Journal of Chromatography
Separation Techniques

Attarde et al., J Chromatogr Sep Tech 2017, 8:1
DOI: 10.4172/2157-7064.1000352

Research Article Open Access

Premnine HCl Estimation in Selected Formulations of '*Dashmul*' and in Chloroform Extract of *Premna integrifolia* L. by a Selective, Validated and Developed HPTLC Fingerprint Method

Attarde DL[1]*, Pal SC[2] and Bhambar RS[1]

[1]*Department of Pharmacognosy, Mahatma Gandhi Vidyamandir's Pharmacy College, Panchavati, Nashik, Maharashtra, India*
[2]*Department of Pharmacognosy, RG Sapakal College of pharmacy, Kalyani Hills, Trimbakeshwar, Nashik, Maharashtra, India*

Abstract

HPTLC technique developed as validated method for estimation of Premnine HCl in *P. integrifolia* chloroform extract (PI-AK) and in selected marketed formulations 'Dashmul arishta','Dashmul kadha' in 3 × 3 batches as per ICH guidelines. Premnine HCl (Pr-s) was isolated as per literature from *P. integrifolia* and focused first time as standard bioactive marker for quantification. Developed mobile phase Toluene: Acetone: Diethylamine (7:2:1) for Pr-S gave Rf 0.59 at λ max 283 nm in densitometric scan, focused for specificity, fingerprint and estimation study in PI-AK and selected formulation successively. Linearity assessed in range of 8 to 16 µg /band with regression coefficient of 0.9983, LOD 0.742 µg/Band, LOQ 2.225, also robust for Pr-S. Accuracy for % recovery performed on extract as well on formulation, further subjected for precision study with application one way ANOVA for finding F value, found within limit therefore no significance of variance. A rapid and selective HPTLC method shows good linearity, recovery and high precision, useful method for analysis of Pr-S and as quality control parameter for raw material as well formulation as per foremost need of WHO, FDA and Pharmacopoeia.

Keywords: *Premna integrifolia;* HPTLC; *Dashmul arishtha; Dashmul kadha;* Premnine HCl

Introduction

Premna integrfolia Linn. (syn. *P. corymbosa* auct., *P. obtusifolia* R., *P. spinosa* Roxb., *P. serratifolia*) is thorny deciduous shrub belonging to Verbenaceae, common along India, Andaman costs, tropical and subtropical Asia, Africa Africa and the specific Islands, known as Agnimanth, Arni, Girikarnika in Sanskrit, Malbau in Malay language useful for inflammation, brochitis, dyspepsia, piles, constipation, fever and root forms an ingredients of '*Dashmul*' which is renowned traditional Ayurvedic remedy tonic for liver, uterus, kidney, also detoxifies and strengthens body. Decoction of root useful for convulsion, rheumatism, and neuralgia [1-3].

Literature survey shows three novel diterpenoids isolated from root bark and simultaneous quantification of it by HPTLC method [4,5] a verbascoside iridoid glycosides from leaves [6], two alkaloid premnine and ganiarine isolated from root [7], volatile constituents isolated from flower buds [8], flavonoids Luteolin 7-O-methyl ether and Apigenin 5,7-O-dimethyl ether isolated from leaves [9], identification of volatile constituents of leaves and roots analysed by GC-MS [10], stem bark and wood alcoholic extracts shows cardiotonic effects and β adrengic effects [11] and also antioxidant effects [12], antimicrobial activity of root extracts [13], wood extract for antiarthritic activity [14], methanolic extract of root evaluated for immunomodulatory effects [15].

Premnine alkaloid shows raise in blood pressure by contracting blood vessels but decreases force of contraction of heart, and dilates pupil [7].

In this original research Premnine alkaloid isolated as per literature and its salt as Premnine HCl first time here focused as unique characteristic bio-active marker for qualitative and quantitative analysis in chloroform extract of *P. integrifolia* and in its marketed formulation as '*Dashmul arishtha*' and '*Dashmul kadha*' with view to develop standardise parameter that will serve as crucial quality control parameter for drug.

Experiment

Equipment

HPTLC Instrument (CAMAG, Switzerland): Linomet Syringe V, TLC scanner V, Digistore- Reprostar 3, Win CATS version 1.4.2. Software, Twin trough Chamber, Pre-coated silica gel 60 F_{254} aluminium plates (0.2 mm thick, Merck, Germany).

Chemicals and solvents

Premnine HCl- isolated as per literature [7].

