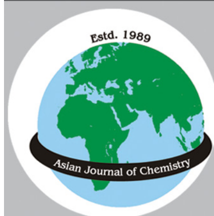

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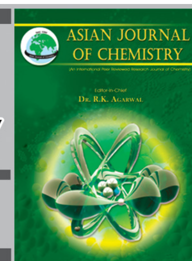

Development and Validation of a New HPTLC Method for Determination of Protopine in Accelerated Solvent Extract of *Fumaria vaillantii* Loisel

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A new, simple and accurate HPTLC method is developed using ICH guidelines for the determination of protopine in methanolic whole plant extract of *Fumaria vaillantii* Loisel. Accelerated solvent extraction (ASE) as an alternative to convention techniques was also explored for the rapid extraction. Chromatographic separation was performed on silica gel 60 F_{254} HPTLC plates in the mobile phase of methyl ethyl ketone:ethyl acetate: formic acid (5:3:2, v/v/v) and the plates were scanned densitometrically at 293 nm (λ_{max} of protopine). The amount of protopine was found in range 1.29-1.41 and 1.47-1.54 mg/g in Soxhlet and ASE 80 ºC extracted plant sample, respectively. The HPTLC method was validated in terms of sensitivity, accuracy, precision and reproducibility. Accelerated solvent extraction method has higher extraction efficiency in less time as compared to conventional Soxhlet extraction. Rapid and reproducible method is useful for routine analysis of protopine and quality control of *F. vaillantii*.

Key Words: Protopine, *Fumaria vaillantii* Loisel, Accelerated solvent extract, Methanol extract, HPTLC.

INTRODUCTION

Fumaria vaillantii Loisel., Fumariaceae [syn. *F. indica* (Haussk.) Pugsley] is an important medicinal plant known as 'Fumitory'. It is a major constituent of many common Ayurvedic and Unani medicinal preparations as well as marketed polyherbal liver formulations[1,2]. The plant has been evaluated for cardiovascular[3], hypoglycemic[4], antipyretic[5], antipsoriatic[6], hepatoprotective activity[7-9], anthelmintic[10], antidiarrhoeal[11] and antiinflammatory, antinociceptive activities[12]. It is a well known source of protopine (CAS-130-86-9), an isoquinoline alkaloid having diverse pharmacological activities like antiplatelet[13], antithrombotic, antiinflammatory[14], antispasmodic[15], anticholinesterase, antiamnesic[16], antidepressant[17] and hepatoprotective[9].

Because of its widespread use in various geographic regions, it is important to standardize whole plant of *F. vaillantii*. Although the HPTLC method for detection of protopine in *F. vaillantii* has been reported[9], the confirmation of effectiveness and validation of method is not yet available. This necessitated developing a new, low-cost and high throughout analytical method for quantification of protopine in quality control of *F. vaillantii*. Hence, the aim of this study was isolation, characterization of protopine and development of a simple, fast, accurate and sensitive HPTLC method for its determination in methanolic whole plant extract using ICH guidelines[18] and also assesses the possible application of accelerated solvent extraction (ASE) in extracting protopine.

EXPERIMENTAL

The whole plant of *F. vaillantii* was collected from wheat fields of Junnar Tehsil, Pune District (Maharashtra state, India) during winter season of 2008-2009. The plant sample was identified, authenticated and deposited in the crude drug repository of Agharkar Research Institute, Pune 411 004, vide voucher specimen number WP-058.

HPTLC plates were purchased from Merck, Darmstadt, Germany. Analytical grade solvents and regents were obtained from sd fine chemicals, Mumbai, India.

