4. IDENTIFICATION OF NATURAL PRODUCT LEAD AND ITS VALIDATION

Chapter IV

4. Identification of Natural Product Lead and its Validation

4.1. Preliminary Screening

Mother earth is comprised of a large reservoir of natural products that has provided many drugs or drug candidates. Hence, there is more perspective of identifying drug and drug candidates from the natural products resources [1,2]. The same trend is also be visible in the case of obesity, especially in the area of PL inhibitors [3,4]. In the recent scenario, the natural products derived PL inhibitors are gaining great attention in the scientific community owing to their large availability and the structural diversity etc. Approximately >800 natural products derived from various natural sources have been investigated for their PL inhibitory potential [4,5]. Orlistat (a commercially available PL inhibitor) a natural product inspired drug has further increased the curiosity in the scientific community for the search of PL inhibitors from the natural sources.

As a part of the continuous search for potential PL inhibitors from natural products, a pool of Indian *Ayurvedic* medicinal plants were selected for the preliminary screening [6]. The criteria involved in the selection of plants for PL inhibition screening mainly relied on their traditional *Ayurvedic* usage and pharmacological studies on obesity (medoroga) and its associated complications. In total, a pool of 15 Indian medicinal plants were selected (Table 4.1) and authenticated (Voucher No: NIP-NPM-CD-235 to CD-250) by Dr. A.S Sandhu, Department of Natural Products, NIPER, SAS Nagar, Mohali. Selected plant materials were dried, powdered, sieved and subjected to sequential extraction (using n-hexane followed by methanol) with three extraction techniques (maceration, continuous hot percolation and ultrasound-assisted extraction) to obtain 90 plant extracts. The PL inhibitory potential of these extracts was evaluated by *in-vitro* PL inhibition assay and the obtained results are summarized in **Table 4.1**.

Screened extracts exhibited potential to poor PL inhibitory activity profile. Amongst the screened extracts, methanol extract of *Alstonia scholaris* stem bark prepared *via* continuous hot percolation exhibited comparatively greater potential (IC_{50} = 12.85 µg/mL) amongst all the screened extracts. Hence, it was selected for further investigation.

Table 4.1. PL inhibitory activity of 90 extracts obtained from 15 plants

		IC_{50} (µg/mL)*					
		Hexane			**Methanol**		
#	Plant (Part)	**Maceration**	**Hot percolation**	**Ultrasound assisted extraction**	**Maceration**	**Hot percolation**	**Ultrasound assisted extraction**
1	*Holarrhena antidysentrica* (Stem bark)	90.63 ± 2.49	19.08 ± 1.04	29.72 ± 0.50	95.38 ± 3.23	20.67 ± 2.48	25.13 ± 0.87
2	*Acacia catechu* (Bark)	92.99 ± 1.55	54.00 ± 1.73	69.98 ± 2.03	85.61 ± 1.69	29.17 ± 1.61	66.61 ± 2.63
3	*Acacia catechu* (Heartwood)	79.03 ± 2.89	74.87 ± 1.04	64.05 ± 1.77	65.40 ± 1.64	30.20 ± 0.45	56.56 ± 1.94
4	***Alstonia scholaris* (Bark)**	40.95 ± 0.98	33.01 ± 1.26	37.61 ± 0.96	45.54 ± 5.10	**12.85 ± 1.57**	28.38 ± 5.11
5	*Ficus glomerata* (Bark)	86.45 ± 3.23	41.84 ± 1.89	51.74 ± 1.23	50.81 ± 2.13	24.45 ± 1.10	36.46 ± 1.31
6	*Ficus glomerata* (Fruits)	80.69 ± 1.57	71.79 ± 2.54	49.40 ± 0.68	74.41 ± 2.99	20.28 ± 0.40	40.01 ± 1.38
7	*Piper longum* (Fruits)	99.87 ± 1.28	42.97 ± 1.47	51.40 ± 1.12	56.49 ± 3.98	26.30 ± 0.96	14.10 ± 0.70
8	*Terminalia chebula* (Fruits)	63.34 ± 1.79	27.60 ± 1.27	27.05 ± 1.31	34.22 ± 2.60	23.62 ± 1.65	20.89 ± 1.13
9	*Thea sinensis* (Leaf)	52.47±1.47	31.66 ± 1.06	27.80 ± 1.02	28.77 ± 1.04	21.49 ± 1.21	20.80 ± 1.16
10	*Tinospora cordifolia* (Stem)	80.25 ± 0.28	61.37 ± 2.17	59.32 ± 0.74	73.67 ± 2.02	23.29 ± 0.57	42.08 ± 1.37
11	*Cuminum cymenium* (Fruits)	34.78 ± 0.75	17.54 ± 0.93	37.03 ± 2.33	68.33 ± 2.03	44.05 ± 1.13	49.50 ± 1.82
12	*Tribulus terrestris* (Fruits)	72.83 ± 1.26	45.87 ± 2.04	37.59 ± 1.92	51.53 ± 2.40	39.10 ± 1.57	15.70 ± 0.78
13	*Adathoda vasica* (Leaf)	73.91 ± 0.91	57.80 ± 2.89	66.24 ± 2.51	73.43 ± 1.37	37.28 ± 1.70	34.31 ± 1.40
14	*Curcuma longa* (Rhizomes)	67.86 ± 0.98	37.35 ± 1.58	33.30 ± 0.76	59.57 ± 1.53	21.64 ± 1.13	23.76 ± 1.18
15	*Garcina indica* (Fruits)	60.68 ± 1.17	50.40 ± 2.51	51.27 ± 2.43	57.00 ± 3.10	36.23 ± 1.96	33.33 ± 1.68

