DEVELOPMENT OF CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF EXTRACTS OF *Phyllanthus maderaspatensis*

W.P.A. Dilushan[1], C. Ranasinghe[1*] and S.R. Weerakoon[2]
[1]Department of Chemistry, [2]Department of Botany,
The Open University of Sri Lanka, Sri Lanka

Phyllanthus maderaspatensis, is traditionally used in Indian medicine to treat various diseases. Phytochemical studies have identified compounds such as phyllanthin and hypophyllanthin, which exhibit hepatoprotective and antioxidant properties. Recent studies done using animal models suggest that methanolic extracts of *P. maderaspatensis* can effectively reduce cholesterol levels, indicating its potential as a natural treatment for hypercholesterolemia. This study focused on developing chromatographic methods (Thin Layer chromatography and High Performance Liquid Chromatography) for the analysis of *P. maderaspatensis* extracts to obtain chromatographic fingerprints/profiles which could be used to compare the phytochemical compositions of similar extracts of the plants from different geographical locations.

Dried whole plants were subjected to sequential extraction with hot hexane and methanol. The methanol extract was further fractionated to reduce the complexity. For Thin Layer Chromatographic (TLC) fingerprinting, 50% dichloromethane in hexane gave the best separation for the hot hexane extract.

The mobile phases which gave the optimal resolution for the dichloromethane, ethyl acetate and ethyl acetate insoluble fractions of the hot methanol extract were 100% dichloromethane, 5% methanol in ethyl acetate and 20% methanol in ethyl acetate, respectively. The High Performance Liquid Chromatographic (HPLC) analysis of the dichloromethane fraction under reverse phase conditions employing isocratic elution with acetonitrile and methanol (60:40) resulted in ten distinct compounds with clear peaks at 250 nm wavelength.

The TLC and HPLC methods developed in this study provided a reliable tool for comparing phytochemical compositions of similar extracts of the plants from different geographical locations.

Keywords: *Phyllanthus maderaspatensis, hypercholesterolemia, chromatographic analysis, phytochemical composition, HPLC fingerprinting*

** Corresponding Author: crana@ou.ac.lk*

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[1]Department of Chemistry, [2]Department of Botany,
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INTRODUCTION

Phyllanthus maderaspatensis, belonging to the family Euphorbiaceae, is known for its medicinal properties but remains underrecognized locally in Sri Lanka. Traditionally used in Indian medicine, it treats liver, kidney, and bladder ailments, and conditions such as bronchitis and Hepatitis B (Jung et al., 2015; Khatoon et al., 2006). Phytochemical analysis reveals that compounds like phyllanthin and hypophyllanthin, contributing to their hepatoprotective and antioxidant activities (Indira & Arumugam, n.d.). Hyperlipidemia, a major risk factor for atherosclerosis and heart diseases, is typically treated with statins, which have significant side effects. Studies conducted using Wistar rats have indicated that methanolic extracts of *P. maderaspatensis* can effectively reduce cholesterol levels, suggesting its potential as a natural and safer alternative for treating hypercholesterolemia (Dissanayaka et al., 2019).

This study is focused on developing methods for Thin Layer Chromatographic (TLC) and High-Performance Liquid Chromatographic (HPLC) fingerprinting of the extracts of *P. maderaspatensis*. This would be the preliminary step of a comparative study on phytochemical compositions of extracts of *P. maderaspatensis* collected from different geographical locations of Sri Lanka.

METHODOLOGY

Sample collection

Fresh plant parts were collected from ‘Gange Wadiya’ (8°15'28.7"N 79°51'51.7"E), a coastal region in Puttalam district. The specimens were identified and authenticated with the assistance of the national herbarium at the Royal Botanical Garden, Peradeniya, Sri Lanka. The collected sample was shade-dried to a constant weight, powdered with a granite mortar and pestle, and stored in airtight containers at room temperature.

Extraction procedures

The plant materials were subjected to two primary extraction processes.

Cold extraction: A sample of air-dried and powdered whole plants of *P. maderaspatensis* (13.42 g) was extracted with hexane (100 mL) by sonicating at room temperature (27–33 ℃, 9 hours). After decanting hexane, the residue was sonicated with methanol (100 mL) for 9 hours at regular intervals. Hexane and methanol extracts were concentrated under vacuum in a rotary evaporator.

Hot extraction: Air-dried and powdered whole plants of *P. maderaspatensis* (79.58 g) were subjected to sequential Soxhlet extraction with hexane (600 mL) at 68 °C for 24-30 hours and methanol (600 mL) at 65 °C for 24-30 hours. Extracts were evaporated to dryness in a rotary evaporator under a vacuum.

Fractionation of the hot methanol extract: Hot methanolic extract dissolved in aqueous methanol was further fractionated using dichloromethane (DCM) and the aqueous layer was further extracted with

ethyl acetate (EtOAc) to yield DCM fraction (DF) and EtOAc fraction (EF). The remaining EtOAc insoluble fraction was labeled as the 'methanol fraction' (MF). The solvents were evaporated under vacuum using a rotary evaporator to obtain dry extracts.

TLC comparison of hot and cold extracts

TLC was carried out on hot and cold, hexane and methanol extracts using precoated silica gel plates. Hexane was used as the developing solvent for the hexane extracts while 5% methanol in DCM was used for the methanol extracts.

TLC comparison of the hot hexane extract and the fractions of hot methanol extract

Different solvents and solvent combinations were tried out to get the best separation of each sample/extract on precoated TLC plates. TLC plates were visualized under UV illumination (254 nm and 365 nm) and by spraying anisaldehyde spray reagent. For the hexane extract, 50% hexane in DCM, for DF 5% EtOAc in DCM and for EF, 5% methanol in EtOAc were used as the developing solvents. For MF, 20% methanol in EtOAc was used.

