SUPPLEMENTARY MATERIAL

Optimization of column chromatography technique for the isolation of tetrandrine from *Cyclea peltata* and LC-ESI-MS based quantification and validation of the method

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Abstract

Reverse phase column chromatography technique was employed to isolate tetrandrine from methanolic extract of *Cyclea peltata* roots. Tetrandrine was efficiently isolated using 0.02% aqueous diethyl amine and methanol (25:75, v/v) as mobile phase with a purity of 98.63%. Compound characterisation was achieved using high-performance liquid chromatography coupled with electrospray ionization mass spectrometry and other spectroscopic methods. In addition, quantification of tetrandrine and validation of the method was carried out following International Conference on Harmonisation guidelines. The study provides a simple and cost effective method to isolate substantially good quantity and pure tetrandrine from *Cyclea peltata.*

Keywords: *Cyclea peltata*; tetrandrine; column chromatography; LC-ESI-MS

Experimental

A. Chemicals, consumables and instrumentation

LC grade methanol, diethylamine and water were obtained from Merck – Lichrosolv grade (Merck, India). Standard tetrandrine was procured from Sigma – Aldrich, India with ≥90% purity and considered as 100% pure throughout the experiment. All other chemicals used in the experiment were of analytical grade and obtained from Merck, India. The glass wares were of Borosil Company (Borosil, India) and LCMS sample vials were purchased from Spinco Biotech (Spincotech, India). Nylon filters with pore size of 0.22 µm were purchased from Himedia (Himedia, India). Glass column was purchased from Vensil (Vensil, India) and silica gel used in the column chromatography was with a mesh size of 100-200, obtained from Sisco Research Laboratory (SRL, India). Thin layer chromatography (TLC) was performed using Silica gel 60 F 254, 200 µm (Merck, India).

LC-ESI-MS analysis was carried out by scan (*m/z* rage: 100-1000) and single ion monitoring (SIM) methods using Shimadzu LCMS 8030 (Shimadzu, Japan) with electrospray ionization (ESI) in positive and negative modes. The instrument was equipped with an autosampler, HPLC pump and UV Detector. The column used was C_{18}G Enable column (250 x 4.6 mm, 5 µm), operated at 40 ºC. The injection volume of the sample was 1µL and the flow rate was 1 mL/min and nitrogen as nebulising gas flow at a rate of 3 L/min. Fragmentation study of the compound was carried out using argon as collision induced dissociation gas (CID gas) at a rate of 230 kPa and the experiment was carried out as per the manufacturer's instruction. Analytical condition of fragmentation study was optimized using multiple reaction monitoring (MRM) mode to obtain the *m/z* of precursor, products, Q1 Pre Bias, CE and Q3 Pre Bias energy.

Fourier transform-infrared spectroscopy (FT-IR) analysis was performed (Shimadzu IR Prestige 21, Japan) in the range of 3000-400/cm. Differential scanning calorimetry (DSC) was performed using Waters, SDT Q600 V20.9 Build 20 (US). NMR spectra for the isolated compound in DMSO were measured at room temperature with tetramethylsilane as an internal reference standard using Bruker AV 400 (US). The UV absorption maximum of the compound was recorded by UV-Vis spectrophotometer-1800 (Shimadzu, Japan).

B. Collection of plant material and extraction of alkaloids

Roots (approximate diameter of 1 cm and above) of flowered *C. peltata* plants were collected from the natural forests of Dakshina Kannada Districts of Karnataka, India (co-ordinate value: 12.8438 ° N, 75.2479 ° E) and, Kasaragod District of Kerala, India (co-ordinate value: 12.5102 ° N, 74.9852 ° E). The plant was identified following the Flora of Madras Presidency (Gamble. 1958) and the voucher specimen (MU/AB/BN-02) has been deposited at the herbarium of Department of Applied Botany, Mangalore University, Karnataka, India.