***** All experiments were performed in triplicate and the values are expressed as mean ± S.E.M.

4.2. Literature Review of *A. scholaris* (L.) R. Br.

The genus *Alstonia* (Apocynaceae) is comprised of more than 60 species [7]. *Alstonia scholaris* (L.) R. Br., also known as Saptaparna, is a large, evergreen tree, with a height of 6-10 m, native to the Indian sub-continent and South Asia. A detailed taxonomical classification of *A. scholaris* is summarised in **Table 4.2**. The bark of *A. scholaris* has a grey to a whitish rough surface **(Fig. 4.1)**, that exudate bitter latex when injured. 'Bhavaprakasha', an ancient *Ayurvedic* text has also explained the medicinal importance of *A. scholaris*. Traditionally it has been used to cure metabolic disorders (diabetes, hypertension), ulcers, skin diseases including bleeding ulcers, asthma, phantom tumors etc [8,9].

Table 4.2. Taxonomical classification of *A. scholaris*

Kingdom:	Plantae	**Subclass:**	Asteridae
Subkingdom:	Viridiplantae	**Superorder:**	Asteranae
Infrakingdom:	Streptophyta	**Order:**	Gentianales
Division:	Tracheophyta	**Family:**	Apocynaceae
Subdivision:	Spermatophytina	**Genus:**	*Alstonia*
Class:	Magnoliopsida	**Species:**	*A. scholaris* (L.) R. Br.

Fig. 4.1. Representative image of *A. scholaris* leaves and stem barks

Previous phytochemical studies of the stem bark resulted in the isolation and identification of various bioactive molecules belonging to various chemical classes such as alkaloids, terpenoids, phlobatanins, steroids, saponins and simple phenolics etc (**Fig. 4.2**). These natural products have diverse pharmacological activities such as antimalarial, antidiabetic, analgesic, anti-inflammatory, lipid lowering and cytotoxicity etc. [8,10,11].