Analytical grade solvents: Ethanol, Conc. HCl, Pet. Ether (60-80°C), chloroform, Toluene, Acetone, Diethyl amine (Merck), Dragendorff's reagent (Freshly prepared).

Plant material

P. integrifolia shrubs are located in Trimbakeshwar forest area of Nashik District, herbarium was prepared of flowering branch. It was deposited to Botanical Survey of India, Pune for identification purpose. Certificates were issued as i.e., Ref.: BSI /WRC/Tech./2012/DVR-1 dt. 02/1/2012 for *Premna integrifolia L.(Verbenaceae).* Stem branches were sliced into small pieces, dried under shade for about 20 days, powdered, passed through sieve 25/30 no.-600 µ. Dried powder packed in air tight container and stored to cool, dry condition for further use.

***Corresponding author:** Attarde DL, Department of Pharmacognosy, Mahatma Gandhi Vidyamandir's Pharmacy College, Panchavati, Nashik, Maharashtra, India, Tel: 9730075256; E-mail: daksha511@rediffmail.com

Received February 05, 2017; **Accepted** February 14, 2017; **Published** February 20, 2017

Citation: Attarde DL, Pal SC, Bhambar RS (2017) Premnine HCl Estimation in Selected Formulations of '*Dashmul*' and in Chloroform Extract of *Premna integrifolia* L. by a Selective, Validated and Developed HPTLC Fingerprint Method. J Chromatogr Sep Tech 8: 352. doi: 10.4172/2157-7064.1000352

Isolation of premnine HCl alkaloid

As per literature [7] stem powder (2 kg) charged in soxhlet assembly in several batches and exhaustively extracted with ethanol. Alcoholic extract concentrated and poured syrupy mass with mechanical stirring into 1% warm HCl and kept for 2 hr on magenetic stirrer. Filtered precipitated black resinous mass. And extracted with chloroform, each chloroform extract fraction washed with 1% HCl and washing returned to acidic alcoholic fraction. pH of acidic alcoholic fraction now adjusted to 9 with dilute ammonia solution and exhaustively extracted with chloroform. Chloroform extract concentrated, dried and yield noted. It was dissolved in few ml of chloroform and gasoline added dropwise to precipitate out blackish resinous mass and filtered. This is repeated several times for purification. This yellowish semi purified alkaloid fraction obtained was divided into two fraction. One fraction subjected for isolation as follow, dissolve into dry ether and few drops of absolute alcohol saturated with HCl gas were added to it drop by drop and stirred continuously. Yellowish precipitate obtained was filtered, dissolved in alcohol and recrystallized this isolated *Premnine* HCl melting point taken, recorded as 212-214°C, matched with reference value of 211-213°C, as per literature no [7]. It was further confirmed as alkaloid with Dragendorff's test. It was designated as Pr-s.

Preparation of purified chloroform extract of *P. integrifolia*

From above procedure second portion of purified chloroform extract was dissolved in few ml of 1% alcoholic HCl, allow to stand for 2 hr and then dried to solid mass, yield noted and designated as PI-AK.

Selected Marketed Formulation and Treatment

***'Dashmul arishtha'*:** Manufacture I: selected 3 batches, Manufacture II: selected 3 batches,

***'Dashmul kadha'*:** Manufacture III: selected 3 batches, batch no., volume, label claim noted and designated with codes. Coded batches were concentrated using rotary evaporator, made hydro alcoholic. pH adjusted to 9 with dilute ammonia solution and extracted with chloroform in several batches. All chloroform extracts were concentrated and dried separately and further dissolved in 1% alcoholic HCl batch wise and allow to stand for 2 hr and then dried to solid mass, yield noted and designated as for Manufacturer I, II, III batches as: DA1, DA2, DA3, SA4, SA5, SA6, BA7, BA8 and BA9 respectively.

Sample preparation for HPTLC

Standard Pr-S solution: 2 mg of standard Pr-S was dissolved in 1 ml of alcohol in volumetric flask (2000 ppm), sonicated for 15 min.

PI-AK solution: 20 mg of purified chloroform extract PI-AK was dissolved in 1 ml of methanol (20000 ppm), sonicated for 15 minutes, filtered through whatman filter paper No.1.

Marketed formulations solution: Coded batches as mentioned and treated above-prepared as 20 mg/ml each in methanol separately and designated as DA1, DA2, DA3, SA4, SA5, Sa6, BA7, BA8 and BA9.