Plant material (1 kg) was washed, dried under shade, powdered to obtain particle diameter if 1-2 mm using lab mill, extracted in 100% methanol at 50 ºC for 2 h, cooled and the extract was collected (Carroll. 2011). Further extraction of the residue was carried out for five times, all the extracts were pooled, concentrated to thick oil at 60-80 ºC and acidified to pH 1 using conc. HCl. The acidic solution was filtered, cooled and the pH was raised to 11-12 using 50% sodium hydroxide. Obtained precipitate was dried at 60-80 ºC, re-extracted 4-5 times in toluene. Toluene soluble fraction was collected, concentrated to dryness at 60 ºC, redissolved in acetone at 50 ºC, and crystallised by slow cooling process (0-10 ºC). The presence of alkaloids in the sample was later detected by TLC with mobile phase of chloroform and methanol (9:1, v/v) and Dragendorff's reagent as detecting agent.

C. Column chromatography for the isolation of tetrandrine

Tetrandrine present in the extract was further isolated using 150 mm glass column packed with 5 g of silica gel (100-200 mesh size) as the stationary phase in 40% aqueous methanol. About 0.5 g of sample in methanol was loaded to the column by dry loading method and eluted with 0.02% aqueous diethylamine-methanol (25:75, v/v) mixture as mobile phase. A total of six different fractions were collected (1-35 mL – fraction 1; 36-50 mL – fraction 2; 51-150 mL – fraction 3; 151-160 mL – fraction 4; 161-190 mL – fraction 5 and 191-210 mL – fraction 6) and dried. Each dried fractions were tested for their purity using TLC as given in section 2.2. The fraction showing single spot in TLC was used for repeated recrystallisation in acetone and the yield was expressed in g/kg (Mean ± SD) of the sample taken. The method was repeated independently for

three times and the pure crystals were used for the compound characterisation and validation of the method.

D. Characterisation of the compound

Characterisation of the isolated compound was carried out using different spectroscopic studies and the data were compared with the available literature to confirm the identity of the compound as well as to determine its purity. LC-ESI-MS/MS analysis for the isolated compound was performed by scan and SIM methods (UV detector λ_{max}: 282) to detect and confirm the *m/z* value of 622. Fragmentation study of the compound was carried out by MRM mode to standardise and confirm the *m/z* of precursor, products, Q1 Pre Bias, CE and Q3 Pre Bias energy.

Further characterisation of the compound was achieved using other spectroscopic studies. FT-IR analysis of the compound (1 mg/mL) to detect the functional groups, DSC (2-5 mg of compound) to find out the melting point and heat flow, UV absorption maxima of the compound in methanol (1 mg/mL) and ^{1}H and ^{13}C NMR spectra in DMSO were measured. All the spectral data recorded in the present study were further compared with the reported literature for standard tetrandrine.

E. Quantification and validation of the method

Presence of tetrandrine in methanol extract and toluene soluble acetone crystallised fraction were quantified by LC-ESI-MS using standard tetrandrine (Sigma – Aldrich, India) linearity curve over the concentration range of 25 to 125 µg/mL. Entire experiment was repeated independently for three times and the concentrations of tetrandrine were expressed in g/kg (Mean ± SD) of the root sample taken.

Validation of the method for the isolation of tetrandrine was performed following International Conference on Harmonisation (ICH) guidelines (Shabir. 2004). Linearity curve for isolated tetrandrine was prepared over a concentration range of 25-125 µg/mL from a stock solution (2 mg/mL of isolated tetrandrine in methanol) to assess the assay range for the isolated tetrandrine and the LC-ESI-MS analysis was carried out using methanol as mobile phase. The experiment was repeated for three times. The calibration curve was constructed using peak area versus concentration of isolated tetrandrine (25, 50, 75, 100, 125 µg/mL) and the linearity was

assessed based on regression coefficient by considering R^2 = 0.99 as acceptable fit. Relative content of tetrandrine in isolated compound was performed in triplicate on "assay as is basis" (100 µg/mL of the isolated compound, 100 µg/mL of standard tetrandrine, n=3) and the purity of the compound was analysed based on area normalisation method for the LC peak area.