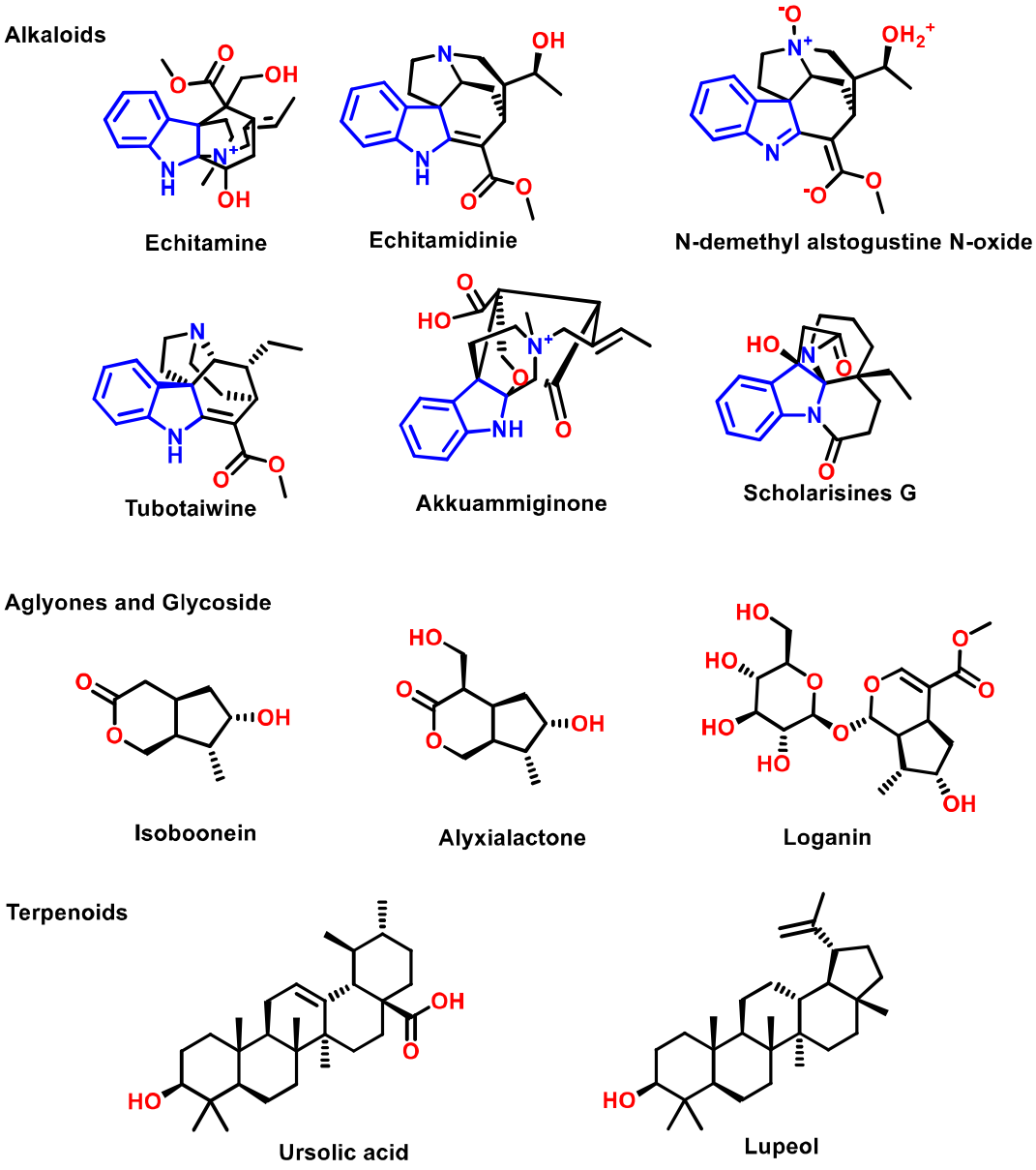

Fig. 4.2. Representative examples of various phytochemicals isolated from *A. scholaris*

4.3. Bioassay-guided fractionation of *A. scholaris* stem bark

The methanol extract of *Alstonia scholaris* stem bark prepared *via* continuous hot percolation exhibited comparatively greater potential (IC_{50} = 12.85 µg/mL), and hence was subjected to the bioassay guided fractionation (**Fig. 4.3**). Methanol extract (500 g) was dissolved in water (1000 mL) and further processed by sequential fractionation. An increasing order of polarity of solvents was used for the sequential fractions in the order of hexane, chloroform and ethyl acetate (3 x 500 mL each). Further, the mother liquor after the ethyl acetate partition was proceeded for Alkaloid Rich Fraction (ARF). The ARF was prepared as per the procedure reported earlier [12]. Briefly, the aqueous phase was acidified to pH 4 using 5% HCl. This aqueous phase was extracted with chloroform (3 x

250 mL) in a separatory funnel and the residual aqueous phase was basified to pH 9 using 25% ammonia solution. The basified aqueous phase was then extracted using chloroform (3 x 250 mL) in a separatory funnel. This alkaloid rich chloroform phase was collected and evaporated to dryness *in-vacuo* resulting in ARF that exhibited a potential PL inhibitory activity of 7.67 µg/mL (IC_{50}).