HPTLC condition

Stationary phase: Pre-coated Silica Gel G 60 F_{254} HPTLC Plates

Mobile Phase: Toluene: Acetone: Diethylamine (7:2:1)

Derivatizing Reagent (Visualising agent): Dragendorff's Spray Reagent

Wavelength: 283 nm (λ max for standard Premnine HCl),

Band application point -X-8 mm, Y-15 mm, Sample Band length-8 mm,

Chamber saturation Time: 10 min, Solvent Run -80 mm, Lamp: Deuterium,

Scanning slit width 6 × 0.45 mm, Scanning speed 20 mm/s, Area Temperature-22+/-2°C Applicator Syringe-100 µl, Sample Application speed 0.2 µl/s.

Spray gas- Nitrogen Inert gas, Scanner- TLC scanner 5 (version 1.14.26),

Photo documentation-Digistore-Reprostar 3.

HPTLC method

The HPTLC analysis was performed using above chromatographic condition, 5, 10, 15 µl of Pr-S applied on pre-coated TLC Silica gel 60 F_{254} plates as band length of 8 mm using Linomat 5 syringe, spots bands were air dried, mobile phase Toluene: Acetone: Diethylamine (7:2:1) poured to CAMAG twin trough chamber and allowed to saturate for 10 min. than developed till 80 mm, dried with dryer, scanned over TLC scanner 5 (1.14.26) with absorption/remission mode at scan speed 20 mm/s at 254 nm initially. Spectral scan done in between 200-400 nm with 100 nm/s speed, spectra of Pr-S wavelength noted, again plates were rescanned at 283 nm in detection scanner mode. Rf (Retention factor), AUC (Area Under Curve) for standard Pr-S noted, used the data for further detection.

Specificity and fingerprinting of Pr-S as standard in PI-AK and in DA1, SA4, BA7 formulations

For specificity and fingerprinting study sample applied as PI-AK (track 1 to 3: 3 µl each), Pr-S (track 4 ,5:5 ,10 µl), DA1(track 6,7:5, 7 µl), SA4 (track 8,9:5, 7 µl), BA7 (track 10, 11:5, 7 µl), respectively sequentially and plate developed as above and scanned in detection at 283 nm λ max for Standard Pr-S. Rf obtained for it is noted, identified and marked in all samples track. The identified and selected bands subjected for spectral scan between 200-400 nm and over laid spectra for confirmation of specificity for standard. The purity of the bands was confirmed at start, middle and end position of chromatogram. Then finally plate derivatized by dipping into freshly prepared Dragendorff's spray reagent, dried, colour visualized as identity for alkaloids at Pr-S Rf and photo documented. Specificity and fingerprinting chromatogram are as shown in Figure 1a and 1b.

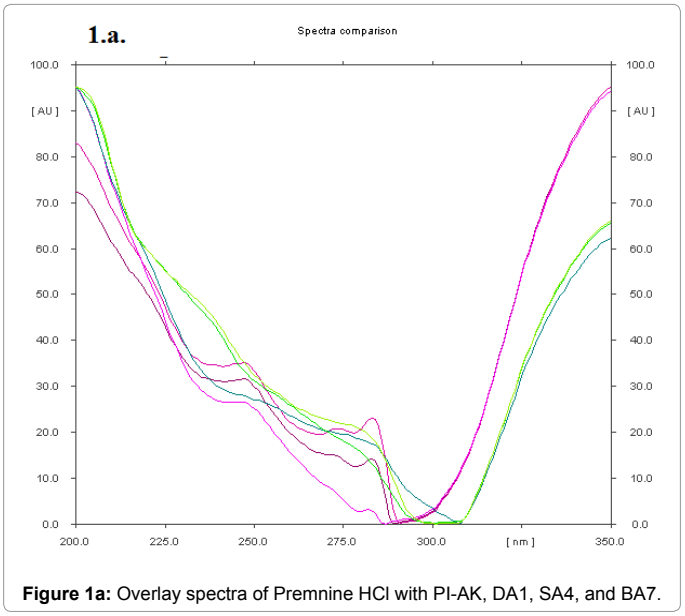

Figure 1a: Overlay spectra of Premnine HCl with PI-AK, DA1, SA4, and BA7.