Intra-day and inter-day precision were calculated as the relative standard deviation (R.S.D.) at target concentration (100 µg/mL, n=3), and assay was assessed and expressed in percentage. The stability of the dissolved sample in methanol was tested by storing the sample solution at 25 °C for 0, 24 and 48 h and by determining the content from the freshly prepared tetrandrine sample. The stability of the isolated compound was expressed as percent deviation in concentration with an acceptable limit of ≤15% to that of freshly prepared sample. Limit of detection (LOD) and Limit of Quantification (LOQ) for the isolated tetrandrine were performed with a linear concentration of 0.1- 125 µg/mL (n=3) of the sample and calculating the LOD and LOQ values based on standard deviation of response and slope of the signal.

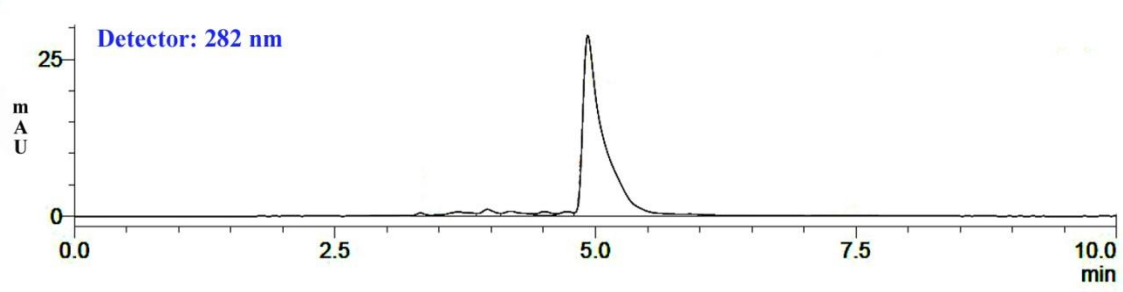

Figure S1. HPLC chromatogram of the isolated compound showing a single sharp peak indicates the purity of the compound.

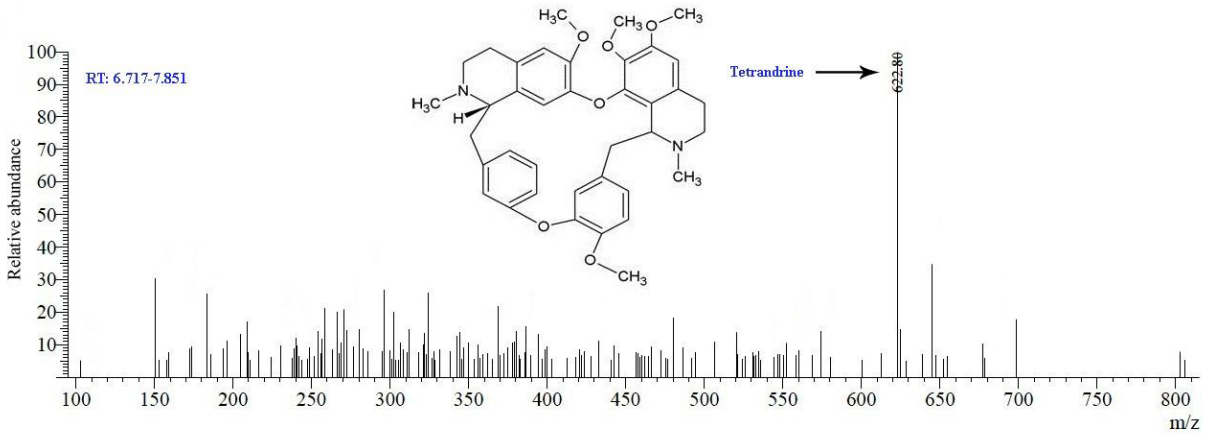

Figure S2. LC-ESI-MS spectrum of isolated compound showing *m/z* 622.8 correspond to tetrandrine. Chemical structure of tetrandrine is given inside the mass spectrum.