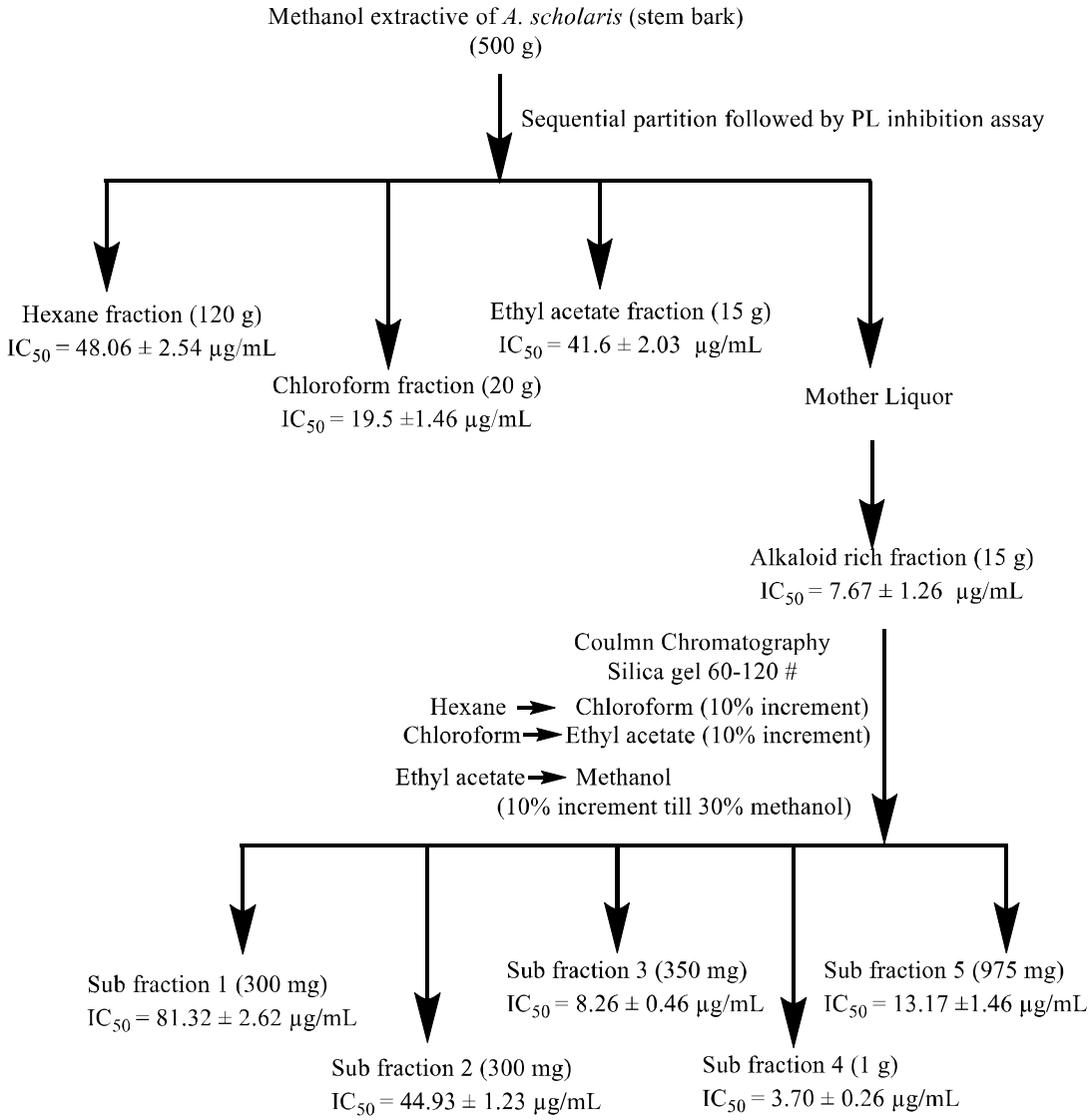

Fig. 4.3. Schematic representation of bioassay guided fractionation of methanol extract prepared from continuous hot percolation technique

Further column chromatography of ARF resulted in 5 subfractions, wherein subfraction 4, exerted the highest PL inhibitory potential (IC_{50} = 3.7 µg/mL). A buff white coloured natural product was obtained from this subfraction. The natural product was further purified by recrystallization and was identified as echitamine (**Fig. 4.4**) by using

spectroscopic techniques such as ^{1}H, ^{13}C, mass spectrometry and by comparing with previously published data [13]. Echitamine exhibited potential PL inhibitory activity (IC_{50} = 10.92 ± 1.26 µM) as compared Orlsitat (IC_{50} = 0.86 ± 0.11 µM) that was used as positive control.

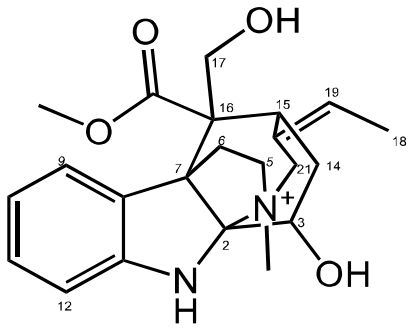

Fig. 4.4. Structure of Echitamine

Echitamine: Buff white crystals: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.97 – 7.56 (d, 1H, H-9), 7.12 (td, *J* = 7.6, 1.2 Hz, 1H, H-11), 6.92 – 6.60 (m, 2H, H-10 & H-12), 5.91 – 5.64 (m, 1H,H-19), 4.42 (s, 1H, H-21α), 4.36 (dt, *J* = 10.9, 4.8 Hz, 1H, H3), 4.17 (d, *J* = 15.0 Hz, 1H, H-21β), 3.86 (d, *J* = 5.2 Hz, 1H, H-15), 3.73 (s, 3H, OMe), 3.58 (dd, *J* = 12.1, 8.3 Hz, 1H, H-5), 3.44 – 3.31 (m, 1H, H-5), 3.28 (s, 3H, NMe), 3.15 (s, 3H, H-17, H-14 β), 2.25 (td, *J* = 13.8, 8.4 Hz, 1H, H-6), 2.04 (dd, *J* = 14.4, 8.5 Hz, 1H, H-6), 1.78 (dd, *J* = 7.0, 2.0 Hz, 3H, H-18), 1.53 (dd, *J* = 14.8, 5.9 Hz, 1H, H-14 α); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.65 (-COO), 147.68 (C-13), 132.55 (C-20), 130.09 (C-19), 129.71 (C-11), 129.40 (C-8), 127.21 (C-9), 120.24 (C-10), 111.13 (C-12), 100.10 (C-2), 69.19 (C-3), 65.28 (C-21), 64.98 (C-17), 62.50 (C-5), 61.08 (C-7), 56.10 (C-16), 52.49 (COOMe), 50.03 (N-Me), 41.57 (C-6), 34.899 (C-15), 31.38 (C-14), 15.40 (C-18). M+H (ESI^{+}) Calculated for $C_{22}H_{29}N_2O_4$ $[M+H]^{+}$ 386.2122, Found 386.2725.

Although echitamine exhibited a potential PL inhibitory activity of 10.92 ± 1.26 µM, the ARF/ methanol extracts exhibited variable PL inhibitory activities in the range of 7.67 ± 1.26 to 45.54 ± 5.10 µg/mL. Thus, we presumed that the difference in the PL inhibitory potential in the extracts/ARF might be due to the variation of echitamine content. Nevertheless, there are no HPTLC based method reported for the quantification of echitamine. Hence, a new HPTLC-HRMS method was developed and validated for the quantification of echitamine in the extracts/ARF.

4.4. HPTLC-HRMS method development and validation of echitamine

Traditionally, chromatographic and related techniques have been reported for the

estimation of various natural products. By considering the advantages of HPTLC technique such as simple method setup, shorter analysis time, low cost per analysis etc., over other chromatographic methods, we proceeded for the HPTLC-HRMS method development and validation [14,15]. The objective of HPTLC-HRMS method development and validation was to identify and quantify the variation in echitamine content in methanol extracts/ ARFs of *A. scholaris* stem bark prepared by different extraction techniques.