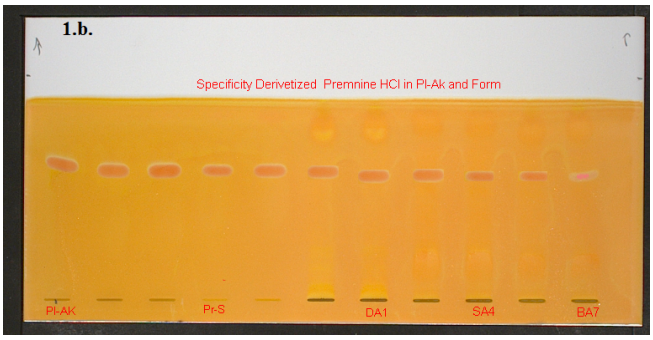

Figure 1b: HPTLC Chromatogram of Standard Premnine HCL with PI-AK, DA1, SA4, BA7 photo documented after derivatization with Dragendorff's reagent.

Parameter	Value
Wavelength, nm	283 nm
Mobile Phase	Toluene:Acetone:Diethylamine (*7:2:1*)
RF Value	0.59
Linearity Range µg/band	8-16
Regression equation	Y=146.57 × - 4.366
Correlation Coeffiecient	0.9983
Limit Of Detection µg/band LOD	0.472 µg/Band
Limit Of Quantification µg/band LOQ	2.225 µg/Band
Specificity	Specific- Overlay Spectra, RF, Derivatization with Dragendorff's reagent

Table 1: Validation Parameter for Estimation of Premnine HCl.

Analyte	Premnine HCl Concentration Obtained
PI-AK	3.9% w/w
Powder Of *P. integrifolia*	0.3590 mg/gm%
Dashmul arishtha	
Manufacturer I Bottle size : 225 ml, Label Claim: Each 100ml contains 0.52 gm of *'Dashmula'* each.	
DA1*	1.231 mg/ml%
DA2*	0.481 mg/ml%
DA3*	0.497 mg/ml%
Dashmul arishtha	
Manufacturer II Bottle size : 220 ml, Label Claim: Each 10 ml contains 0.50 gm of *'Dashmula kwath'*	
SA4**	2.73 mg/ml %
SA5**	2.52 mg/ml %
SA6**	3.04 mg/ml %
Dashmul kadha	
Manufacturer III Bottle size : 227 ml, Label Claim: Each 100ml contains 418.91 mg of '*Dashmula*' each	
BA7***	1.58 mg/ml %
BA8***	1.046 mg/ml %
BA9***	2.66 mg/ml %

PI-AK- *Premna integrifolia* chloroform extract
*Manufacutre -1: Three Batches Coded As DA1, DA2, DA3
** Manufacutre -2: Three Batches Coded As SA4, SA5, SA6
*** Manufacture-3: Three Batches Coded As BA7, BA8,BA9

Table 2: Quantification of premnine HCl in PI-AK extract and selected marketed dashmula formulations.

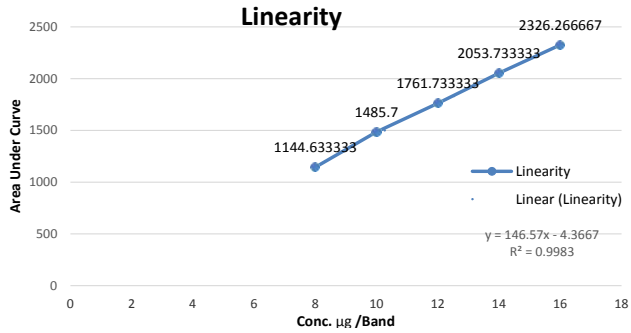

Figure 2: Linearity Curve of Premnine HCl standard.

Linearity assessment for Pr-S standard and its estimation in PI-AK and in selected marketed formulation

For linearity samples applied as Pr-S: 4, 4, 5, 6, 7, 8 µl (8 to 16 µg/band), for estimation on same plate sample applied as PI-AK- 2 µl, DA1, DA2, DA3- each 8 µl SA4, SA5, SA6- each 6 µl BA7, BA8 and BA9 each 7 µl over track 1 to 16 respectively with band length 8 mm. As per above condition chromatogram developed till 80 mm in above mobile phase, scanned at 283 nm and recorded at Rf 0. 59 for Pr-S, repeated thrice and average AUC noted. Plates were derivatized with Dragendorff's reagent for confirmation of Pr-S in samples. Standard linearity curve prepared by plotting Area Under Curve vs conc. in ng as shown Figure 2 and using calibration curve of concentration of Pr-S in PI-AK and in marketed formulations DA1, DA2, DA3, SA4, SA5, Sa6, BA7, BA8 and BA9 calculated as shown in Tables 1 and 2, Figures 3a-3j.