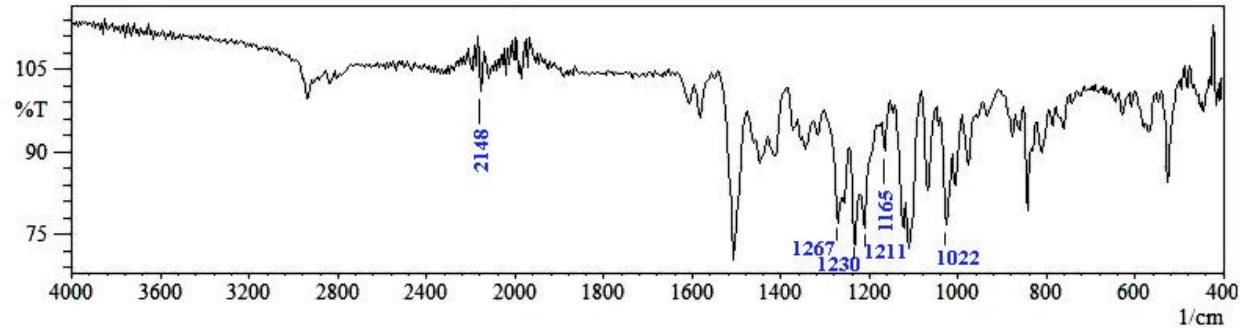

Figure S3. FT-IR analysis of isolated compound exhibits absorption bands for C-O (1022-1267 cm^{-1}) groups.

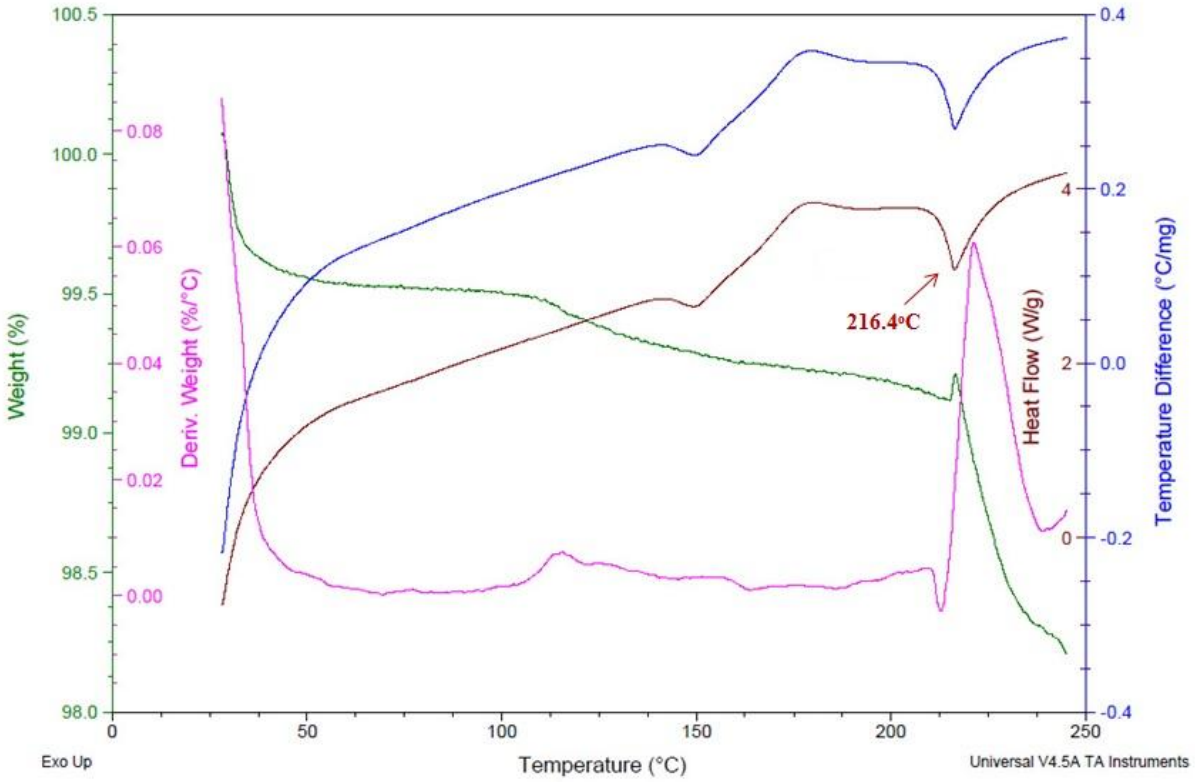

Figure S4. DSC profile of isolated compound showing a sharp endothermic peak at 216.4 °C.