4.4.1. Materials and Methods

4.4.1.1. HPTLC densitometry

The quantification of echitamine was performed using a HPTLC System (CAMAG, Switzerland), equipped with Linomat 5 sample applicator, Hamilton syringe (100 µL), Twin Trough Development chamber (10 x 10 cm) and TLC Scanner 3, operated through winCATS (v 1.4.10) software. Chromatography of the samples was performed in triplicate on TLC plates pre-coated with silica gel G60 F_{254} (0.20 mm layer thickness). Prior to the experiments, the TLC plates were washed with methanol and activated at 120°C for 20 min. Sample solutions (5 µL) were applied in the form of 5 mm bands at 10 mm from the lower and left edges respectively, and 10 mm of track distance using N_2 as a nebulizer gas. Linear ascending development was carried out in pre-saturated vertical twin trough glass chamber saturated with the mobile phase. The mobile phase consisted of chloroform: methanol (80:20, %v/v) with 0.04 % of formic acid and TLC separation of echitamine was achieved without interference from sample matrix components under laboratory conditions (temperature 25 ± 2ºC and relative humidity 60 %). The plates were dried under a stream of air. Further, the plates were scanned and quantified densitometrically at 294 nm using TLC Scanner 3. The scanning was performed in the absorption mode, with a slit width of 3.00 mm × 0.1 mm, scanning speed of 20 mm/s and data resolution of 100 µm/step. Savitsky Golay-7 was used for data filtering.

4.4.1.2. HPTLC-HRMS analysis

HPTLC band (R_f =0.38) was extracted using HPLC grade methanol from the HPTLC plates by using Advion plate eXpress with a flow rate of 0.30 mL/min. The solvent was filtered through 0.2 µM PTFE membrane filter. The filtered sample was used for HRMS analysis.

HRMS analysis was performed using 1250 infinity II series LC system coupled to a 6545 series electrospray ionization (ESI) - quadrupole time-of-flight (QTOF) high-resolution mass spectrometer from Agilent Technologies. Sample was passed through Eclipse Plus C18 RRHD column (1.2 µm, 50 mm x 2.1 mm). The column temperature was set at 28ºC. The mobile phase was composed of water (Solvent A) and acetonitrile (Solvent B). An isocratic gradient of 40% solvent A and 60% of solvent B was used with a flow rate of 300 µL/min. Total run time was 3 min. Injected volume of the sample was 0.3 µL.

ESI-QTOF parameters were as follows: positive mode, 4 GHz mode for resolution, mass range 100–1200 *m/z*, drying gas temperature 325 °C, gas flow 5 L/min, nebulizer pressure 20 bars, and capillary voltage−4000. Nitrogen was used as the nebulizer gas. Data acquisition and HRMS-MS data analysis was carried out by MassHunter® software.

4.4.1.3. Method validation

The validation of the developed HPTLC method was performed as per the Q2 (R1) guidelines of the International Conference on Harmonization (ICH) for Instrumental Precision, Specificity, Sensitivity, Linearity, Precision, Accuracy, and Robustness [16]. Instrumental precision for Scanner 3 was evaluated by scanning a single spot of echitamine (500 ng/spot) seven times (*n*=7). Linomat 5 sample applicator was checked for its suitability by spotting echitamine (500 ng/spot) seven times (*n*=7). The specificity of the developed method was ascertained by co-analysing echitamine and different extracts. The sensitivity of the developed method was established by determining the Limit of Detection (LOD) and Limit of Quantification (LOQ). For this, signal-to-noise (S/N) ratio with the different concentrations (150-1000 ng/spot) of echitamine were spotted along with the methanol as a blank and were evaluated. LOD was measured as 3.3 times the noise level, while LOQ was calculated as 10 times the noise level. The linearity was evaluated at different concentration levels (200-1500 ng/spot) of echitamine by application of 5 µL of the respective stocks (40-300 ng/µL). The experiment was performed thrice (*n*=3), and the calibration curves were obtained by plotting peak area (Y-axis) *Vs*. concentration of echitamine per spot applied (X-axis). Precision and accuracy were evaluated as Intra-Day (*n*=3) and Inter-Day (*n*=3) at three levels using Low (300 ng/spot), Medium (900 ng/spot), and High (1450 ng/spot) concentrations of the calibration curves and the obtained peak areas were used to calculate % RSD and % CV respectively. The recovery studies were performed by spiking three concentration levels (300,900 and 1200 ng) of echitamine into pre-analysed samples containing echitamine and the % recovery of echitamine was

calculated (*n*=3). The robustness of the method was evaluated by implementing small changes in various parameters of the HPTLC determinations, such as mobile phase polarity, mobile phase volume, saturation time of the development chamber and solvent front position. Each experiment was carried out thrice (*n*=3) and the amount of echitamine and its % RSD (Relative Standard Deviation) was calculated.

4.4.2. Results and Discussion

4.4.2.1. HPTLC -HRMS method development

Various mobile phases and different ratios of various solvents were tried to develop a HPTLC method with an aim of maximum resolution of the peak by keeping a minimum interference from solvent matrix. After several trials with various solvents, the mobile phase composition was optimised as chloroform: methanol (80:20, %v/v) with 0.04 % formic acid. Echitamine exhibited a retardation factor (R_f) of 0.38. However, the spots could not be visualised at 254 nm. The spectral scan of echitamine revealed the maximum absorption (λ_{max}) at 294 nm (**Fig. 4.5**). Echitamine present in extracts at a R_f value of 0.38 was further confirmed by extracting the corresponding band using Advion plate eXpress with methanol as solvent and LC-HRMS analysis was performed. The mass spectrum of the extracted compound from HPTLC is shown in **Fig. 4.5, (**HRMS m/z: for $C_{22}H_{29}N_2O_4$ $[M+H]^+$ calcd. 386.2122, found 386.2725). The obtained λ_{max} of 294nm was used for the remaining studies.