Validation of HPTLC method

As per International Conference on Harmonization Guidelines-Q2 (R1), 2005 the above estimation method was validated for various parameters as follows.

Linearity for Pr-S standard was assessed in range of 8-16 µg /band. The calibration curve established by plotting Peak area (AUC) *vs.* Conc. in µg/band, regression equation with slope, intercept and coefficient of correlation was calculated.

Using following formula, the Limit of detection (LOD) and limit of quantification (LOQ) were calculated as: LOD=3.3 × σ/S (1)

LOD-10 × σ/S (2)

Where σ=standard deviation of the response; S=slope of the regression line.

Repeatability precision

The repeatability of the method was assessed by three concentration 8, 12, 16 µg/band in triplicate for Standard Pr-S**.** The percentage relative standard deviation was calculated and expressed as Table 3.

Accuracy (% recovery)

The accuracy of the method was calculated by recovery experiments over known concentration on Extract–PI-AK and formulation SA4 at 80%, 100% and 120% spike of Standard Pr-S in triplicate for each experiment and analyzing it with %RSD. As per Table 4a, 3D graph of accuracy study shown in Figures 4a and 4b.

Intermediate precision

Precision study for Standard Pr-S done for three concentration as 8,

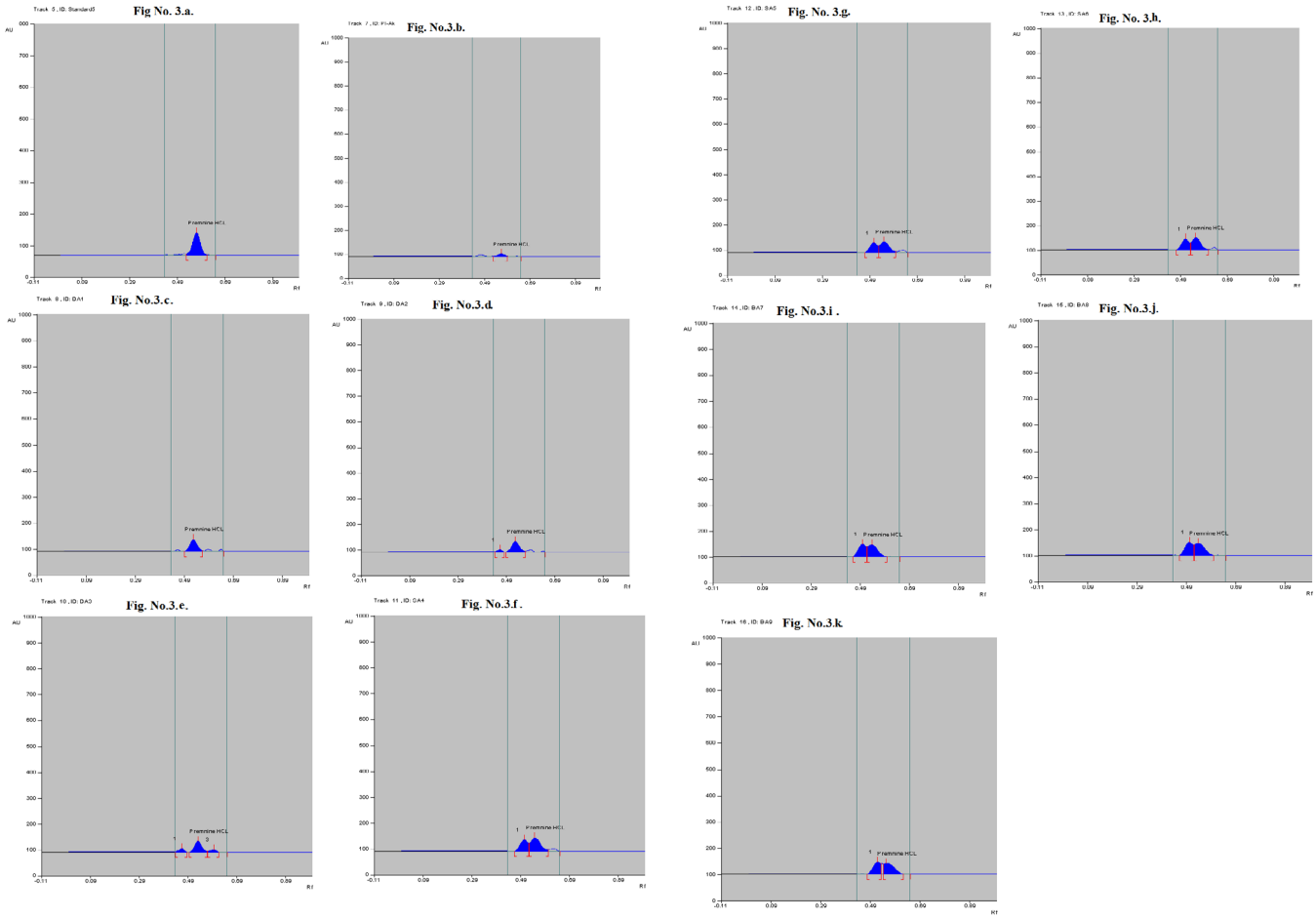

Figure 3 a-k: HPTLC resolution chromatogram of standard Premnine HCl, PI-AK extract, DA1, DA2, DA3, SA4, SA5, SA6, BA7, BA8 and BA9 marketed formulation respectively.

Concentration[T] (µg/band)	Average Concentration[a] (n=3) Intraday (µg band)	Intra day %RSD	Average Concentration[a] (n=3) Interday (µg /band)	Inter day %RSD