Isolation and characterization of protopine: Protopine was isolated from whole plant of *F. vaillantii*[19]. Freshly collected plant material was dried in shade and powered. The powdered material (500 g) was extracted with methanol (1000 mL × 3 mL) by cold percolation method. The extracts were filtered and the combined extract was concentrated *in vacuo* to give a dark green-brown gummy residue (16.6 g). The residue was dissolved in a mixture of water (300 mL) and concentrated hydrochloric acid (10 mL). The solution was kept in refrigerator (4 ± 2 ºC) for 3 days and filtered for removal of sediments. The acidic solution was extracted exhaustively with chloroform (300 mL × 5 mL) to separate chloroform soluble

hydrochloride. Chloroform extract was discarded. The filtrate was concentrated and repeatedly boiled with dilute hydrochloric acid (2 %, 30 mL) and the solution was extracted with ether to separate ether soluble portion. Ether extract was discarded and the acidic solution was then basified with excessive potassium hydroxide for precipitation. Precipitates were filtered off after standing for a while, washed with water and dissolved in a mixture of chloroform and methanol. Crude protopine (265 mg) was obtained by removal of solvent under reduced pressure. Repeated crystallization of the crude product by chloroform and methanol mixture was carried out to obtain pure protopine (112 mg). The isolated compound was analyzed by IR, UV and ^{1}H NMR and identified by comparison with the available spectral data[20].

Sample extraction procedure for HPTLC analysis

Soxhlet extraction: Accurately weighted (2.5 g) powdered whole plant sample was extracted exhaustively with methanol (50 mL) using Soxhlet apparatus for 360-600 min. The optimum yield was obtained in methanol with extraction time of 480 min. The extract was concentrated under reduced temperature and pressure using rotary evaporator. Concentrated extract (100 mg) were weighed out and dissolved in 5 mL of methanol (analytical grade), filtered through 0.45 μm membrane filters to get final sample solution and were subjected to HPTLC analysis for quantitative determination of the compound.

Accelerated solvent extraction: Plant material (5 g) was placed in the stainless-steel cell of a Dionex (Sunnyvale, CA, USA) ASE 100 accelerated solvent extractor and extracted with methanol. Extraction was performed at 100 bar at 60, 80 and 100 ºC, for 15 min (1 cycle). In the experiment at 80 ºC five replicate cycles were performed. Extract was concentrated under reduced pressure, dissolved in small portion of methanol and transferred to a 10 mL graduated flask. Concentrated extract (100 mg) was weighed out and dissolved in 5 mL of methanol (analytical grade), filtered through 0.45 μm membrane filters to get final sample solution and were subjected to HPTLC analysis for quantitative determination of the compound.

Chromatographic experiments: HPTLC was performed on aluminium backed HPTLC plates 10 cm × 10 cm with 0.2 mm layers of silica gel 60 F_{254} (E. Merck, Germany). Samples were applied the plate with band width 6 mm employing Linomat IV sample applicator (Camag, Switzerland) fitted with a microlitre syringe. Linear ascending development of the plates to a distance of 80 mm was performed with mobile phase methyl ethyl ketone:ethyl acetate:formic acid (5:3:2, v/v/v) in a twin-trough glass chamber previously saturated with mobile phase vapour for 10 min at 25 ºC. The dried plate was scanned at wavelength of 293 nm (λ_{max} of protopine) using a Camag TLC scanner 3 with CATS 4 software. A variety of mobile phases were tried for analysis of protopine in methanol extracts of whole plant. This included methyl ethyl ketone:ethyl acetate:formic acid (5:2.5:2.5, v/v/v), methyl ethyl ketone:ethyl acetate:formic acid (4:3:3, v/v/v), methyl ethyl ketone:ethyl acetate:formic acid (4:4:2, v/v/v).

Preparation of protopine standard solution and calibration plot: A stock solution of protopine (1 mg/mL) was prepared by dissolving 10 mg, accurately weighted protopine diluting to 10 mL methanol in volumetric flask. Working standard protopine solutions 5, 10, 15, 20, 25 and 30 μg/mL of different concentrations; 100, 200, 300, 400, 500 and 600 ng, respectively were prepared by diluting the stock solution.