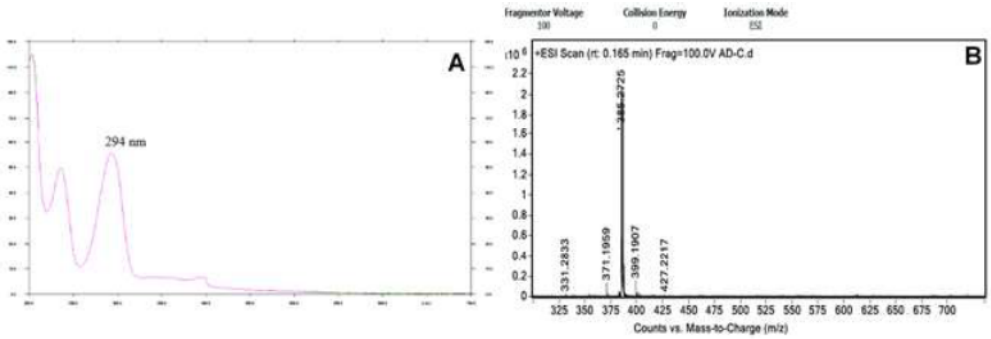

Fig. 4.5. A) Spectral scan chromatogram of echitamine highlighting the λ_{max} at 294 nm; **B)** HRMS spectrum of echitamine

Post-chromatographic derivatisation of TLC plates by anisaldehyde-sulphuric acid reagent [17] resulted in the visualisation of echitamine in standard solution as well as in different extracts as pink color spots (**Fig. 4.6**). Post chromatographic derivatization further confirmed that the echitamine spot was clearly resolved from other natural products present in the extracts.

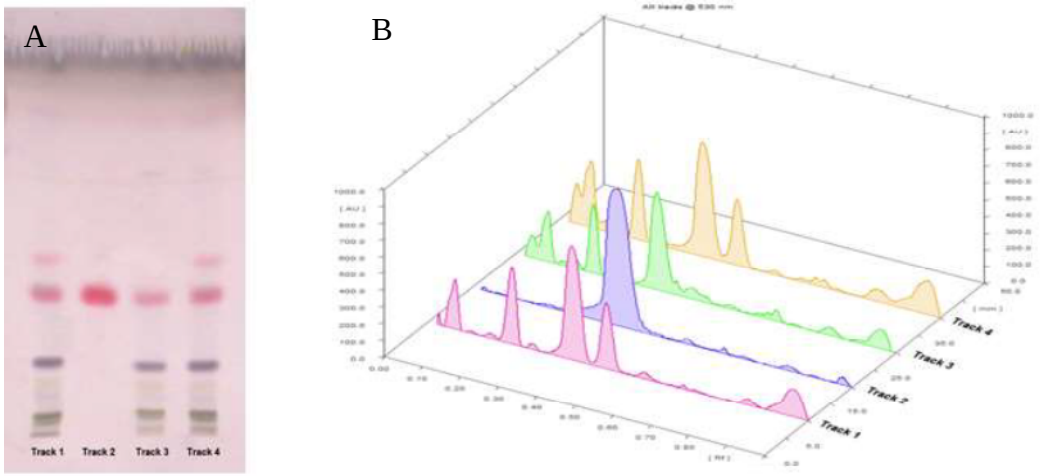

Fig. 4.6. A) TLC of echitamine (Track 2) and different extracts (Track 1,3 and 4) prepared from different extraction techniques, derivatised used anisaldehyde-sulphuric acid; B) HPTLC chromatograms of echitamine, different extracts and scanned densitometrically at 530 nm.

Track Details – 1: continuous hot percolation, 2: Echitamine, 3: cold-maceration, 4: Ultrasonic extraction; Mobile phase – Chloroform: methanol (80:20, %v/v) with 0.04 % of formic acid

4.4.2.2. HPTLC method validation

The validation of the developed HPTLC method was performed as per the Q2 (R1) guidelines of the International Conference on Harmonization (ICH) for Instrumental Precision, Specificity, Sensitivity, Linearity, Precision, Accuracy, and Robustness [16] and the obtained results are summarised in **Table 4.3**.

Table 4.3. Summary of the validated HPTLC parameters for echitamine.