Table S1. ^{1}H NMR (400MHz) and ^{13}C NMR (100MHz) spectroscopic data of isolated tetrandrine. ^{1}H and ^{13}C chemical shifts were expressed in ppm.

No.	^{1}H NMR	^{13}C NMR
1	3.50 (d, J = 9.6 Hz, 1H)	61.08
NCH_3	2.49 (s, 3H)	41.92
3-α	2.83 (m, 1H)	44.73
3-β	3.40 (m, 1H)	-
4- α	2.58 (m, 1H)	21.07
4- β	2.84 (m, 1H)	-
5	6.30 (d, J = 6.8 Hz, 1H)	106.29

6	-	148.87
7	-	137.42
8	-	147.87
9	-	135.62
10	6.60 (s, 1H)	115.38
11	-	150.90
12	-	147.64
13	6.76 (d, J = 8.4 Hz, 1H)	111.99
14	6.90 (d, J = 8.0 Hz, 1H)	122.82
1′	3.89 (dd, J = 10.0 Hz, 5.6 Hz, 1H)	62.60
N′CH_3	2.49 (s, 3H)	42.21
3′-α	2.81 (m, 1H)	43.22
3′-β	3.39 (m, 1H)	-
4′- α	2.64 (m, 1H)	25.15
4′- β	2.67 (m, 1H)	-
5′	6.38 (s, 1H)	112.82
6′	-	147.65
7′	-	143.18
8′	5.90 (s, 1H)	119.56
9′	-	134.29
10′	6.35 (s, 1H)	132.48
11′	6.66 (d, J = 8.0 Hz, 1H)	122.37
12′	-	152.87
13′	7.07 (d, J = 8.0 Hz, 1H)	121.43
14′	7.43 (d, J = 8.0 Hz, 1H)	130.43
α1	2.36 (d, J = 16.0 Hz, 1H)	41.46
α2	2.28 (d, J = 13.2 Hz, 1H)	-
α′1	2.75 (m, 1H)	39.90
α′2	3.17 (dd, J = 12.4 Hz, 6.0 Hz, 1H)	-
4a	-	128.20

8a	-	127.76
4′a	-	128.20
8′a	-	128.62
6-OCH_3	3.66 (s, 3H)	55.54
7-OCH_3	3.01 (s, 3H)	59.33
12-OCH_3	3.79 (s, 3H)	55.65
6′-OCH_3	3.27 (s, 3H)	55.61

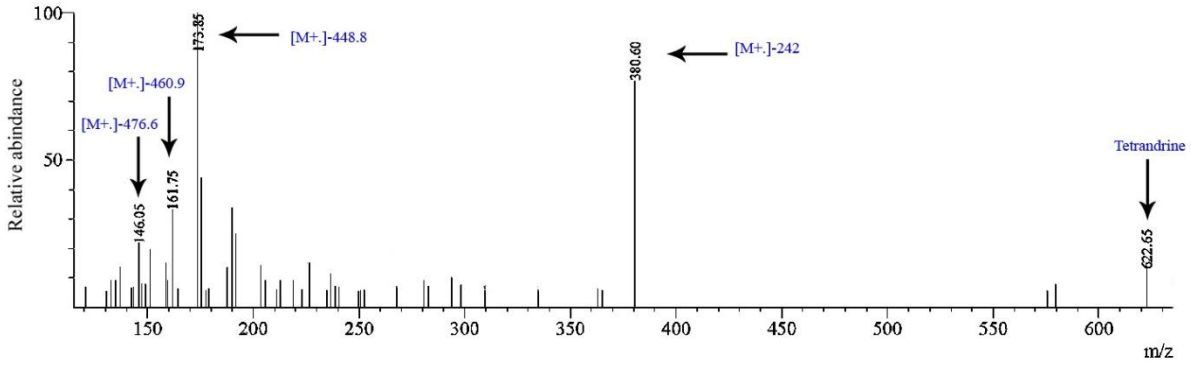

Figure S5. Fragmentation study of isolated compound showing four major fragment peaks at *m/z* 380.6, 173.85, 161.75, 146.05 and a small peak of tetrandrine at *m/z* 622.65.