Validation: The method was validated according to the ICH guidelines[18] by determining peak purity, limit of detection (LOD), limit of quantification (LOQ), instrumental precision, robustness, accuracy and repeatability of protopine from sample extracts. LOD and LOQ were determined by diluting known concentrations of standard stock solution until the average responses were approximately three or ten times the responses of blank. Instrument precision was checked by repeated scanning of protopine band (400 ng) and was expressed as relative standard deviation (RSD, %). Precision was studied by analyzing six bands of sample solution per plate on three plates (intra-day precision) and by analyzing six bands of sample solution per plate on three consecutive days (inter-day precision) at three different amount (300, 400, 500 ng) and calculated % RSD. Repeatability was tested by analyzing the bands of protopine after application of solutions to the plate (n = 3) and was expressed as RSD (%). The specificity of the method was determined by acquiring the *in situ* reflectance spectrum of protopine standard and the corresponding peak in the test samples, in the range 200-400 nm, by means of a reflectance spectrometer. The spectra were completely superimposable, confirming the purity of the peaks obtained from the test solutions. The accuracy of the method was tested by determination of recovery at three levels, after addition of 50, 100 and 150 % protopine to the sample. The protopine content was quantified and the percentage recovery was calculated. The robustness of the method was studied at three different concentrations - 300, 400, 500 ng/band protopine by introducing small deliberate changes in mobile phase composition methyl ethyl ketone:ethyl acetate:formic acid (4.8:2.8:2.1, 5.2:2.8:2.2, 5.1:2.9:2, v/v/v). LOD and LOQ were determined by the standard deviation (SD) method from the slope (S) of the calibration plot and the SD of a blank sample (blank methanol was spotted three times), by use of the equations LOD = 3.3 × SD/S and LOQ = 10 × SD/S.

Analysis of protopine in *F. vaillantii* extracts: Test solution of extract was applied in replicates on silica gel F_{254} HPTLC plates. The plates were developed and scanned using predetermined conditions for quantification of protopine in each extract. The amounts of protopine present in samples were calculated.

RESULTS AND DISCUSSION

The melting point of compound was 208 ºC in agreement with reported data[20]. The infrared spectrum contained sharp peaks at 2900 (CH stretching), 1720 (C=O), 1560, 1450 (aromatic) cm^{-1}. In the UV spectrum absorbance maxima was observed at 293 (log ε = 3.28). The ^{1}H NMR spectrum contained peaks at δ 1.1-1.6 (8H, m, C_7H, $C_{10}H$, $C_{11}H$, $C_{12}H$), 1.91 (3H, s, $C_{20}H$), 5.94 (1H, s, $C_{18}H$), 5.96 (1H, s, $C_{19}H$), 6.66 (1H, s, C_4H), 6.67 (1H, d, *J* = 7.6 Hz, $C_{14}H$), 6.70 (1H, d, *J* = 7.6 Hz, $C_{15}H$), 6.89 (1H, s, C1H), in agreement with reported data[20]. The molecular formula and molar mass was $C_{20}H_{19}NO_5$

and 353, respectively. These results were confirmed that the isolated compound was protopine.

The HPTLC procedure was optimized to quantify protopine from whole plant extract of *F. vaillantii*. Accelerated solvent extraction (ASE) was studied for the development of rapid sample preparation method, along with Soxhlet extraction. Accelerated solvent extraction has been applied for the first time for the extraction of protopine in whole plant of *F. vaillantii*. Accelerated solvent extraction at 80 ºC gave almost the same results for 15 min as Soxhlet for 480 min. Of the various mobile phases tried, the one containing methyl ethyl ketone:ethyl acetate:formic acid (5:3:2, v/v/v) gave optimum results with sharp, symmetrical and well resolved peak of protopine at R_f 0.36 in presence of other compounds in the sample extracts (Fig. 1). A linear relationship was obtained between response (peak area) and amount of protopine in the range 100-600 ng/spot (n = 3); the correlation coefficient was 0.9947 with the standard error of mean 0.0021. No significant differences were observed in the slopes of the standard curve. The amount of protopine was found in range 1.29-1.41 and 1.47-1.54 mg/g in Soxhlet and accelerated solvent extraction at 80 ºC extracted plant sample, respectively. Compound yield of Soxhlet extraction was found to be almost similar efficiency. However, taking into consideration the time of extraction and solvent consumption, accelerated solvent extraction proves to be a promising alternative to Soxhlet.