8	7.78	1.9842	7.79	0.6465
12	12.22	0.5664	12.02	1.4634
16	15.79	1.6727	15.88	0.5044

T- Therotical, a- Obtained, % RSD- Relative Standard Deviation.

Table 3: Intermediate Precision Study for Standard Premnine HCl.

	Level of % Recovery	Amount of Pr-S in PI-AK (µg)	Amount of Pr-S Added (µg)	Total amount of Pr-S taken (µg)	Total amount of Pr-S obtained (µg)	%recovery ± S.D.(n=3)
In PI-AK + Pr-S						
	80%	3.95	3.16	7.11	7.37	103.65 ± 0.956
	100%	3.95	3.95	7.9	7.74	97.97 ± 0.535
	120%	3.95	4.74	8.69	8.20	94.91 ± 0.563
In SA4						
+ Pr-S	80%	5.14	4.11	9.25	9.23	99.78 ± 0.936
	100%	5.14	5.14	10.28	8.31	103.21 ± 0.526
	120%	5.14	6.16	11.3	11.34	100.35 ± 1.27

(a) PI-AK- *Premna integrifolia* chloroform extract, (b) SA4-Manufacture-2 -coded Batch, (c) Pr-S- Premnine HCl Standard

Table 4a: Accuracy (%Recovery) for PI-AK extract and SA4 extract with spike of Standard Premnine HCl (Pr-S) at 80% ,100% and 120% level.

(µg) Amount	(n=3)	Amount of Standard Obtained in µg			WMS	BMS	F value
PI-AK + Pr-S		Day 1	Day 2	Day 3			
80 %	Mean	7.3	7.26	7.27	0.017189	0.000933	0.054299
(7.11)	%RSD	1.883	1.641	1.86			
100%	Mean	7.75	7.9	7.77	0.009922	0.018433	1.857783

(7.9)	%RSD	0.858	1.74	1.031			
120%	Mean	8.43	8.44	8.47	0.0231	0.001378	0.059644
(8.69)	%RSD	1.44	1.899	1.996			
SA4 + Pr-S							
80%	Mean	9.23	9.25	9.07	0.037567	0.028078	0.747412
(9.25)	%RSD	1.54	1.828	1.877			
100%	Mean	10.57	10.56	10.32	0.031556	0.060233	1.908803
(10.28)	%RSD	1.643	1.640	1.79			
120%	Mean (n=3)	11.14	11.34	11.31	0.029256	0.035233	1.20433
(11.3)	%RSD	1.86	0.759	1.70			

Table 4b: Precision study for PI-AK and SA4 with spike of Standard Premnine HCL at 80% ,100% and 120% level. (One Way ANOVA).