S. No.	Parameter	Range
1	Instrumental precision (% CV, *n*=7)	Linomat 5 (1.81); Scanner 3 (2.66)
2	Specificity (*n*=8)	Specific with R_f of 0.38 ± 0.02
3	Limit of Detection (LOD)	64 ng/spot
4	Limit of Quantification (LOQ)	194 ng/spot
5	Linearity Range	200-1500 ng/spot
6	Regression equation (r^2)	y=7.7888x + 1260.3 (0.9989)
7	Precision - % RSD range (Intra-day; Inter-Day)	1.60-2.83; 0.21 - 3.65
8	Accuracy- % CV (Intra-day; Inter-Day)	6.21 – 9.54 %; 4.75 – 6.35 %
9	Recovery studies	96.80 – 104.77 %
10	Robustness (% RSD Range)	0.40 - 1.59 %

CV - Co-efficient of Variation; *n* – number of replicates; RSD – Relative Standard Deviation

In the developed method, echitamine (R_f = 0.38) was separated with no interference from other natural products in the sample matrices. The sensitivity of the method was evaluated by calculating LOD and LOQ of echitamine, that was found to be 64 and 194 ng/spot, respectively. Further, echitamine showed a good linearity (0.9989) of correlation coefficient (r^2) in the concentration range of 200-1500 ng/spot with a regression equation y=7.7888x+ 1260.3 (**Fig. 4.7)**.

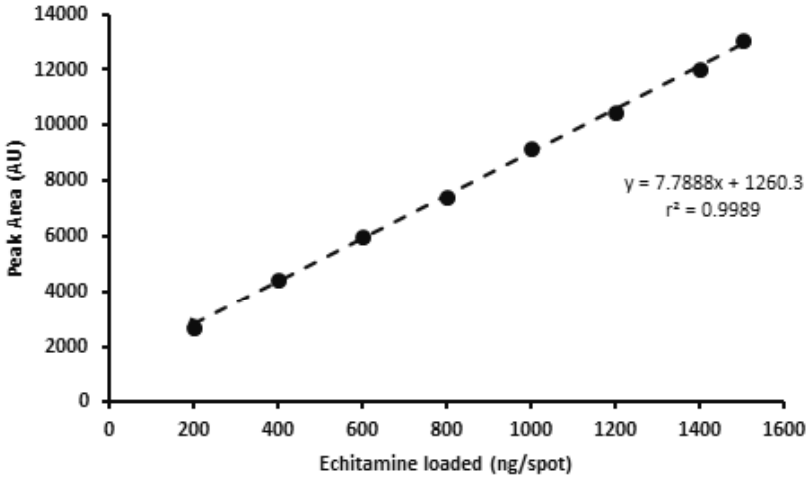

Fig. 4.7. Regression plot representing the linearity of the validated HPTLC method.
All values expressed as mean ± S.E.M (n=3)

Accuracy and precision of the method was evaluated by intra and inter day, wherein, % CV existed in the range of 4.75 - 9.54 % while % RSD existed in the range of 0.21-3.65 % **(Table 4.4)**.

Table 4.4. Summary of intra- and inter-day accuracy and precision studies

Echitamine Spotted (ng/spot)	Amount Detected (ng) ± SD	% RSD	% CV
Intra-Day (*n*=3)			
300	308.18 ± 23.65	2.72	7.67
900	885.59 ± 34.49	0.43	9.54
1450	1408.86 ± 30.68	2.83	6.21
Inter-Day (*n*=3)			
300	310.96 ± 17.89	3.65	5.75
900	898.05 ± 57.09	0.21	6.35
1450	1427.31 ± 67.91	1.56	4.75

ng – nanogram; *n* – number of replicates; SD – Standard Deviation.
RSD – Relative Standard Deviation; CV - Co-efficient of Variation

The recovery study of the method was performed by spiking three known concentrations of echitamine into the pre-analysed extracts. The % average recovery existed in the range of 96.80 - 104.78 % **(Table 4.5)**.

Table 4.5. Recovery study of the developed method summarizing the % recovery values

Extraction technique	Echitamine added (ng)	Echitamine recovered (ng)	% Recovery ± SD (*n*=3)
Hot percolation	300	312.42	104.14 ± 9.33
	900	893.63	99.30 ± 5.48
	1200	1161.59	96.80 ± 4.49
	% Average Recovery ± SD		100.07 ± 6.43
Sonication	300	314.32	104.78 ± 8.38
	900	908.89	100.98 ± 5.91
	1200	1182.45	98.53 ± 4.03
	% Average Recovery ± SD		101.43 ± 6.11
Maceration	300	302.57	100.86 ± 6.08
	900	887.64	98.63 ± 1.56
	1200	1200.77	100.06 ± 3.93
	% Average Recovery ± SD		99.85 ± 3.86
ARF	300	297.02	99.00 ± 4.24
	900	882.91	98.10 ± 3.03
	1200	1178.75	98.23 ± 5.25
	% Average Recovery ± SD		98.44 ± 4.37

Robustness of the method was validated by incorporating small changes in the variables involved in the method, such as, change in the mobile phase polarity and volume, solvent front position and saturation time of the development chamber. Echitamine exhibited a R_f of 0.38 and 0.59 respectively, with 80:20 and 75:25, % v/v of chloroform: methanol with 0.04 % formic acid, while the % RSD existed in the range of 0.15 – 1.59 % **(Table 4.6)**.

Table 4.6. Summary of robustness results of the developed method.

Parameter	Echitamine spotted (ng)	Echitamine detected (ng)[a]	% RSD
Mobile phase composition chloroform: methanol (80: 20, v/v)	900	914.24 ± 36.32	1.58
Mobile phase composition chloroform: methanol (75: 25, v/v)	900	914.08 ± 41.08	1.56
Mobile phase volume (10 mL)	900	896.34 ± 16.95	0.40
Mobile phase volume (15 mL)	900	914.37 ± 30.00	1.59
Development chamber saturation time (20 min)	900	910.69 ± 34.18	1.18
Development chamber saturation time (40 min)	900	905.97 ± 28.68	1.02
Solvent front position (90 mm)	900	913.19 ± 33.69	1.47
Solvent front position (100 mm)	900	898.63 ± 47.87	0.15

[a] Values represented as Mean ± SD (*n*=3).