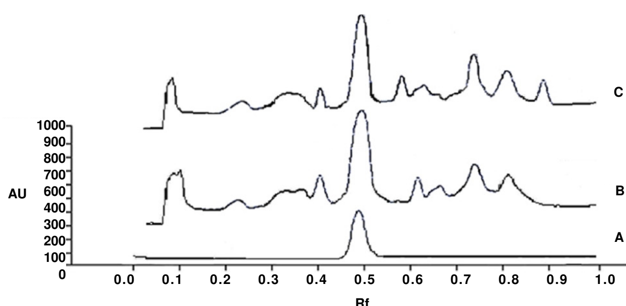

Fig. 1. HPTLC profiles (overlay) of extracts of *F. vaillantii* with protopine as marker. A. Standard protopine; B. Soxhlet extraction-whole plant methanol extract; C. ASE 80 ºC-whole plant methanol extract. Mobile phase-methyl ethyl ketone: ethyl acetate:formic acid (5:3:2, v/v/v), detection: 29

The method was validated in terms of peak purity, precision, LOD, LOQ and accuracy (Tables 1-3). The method was specific for analysis of active principle protopine because it resolved the compound at R_f 0.36. The purity of the protopine peak was checked from the samples by recording UV spectra.

TABLE-1
VALIDATION DATA OF HPTLC METHOD FOR THE ESTIMATION OF PROTOPINE

Parameter	Value
Instrumental Precision (% RSD, n = 6)	0.63
Calibration range (ng/spot)	100-600
Correlation coefficient	0.9947
Repeatability of standards (% RSD , n = 6)	0.67
Repeatability of samples (% RSD, n = 6)	0.93
Limit of detection (LOD) (ng/spot)	33.34
Limit of quantitation (LOQ) (ng/spot)	100.78
Robustness (% RSD, n = 3)	0.81

TABLE-2
RESULTS FROM STUDY OF INTRA-DAY AND INTER-DAY PRECISION FOR PROTPINE

Concentration (ng/band)	Intra day (% RSD, n = 6)	Inter days (% RSD, n = 6)
300	1.09	0.94
400	0.89	0.83
500	1.18	1.23

The identified protopine spot was confirmed from samples extract by overlaying UV absorption spectrum of samples with standard at 293 nm (λ_{max} of protopine). Instrument precision was studied by scanning the same spot of protopine six times (% RSD = 0.63). Intra- day and inter-day precision were studied by triplicate assay of three different concentrations of protopine (300, 400 and 500 ng/band) on the same day and on different days. Low RSD values (Table-2) indicated the method was precise. Small changes in mobile phase composition had no significant effect on the chromatography. The low RSD values of the peak areas calculated indicate the robustness of the method (Table-1). The accuracy of the method was determined at three levels (50, 100 and 150 %) by adding known amounts of protopine to samples extract. Recovery of protopine at the three levels is represented in Table-3. High recovery indicated the proposed method was reliable and reproducible. The LOD and LOQ were 33.34 and 100.78 ng/spot, respectively.

A new, simple, sensitive and rapid HPTLC method is first time reported for estimation of protopine in whole plant methanol extract of *F. vaillantii*. Comparison of the extraction yields of the protopine reveals ASE is a simple and efficient method which helpful for standardization and routine quality control of raw materials and herbal products containing *F. vaillantii* whole plant as an ingredient. It provides significant advantages in terms of greater specificity and rapid analysis.

TABLE-3
RECOVERY STUDY OF THE METHOD FOR PROTOPINE

Compound	Amount of protopine in sample [ng][a]	Amount of protopine added [ng][a]	Amount of protopine found in mixture [ng][a]	Recovery (%)[a]	Average recovery (%)[a]
Soxhlet extraction-whole plant methanol extract					
Protopine	117 ± 5.34	58.2	167.9 ± 4.33	95.66	94.64
	117 ± 5.34	117	215.5 ± 5.87	92.09	
	117 ± 5.34	175.5	281.3 ± 4.03	96.17	
ASE 80 ºC-whole plant methanol extract					
Protopine	117 ± 5.34	58.2	163.2 ± 3.98	92.99	94.12
	117 ± 5.34	117	220.7 ± 4.45	94.31	
	117 ± 5.34	175.5	277.9 ± 4.14	95.06	

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