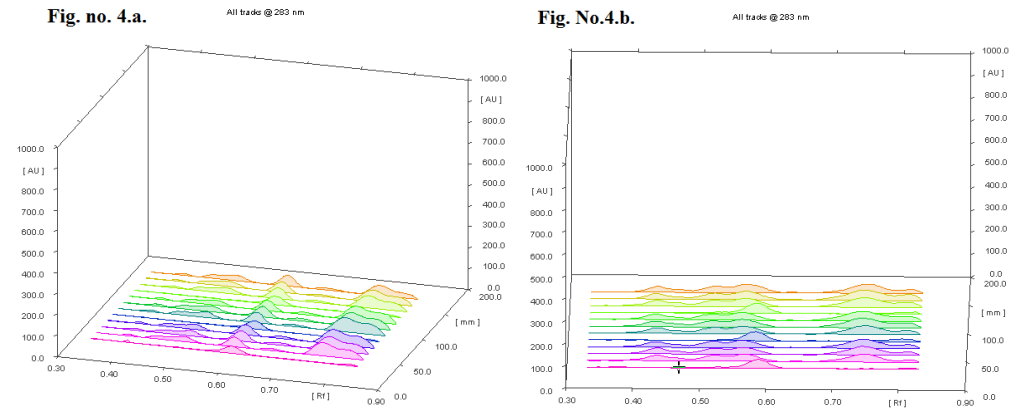

Figure 4 a and b: Graph 3D resolution chromatogram for PI-AK and SA4 respectively for Accuracy (%Recovery) with spike of Standard Premnine HCl at 80% ,100% and 120% level.

12, 16 µg /band, done in triplicate for intraday (Three times a day with 2 hr' interval) and inter day precision

(Three day consecutively).

Precision study also checked for extract and formulation as for PI-AK and SA4 (selected for recovery and precision study) done separately in triplicate for 80%, 100% and 120% triplicate for intraday and inter day precision with 3 × 3 model for each experiment and analyzing it with % RSD.

Further result obtained were subjected for one way analysis of variance and with-day mean square compared to between -day mean square by F test [25] (Table 4b).

Robustness

Mid concentration of 12 µg/band in triplicate of standard Pr-S subjected for robustness study using variability like wavelength 283-5 nm, slit width change as 6 × 0.3 mm, scan speed change as 40 mm/s, mobile phase change of saturation time as 12 min., and mobile phase change in composition as Toluene: Acetone: Diethylamine (7.2:1.8:1) and (6.8:2.2:1).

Results and Discussion

For specificity and fingerprinting study from each manufacturer one batch selected as DA1, SA4 and BA7 and letter on for estimation study all selected nine batches were subjected for study. In specificity study with developed chromatographic condition in mobile phase Toluene: Acetone: Diethylamine (7:2:1), Rf obtained 0.59 for Pr-S HCl standard at λ max 283 nm. There is single spot over track for standard. Track of PI-AK, DA1, SA4, KA7 shows spots with similar Rf for Pr-S. Spectral scan of this selected spots gave specific overlay for of wavelength at 283 nm in all selected batches of different manufacturer. Derivetized plate with dragendorff's reagent gave orange red spot for Pr-S all sample at Rf 0.59 that confirms presences and helped for fingerprinting of Pr-S bioactive alkaloid in PI-AK and in selected formulations as shown in Figures 1a and1b. The calibration curve of Pr-S was found to be linear in range of 8-16 µg/band with good regression coefficient 0.9983. Table 1 summarize the validation parameter.

Estimation analysis of Pr-S as bio active marker for Extract PI-AK and formulation as shown in Table 2: Variability observed between manufactures and even within batches. Pr-S was selected as standard marker due to its bioactive properties and also specific content of *Premna* species that it can selectively justify presence of raw material in formulation.

Resolution of Pr-S peak in PI-AK extract and formulations while estimating are precise, checked for peak purity of the bands at start, middle and end position of chromatogram as shown in Figures 3a-3j, though co-eluted compound as peak no.1 are observed in resolution chromatogram of Figures 3f-3k, may be having structural similarity with standard Premnine HCl, but it is not alkaloid as do not give Dragendorff's test. And the peak area scanned for these peak and peak corresponds to standard marked as separate peak No. and integrated with red mark line as shown in Figures 3f-3k, noted as different with peak area by Camang -TLC scanner 5 software, therefore that value correspond to Premnine only consider for calculation. Precision study with repeatability performed over low, mid high concentration over Premnine HCl expressed as % RSD inter and intraday as per Table 3. Accuracy study with % recovery at three concentration level performed in PI-AK and in one of selected formulation SA4 shows result as per Table 4a.

Accuracy with % recovery at 80, 100,120% level with spike of Pr-S subjected for intraday and inter day, results at each level subjected

to one way analysis of variance and the F value for each level were determined as per Table 4b.

F value as ratio of BMC/WMC, compared with tabulated $F_{(2,6)}$ value which is 5.14 and all calculated values are below it [16,17], therefore there was no significant difference between intra and inter day variability, suggesting good intermediate precision of the method.