HPLC analysis of the DCM extracts

High-Performance Liquid Chromatography (HPLC) was used to further analyze DF. HPLC analysis was carried out using a 1260 Infinity II HPLC system (Agilent technology), coupled with a 1260 DAD WR (DEAC607928) detector and Poroshell 120 EC-C18 (4.6x250 mm, 4 µm) column. The samples were filtered using a nylon membrane filter (0.45 µm, 47 mm). Data acquisition and processing were performed by ChemStation software (Agilent G2175BA). CT-2600 model UV-vis spectrophotometer was used to conduct UV-vis spectroscopy.

HPLC analysis coupled with diode array detection was conducted at room temperature. The detection wavelength was set at 250 nm wavelength. Optimization of chromatographic conditions was done by performing isocratic and gradient elution with different mobile phases.

RESULTS AND DISCUSSION

Dry weights and percentage yields of the hot and cold extracts of *P. maderaspatensis* are given in Table 1.

Table 1: Dry weights and percentage yields of the extracts

	Cold extracts		**Hot extracts**			
	Hexane	**Methanol**	**Hexane**	**DF**	**EF**	**MF**
Weight (g)	0.03 g	0.37 g	0.31 g	0.13 g	0.25 g	2.18 g
% yield	0.27%	2.76%	0.39%	5.04%	9.69%	84.50%

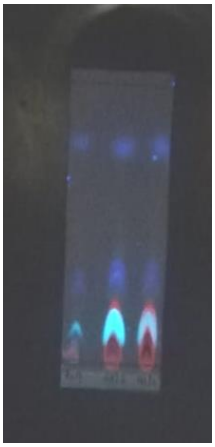
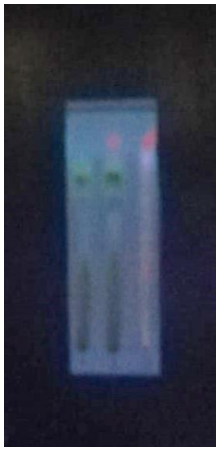
TLC comparison of Cold and Hot extracts

Compared to the hot extracts, yields of both the hexane and methanol cold extracts were very low. The pattern of spots given by the cold extracts on TLC showed the presence of fewer components in them in minor quantities than the hot extracts. Therefore, further analysis on cold extracts of *P. maderaspatensis* was not carried out.

TLC comparison of hexane extract and DF, EF and MF of methanol extract

The TLC fingerprints of hexane, DF, EF, and MF under UV illumination (365 nm) are given below in Table 2.

Table 2: TLC patterns of hot hexane and fractions of hot methanol extract observed under UV (365 nm)

Hexane extract Eluent: 50% DCM in hexane	**DF** Eluent: 100% DCM	**EF** Eluent: 5% methanol in EtOAc	**MF** Eluent: 20% methanol in EtOAc
			
R_f values of the spots: 0.062, 0.108, 0.246, 0.800	R_f values of the spots: 0.138, 0.338, 0.923	R_f values of the spots: 0.403, 0.731, 0.776	R_f values of the spots: 0.118, 0.309

HPLC analysis of the DF

The DCM fraction of the methanol extract of *P. maderaspatensis*, which carries the compounds of medium polarity, showed four major spots on TLC. This was selected for the HPLC analysis.

HPLC analysis validated the TLC findings and offered a finer resolution of phytochemical differences. Out of the several mobile phases tried out under gradient and isocratic elution, the optimal resolution was given by isocratic elution with acetonitrile: methanol (60:40). On the HPLC chromatogram, ten clear peaks due to pure compounds could be observed under a running time of less than 10 minutes. See Figure 1.

Peak no.	**Retention time**	**Area under the peak/units**
1	2 m 50 s	4.4465
2	3 m 03 s	15.1777
3	3 m 35 s	61.8975
4	4 m 17 s	5.5394
5	4 m 32 s	8.2231
6	5 m 50 s	1632.9144
7	6 m 08 s	167.6836
8	6 m 25 s	167.7157
9	6 m 36 s	173.5017
10	7 m 09 s	102.7032

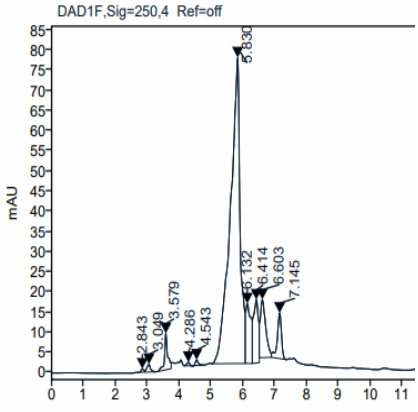


Figure 1: HPLC profile of the DCM fraction of the methanol extract of *P. maderaspatensis* and details of its peaks

CONCLUSIONS/RECOMMENDATIONS

The study demonstrated that hot extraction yielded a higher number of phytochemicals compared to cold extraction, making it more effective for TLC and HPLC analysis.

The HPLC method developed, successfully resolved the compounds present in the DCM fraction of the methanol extract to give ten clear peaks. HPLC fingerprints thus obtained can be used to compare the phytochemical compositions of similar extracts obtained from *P. maderaspatensis* collected from different geographical locations can be easily compared by using their HPLC profiles. However, for any quantification studies, this HPLC method should be validated by determining the parameters such as specificity, linearity, sensitivity, accuracy, precision and robustness.

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