4.4.2.3. Echitamine quantification and its correlation with PL inhibitory activity

Echitamine exhibited potential PL inhibitory activity (IC_{50} = 10.92 ± 1.26 µM) and the PL inhibitory activities of extracts/ ARF were in the range of 7.67 – 45.54 µg/mL. However, a significant variation in PL inhibitory activity was identified among different extracts with respect to the echitamine content. Thus, echitamine was quantified in various extracts by using the validated method to understand the role of extraction techniques on the content of echitamine and its correlation with PL inhibitory profiles. Previously, a High-Performance Liquid Chromatography (HPLC) based quantification of echitamine has been reported [18]. The echitamine content in the extracts was higher with continuous hot percolation, followed by ultrasound-assisted extraction and least with maceration technique. As per the previously reported HPLC quantification method, echitamine yield in extracts was found to be 0.088, 0.270, 0.085 % w/w in cold extraction, ultrasonic wave assisted extraction and hot percolation techniques, respectively. In the present study, the % yield of echitamine was comparatively higher than that of the previous report. The extractive yield of echitamine, was 0.20, 0.38 and 0.61 % w/w from maceration, ultrasound-assisted extraction and hot percolation extracts, respectively. In the present case the longer extraction time may have resulted in improved extraction of echitamine from the stem bark sample. Further, in ARF sample, the echitamine content was found to be 3.61 % w/w. From this data, it was evident that the echitamine content is directly proportional to the PL inhibitory potential of the extracts (**Fig. 4.8** & **Table 4.1**). Pearson correlation analysis exhibited a significant correlation between PL inhibitory activity with their respective echitamine content ($p < 0.05$, Pearson's $r = -0.9409$). Thus, the present study highlighted that the extraction techniques have a significant role in the extractive

yield of echitamine, wherein continuous hot percolation technique resulted in its higher yield.

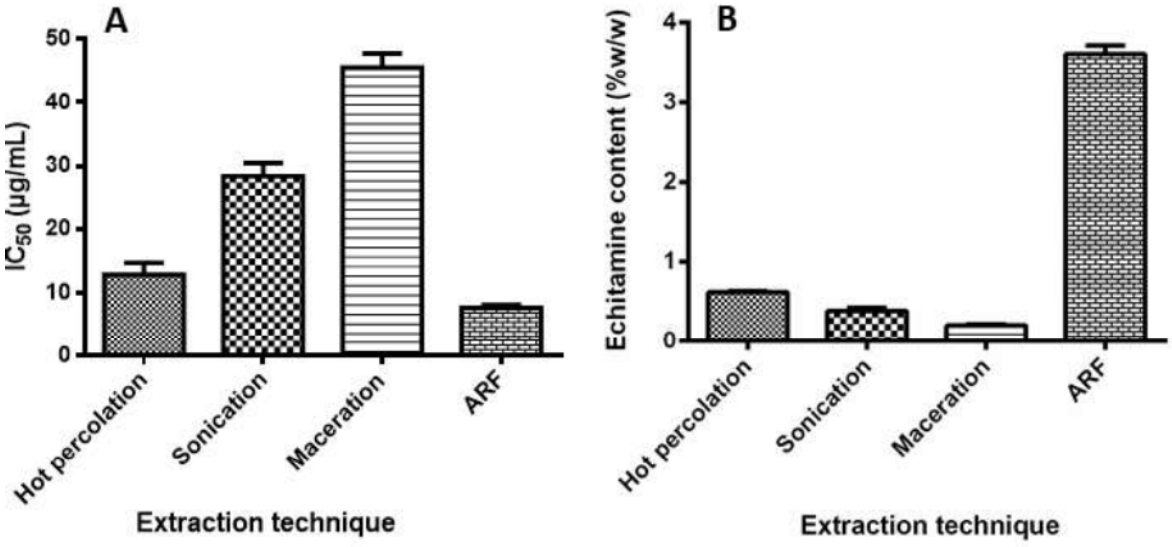

Fig. 4.8. Representation of PL inhibitory activity (IC_{50}) (A) and extractive yield of echitamine (B) from extracts prepared via different extraction techniques in the stem bark of *A. scholaris* on dry weight basis.

To summarize, the present chapter focused on the identification of potential PL inhibitory natural product lead from the Indian medicinal plants. The methanol extract from the stem bark of *A. scholaris* exerted a potential PL inhibition, hence was subjected to bioassay guided fractionation. The study resulted in the isolation and identification of echitamine as a potential natural product lead, that exhibited an IC_{50} value of 10.92 µM. Further, a HPTLC-HRMS method was developed and validated for the quantification of echitamine. Moreover, quantification of echitamine in numerous extracts revealed a direct correlation between its quantity and PL inhibitory potential.

Although echitamine exhibited a potential PL inhibition, it was felt that further structural modification or synthesis of echitamine inspired analogues can result in potent PL inhibitory activity. The coming chapters will discuss the attainment of the same.

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