D. montana,* was found to be have significant tumour inhibitory activity [1,5]. Apart from anticancer activity, the compound was also found to possess antileishmanial and antiplasmodial properties [12,13]. A literature survey revealed that, there was no method reported for the estimation of diospyrin content from the plant material. Preliminary experiments showed that it was possible to separate diospyrin from other constituents in the stem bark extract by TLC.

Diospyrin was isolated from the stem bark of *D. montana* (yield: 0.225%, w/w, m.p.: 256–258°C) and the identity of the compound was established from the following spectral data:

UV: $\lambda_{max}=223$, 262 and 442 nm (CAMAG TLC scanner 3), λ_{max} in EtOH=222, 254 and 438 nm (Jasco UV–Vis spectrophotometer).

IR: 3400 (OH), 1662 & 1640 (C=O), 2800–2900 (C–CH).

LC–MS: 375 [M+1], 373 [M−1] (ca. molecular mass 374).

The UV, IR and mass (by LC–MS) spectral data were found to be comparable with the reported data for diospyrin [10].

The purity of the compound was established by the following: (1) the analysis by differential scanning calorimetry gave a single peak with a melting point of 257.34°C (ca. m.p. 256–258°C on melting point apparatus). (2) The TLC chromatogram showed a single peak (Fig. 1a). UV absorption spectra that were recorded (on the CAMAG TLC scanner 3) at the start, middle and end positions of the band completely overlapped (Fig. 2).

The isolated diospyrin was used for developing method for analysis of diospyrin in stem bark by HPTLC. Of the different solvent systems tried, toluene–ethyl acetate–cyclohexane–glacial acetic acid (6:1:1:0.1, v/v) was found to be the most suitable one. In this system, diospyrin was resolved ($R_F=0.64$) in the presence of other compounds in the sample extract (Fig. 1b). The band of diospyrin from the sample was confirmed by comparing the UV spectrum of standard band with the corresponding sample band on the CAMAG TLC scanner (Fig. 3).

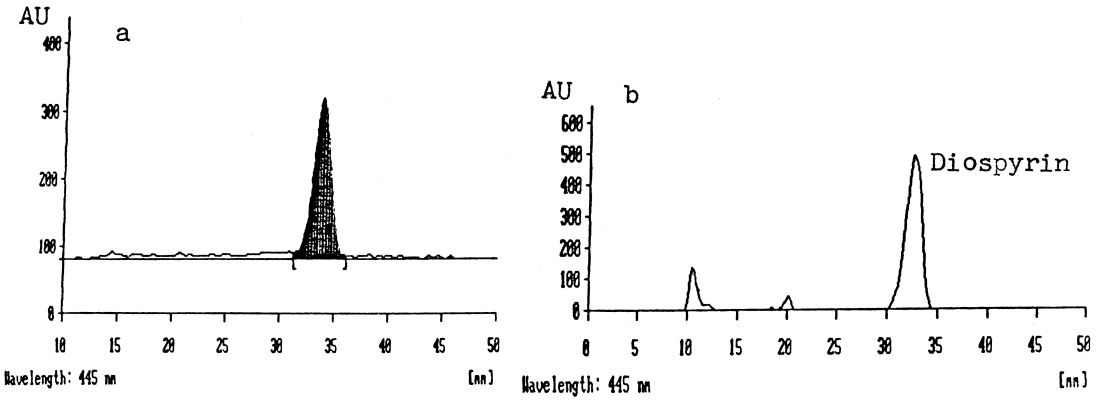

Fig. 1. TLC chromatogram of (a) diospyrin standard (100 ng per spot); (b) resolution of diospyrin in the sample.

3.1. Validation

The HPTLC method was validated in terms of precision, accuracy and repeatability (Table 1). The method is specific as it well resolved diospyrin with an R_F value of 0.64, in the presence of other

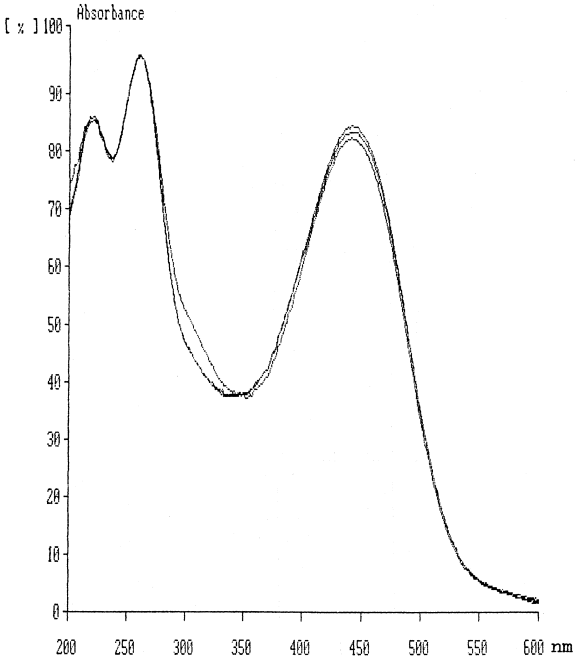

Fig. 2. Overlay spectra (at start, middle and end positions) of diospyrin band in absorption mode in the UV range, taken on the CAMAG TLC scanner 3.