Robustness study with change in wavelength by 5 nm, slit width, scan speed for mid concentration, for Pr-S shows %RSD value as 2.003, 1.4321, 1.756 respectively. Changes in mobile phase saturation time for 10 to 12 min and composition change as 7.2: 1.8:1, 6.8:2.2:1 do not show change in Rf from 0.59 and average ng values found as 12.29, 12.34, 12.24 and % RSD 1.74,1.24,1.76 respectively. Therefore, shows no significant changes in values so method is robust.

Conclusion

With present research study of development, validation and estimation conclusion is drawn as, HPTLC method is simple, precise, rapid, robust and selective for estimation of Pr-S alkaloid in Dashmul-*P. integrifolia* chloroform extract and its marketed formulations.

Pr-S selected as standard marker due to its bioactive properties and that has proven fingerprinting and specificity parameter as unique content in *Premna* species that it can etch presence of raw material in formulation.

Although variance is observed in % content of Pr-S alkaloid between manufactures and even in batches but method is precise and accurate even on extract and formulation through validation study therefore can be recommended as method for qualitative and quantitative analysis for quality control parameter for 'dashmul' *P. integrifolia* and its formulation.

Acknowledgement

We are thankful to our Institute and management for providing facility to work over HPTLC instrument.

References

1. The Wealth of India (1972) Dictionary of Indian raw materials and industrial products, New Delhi: Council of Scientific and Industrial Research 8: 239-240.
2. Nadkarnis KM (1976) Dr. Nadkarni's KM 'The Indian Materia Medica' Bombay Popular Prakashan I: 1009.
3. Kirtikar KR, Basu BD (1984) An ICS. Indian Medicinal Plants. Dehradun, India.
4. Deepti Y, Neerja T, Madan G (2010) Diterpenoids from Premna integrifolia. Phytochemistry Letters 3: 143–147.
5. Yadav D, Tiwari N, Gupta MM (2011) Simultaneous quantification of diterpenoids in Premna integrifolia using a validated HPTLC method. Journal of Separation Science 34: 286-291.
6. Otsuka H, Watanabe E, Yusana K, Ogimi CA (1993) A verbascoside iridoid glucoside conjugate form Premna corymbosa Rott. Phytochem 32: 983-986.
7. Basu NK, Dandiya PC (1947) Chemical investigation of Premna integrifolia linn. Journal of the American Pharmaceutical Association 36: 389–391.
8. Taivini T, Bianchini J, Lafontaine AC, Cambon A (1998) Volatile constituents of the flower buds concrete of Premna serratifolia L. Journal of Essential Oil research 10: 307-309.
9. Saidatul HBS (2005) Isolation, identification of flavonoid components and antioxidant activities determination from Morinda citrifolia (Meng kudu) and Premna serratifolia (Bebuas), Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science.
10. Ravinder SC, Nelson R, Krishnan PM, Pargavi B (2011) Identification of Volatile Constituents from Premna serratifolia L. Through GC-MS. International Journal of Pharm Tech Research 3: 1050-1058.
11. Rajendran R, Suseela L, Meenakshi SR, Saleem BN (2008) Cardiac stimulant activity of bark and wood of Premna serratifolia. Bangladesh J Pharmacol 3: 107-113.
12. Rajendran R, Basha N, Ruby S (2009) Evaluation of *In Vitro* Antioxidant Activity of stem-bark and stem-wood of Premna serratifolia Lin., (Verbenaceae) Research. J Pharmacognosy and Phytochemistry 1: 11-14.
13. Rajendran R, Basha S (2010) Antimicrobial activity of crude extracts and fractions of Premna serratifolia Lin. Root. International Journal Of Phytomedicines and Related Industries 2: 33-38.
14. Rekha R, Ekambaram K (2010) Antiarthritic activity of premna serratifolia linn. Wood against adjuvant induced arthritis. Avicenna J med Biotech 2: 101-106.
15. Gokani RH, Lahiri SK, Santani DD, Shah MB (2012) Evaluation of Anti-inflammatory and Antioxidant Activity of Premna integrifolia Root. Journal of Complementary and Integrative Medicine 1: 1553-3840.
16. Reich E, Schibli A (2006) High Performance Thin Layer chromatography for the analysis of medicinal plants. Thieme New York.
17. Balton S, Bon CC (2005) Pharmaceuticals Statistics practical and clinical Applications, Marcel dekker Inc, pp: 215-220.