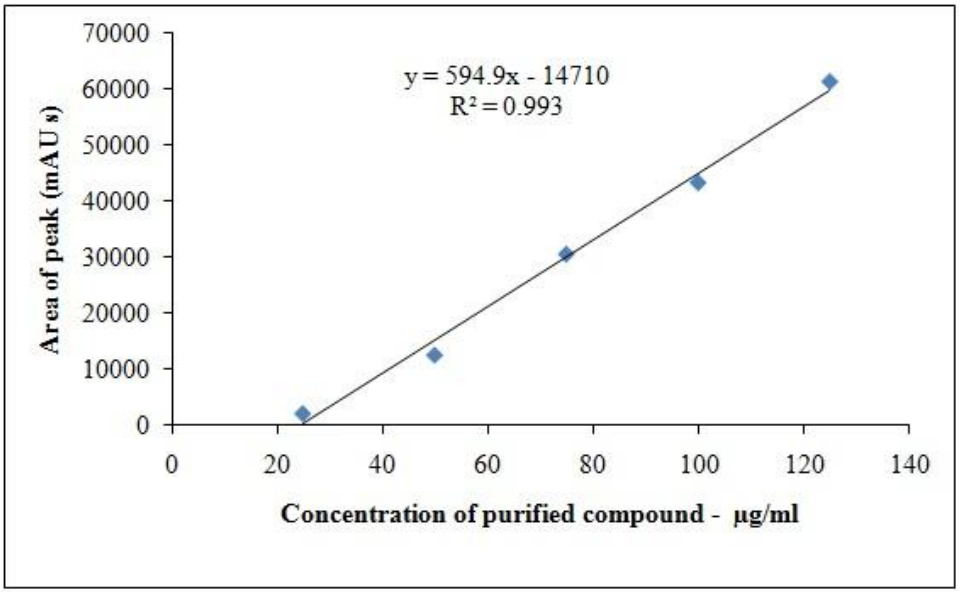

Figure S6. Linearity curve of isolated tetrandrine showing proportional relationship of response versus analyte concentration over the working range of 25-125 µg/mL.

Table S2 Assay on as is basis and intra and inter –day precision of isolated tetrandrine

Standard tetrandrine (100 µg/mL)	Isolated compound (100 µg/mL)							
	Assay on as is basis				Intra and inter–day precision			
Average peak area (mAU s)	Trial	Peak area (mAU s)	Relative content (%)	% purity	Day	Relative content (%)	SD	RSD %
30240	1	26804	88.64	91.01	1	92.94	4.06	4.3
	2	28260	93.45	95.50				
	3	29248	96.72	95.88				
		Mean	92.94	94.13	2	80.45	1.73	2.15
		SD	4.06	2.31				
		RSD%	4.3	2.4				

Table S3 Stability of isolated tetrandrine

	0 hr				24 hr				48 hr			
	RT (min)	Peak area (mAUs)	Peak height (mAU)	Relative content (%)	RT (min)	Peak area (mAUs)	Peak height (mAU)	Relative content (%)	RT (min)	Peak area (mAUs)	Peak height (mAU)	Relative content (%)
Mean	7.523	25792.8	604.4	84.65	7.988	23434.6	502.3	81.59	8.246	23032.8	509.6	81.53
SD	0.098	2112.1	50.27	5.8	0.144	2412.1	43.35	9.2	0.305	2033.19	141.1	7.0
RSD%	1.3	8.2	8.3	6.8	1.8	10.2	8.6	11.3	3.7	8.8	25.7	7.4

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