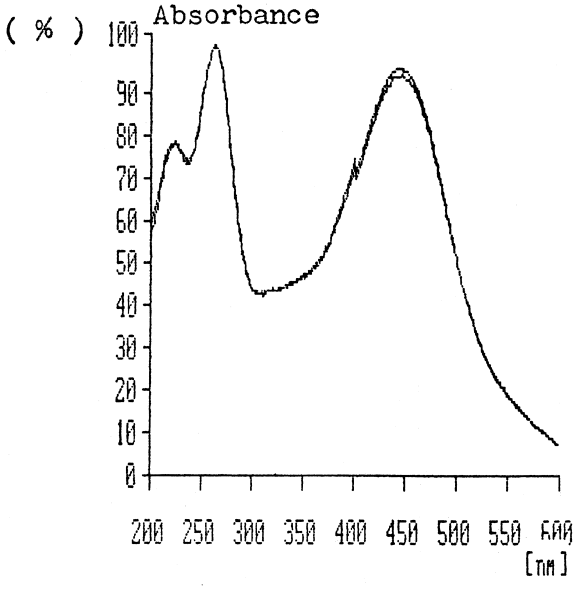

Fig. 3. Overlay spectra of diospyrin standard and diospyrin in sample in absorption mode in the UV range, taken on the CAMAG TLC scanner 3.

Table 1
Method validation parameters for the estimation of diospyrin by the HPTLC method

Serial No.	Parameter	Result
1	Instrumental precision (CV, %) (n=7)	0.086
2	Repeatability (CV, %) (n=5)	0.937
4	Limit of detection (ng/spot)	30
5	Limit of quantification (ng/spot)	100
6	Specificity	Specific
7	Linearity (correlation coefficient)	0.995
8	Range (ng/spot)	100–500

Table 2
Intra- and inter-day precision study

Concentration (ng/spot)	Intra-day precision (RSD, %, $n=3$)	Inter-day precision (RSD, %, $n=3$)
100	1.85	2.95
300	1.09	1.80
500	0.72	1.06

components in samples of stem bark. A linear relationship was obtained within the concentration range of 100–500 ng/spot for diospyrin with a correlation coefficient of 0.995. The instrumental precision was studied by repeated scanning of the same spot seven times (% CV=0.086). Repeatability of the method was tested by analysing the standard solution (300 ng/spot) five times (% CV=0.937). Variability of the method was studied by analysing aliquots of different concentrations on the same day (intra-day precision) and on different days (inter-day precision) and the RSD indicated that the method was precise. The results are shown in Table 2.

Accuracy of the method was determined at two levels (50% and 100% addition) by adding a known amount of diospyrin to the powder of stem bark and the mixture was analysed. The recoveries were found to be 97.59% and 98.15% at the two levels, respectively and the average recovery was 97.87% (Table 3).

3.2. Application of the method

The diospyrin content of three different samples of *D. montana* was estimated by the above validated HPTLC method (Table 4). The amount of diospyrin in these samples was found to vary from 0.35 to 0.47% (w/w). Further, diospyrin was isolated from the stem bark sample from Shimoga, the yield (0.225%, w/w) of which is also fairly comparable with the value obtained by the HPTLC method.

Table 4
Diospyrin content in different samples of *D. montana* by the HPTLC method

Sample[a]	Content of diospyrin[b] (%, w/w)
DM 1 (Shimoga)	0.364±0.0036
DM 2 (Sagar)	0.414±0.0059
DM 3 (Sringeri)	0.465±0.0017
DM 4 (Soraba)	0.346±0.0012

[a] DM=*D. montana* stem bark; source of the sample is in parentheses, $n=3$.
[b] Mean±SD.

4. Conclusion

The proposed HPTLC method was found to be simple, specific, precise, accurate and sensitive. It can be used for the estimation of diospyrin from plant material.

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Table 3
Recovery study of diospyrin by the HPTLC method ($n=3$)

Serial No.	Amount of diospyrin present in stem bark powder (mg)	Amount of diospyrin added (mg)	Amount of diospyrin found in mixture[a] (mg)	Recovery[a] (%)
1	5.05	2.5	7.368±0.051	97.59±0.68
2	5.05	5.0	9.864±0.035	98.15±0.35

[a] Mean±SD.

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