

## Validation and Development of HPTLC Method for Simultaneous Estimation of Apigenin and Luteolin in Selected Marketed Ayurvedic Formulations of 'Dashmula' and in Ethyl Acetate Extract of *Premna integrifolia* L.

Attarde DL<sup>1\*</sup>, Pal SC<sup>2</sup> and Bhambar RS<sup>1</sup>

<sup>1</sup>Department of Pharmacognosy, Mahatma Gandhi Vidyamandir's Pharmacy College, Panchavati, Nashik, Maharashtra, India

<sup>2</sup>Department of Pharmacognosy, RG Sapakal College of Pharmacy, Kalyani Hills, Trimbakeshwar, Nashik, Maharashtra, India

### Abstract

Dashmula are specific ayurvedic combination of ten roots used for various disorders of liver, kidney, uterus. Standardisation of herbals are necessity for efficacy and quality parameter as per WHO guidelines. Therefore, through this original research attempt was made to standardise one of 'Dashmula', *P. integrifolia* and selected batches of marketed formulation using Apigenin and Luteolin as active biological marker for simultaneous quantification and fingerprinting through developed and validated HPTLC techniques. Developed mobile phase Toluene: Ethyl acetate: Formic acid (6:4:0.15) gave R<sub>f</sub> (Retention factor) 0.39 and 0.29 for Standard Apigenin and Luteolin respectively at 347 nm iso absorptive wavelength. The ethyl acetate extract of *P. integrifolia* (PI-ET), 'Dashmularishtha': Manufacturer 1; 3 coded batches as DF1, DF2, DF3, Manufacturer 2; 3 coded batches -as BF4, BF5, BF6, 'Dashmulkadha': Manufacturer 3; 3 coded batches as KF, KF8, KF9 were found to contain : 12.8% w/w, 0.294 mg/ml%, 0.429 mg/ml%, 0.314 mg/ml%, 0.077 mg/ml%, 0.071 mg/ml%, 0.145 mg/ml%, 0.176 mg/ml%, 0.242 mg/ml%, 0.098 mg/ml% of Apigenin and 4.7% w/w, 0.542 mg/ml%, 0.365 mg/ml%, 0.569 mg/ml%, 0.343 mg/ml%, 0.311 mg/ml%, 0.607 mg/ml%, 0.812 mg/ml%, 0.828 mg/ml%, 0.439 mg/ml% of Luteolin respectively. In stem powder of *P. integrifolia* 19.84 mg/gm Apigenin and 7.433 mg/gm Luteolin was calculated. The estimation shows variance in manufacturers and even within batches, but will be quality control parameter. The method was validated for specificity, linearity, accuracy, precision, and robustness. It was found to be linear in range of 40-120 ng/band with regression coefficient 0.9983, 0.9997 for Apigenin and luteolin. Percentage recovery study carried out for extract PI-ET and DF1 with spike of Apigenin and Luteolin at 80, 100 and 120% level, carried out for inter and intraday precision, subjected for one way ANOVA and found F value is below tabulated F<sub>(2,6)</sub> value 5.14, therefore there is no significance variance of obtained values.

**Keywords:** Apigenin; Luteolin; HPTLC validation; Dashmoola; *Premna integrifolia*

### Introduction

Dashmool (dashmul, dashamula) are specific combination of ten roots, famous Ayurvedic remedy mainly for strengthening body, promotes healthy elimination of toxins, tonic for kidney, liver, tonic in infertility, uterine tonic, used for anorexia, edema, anaemia, potent antioxidant and in disorders of nerves, bones, joints and muscles. It contains ten roots in equal proportion under category as '**Brihat Panchmul**' includes; *Aegle marmelos*, *Premna integrifolia*, *Oroxylum indicum*, *Stereospermum suaveolens*, *Gmelia arborea* While '**Laghu panchmul**' includes; *Solanum indicum*, *Solanum xanthocarpum*, *Uraria picta*, *Desmodium gangeticum* and *Tribulus terrestris* [1].

More number of people worldwide switching once again towards the alternative system of medicines to avoid harmful side effects of synthetic drugs. As per WHO it is essential to establish quality standards for herbals. Thus, standardization of crude drugs and their formulations are at urgent need [2].

### *P. integrifolia* synonym(s)

*P. serratifolia* L., Large, thorny, deciduous shrub a member of Brihatpanchamula groups, known as Agni-manth, in Sanskrit; Agetha in Hindi, distributed along the coasts and islands of tropical and subtropical country. The leaves and roots have cardiotoxic, astringent, anti-inflammatory, antibacterial, anti-hypoglycemic, anticoagulant, anti-arthritis and cardio protective properties [3-7].

As per literature, diterpenoids reported from the root bark, a verbascoside iridoid glucoside from leaves [8], two alkaloids, Premnine

and Ganiarine, have been isolated from it and their physical and chemical properties described [9]. Volatile oil from flower bud, distillate and its fractions were analyzed by GC and GC/MS and the major components are 1-octen-3-ol (16.9%), (Z)-3-hexenol (10.2%) [10-12]. Isolation and evaluation of flavonoid i.e., Luteolin 7-O-methyl ether and apigenin 5,7-O-dimethyl ether from *P. serratifolia*'s leaves [13-18].

Cardiac stimulant activity of bark and wood of *P. serratifolia* [14], root extract for its anti-inflammatory, antioxidant activities, antimicrobial, Anti-Arthritic Activity [16], Cardioprotective effect of ethanol extract of stem-bark and stem-wood of *P. serratifolia* Lin., (Verbenaceae) were evaluated Tables 1 and 2.

Apigenin, 4', 5', 7-tetrahydroxy flavone, is recognized as a bioactive flavonoid shown to possess exerts anxiolytic effects by acting as a benzodiazepine ligand, anti-inflammatory, antioxidant, anticancer properties particularly cancers of the breast, digestive tract, skin,

**\*Corresponding author:** Attarde DL, Department of Pharmacognosy, Mahatma Gandhi Vidyamandir's Pharmacy College, Panchavati, Nashik-422 003, Maharashtra, India, Tel: 9730075256; E-mail: [daksha511@rediffmail.com](mailto:daksha511@rediffmail.com)

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| Parameter                           | Apigenin   | Luteolin  |
|-------------------------------------|--|---|
| Wavelength, nm                      | 340 nm   | 353 nm  |
| Isoabsorptive Wavelength            | 347 nm   | 347 nm  |
| RF Value                            | 0.39   | 0.29  |
| Linearity Range ng/band             | 40-120   | 40-120  |
| Regression equation                 | Y=37.395x+636.27   | Y=30.455x-588.62  |
| Correlation Coefficient             | 0.9983   | 0.9997  |
| Limit of Detection ng/band LOD      | 2.01 ng/Band   | 1.82 ng/Band  |
| Limit of Quantification ng/band LOQ | 5.98 ng/Band   | 5.52 ng/Band  |
| Specificity                         | Specific- Overlay Spectra, Retention factor, Derivatized Colour Parrot green at 366 nm | Specific- Overlay Spectra, Retention Factor, Derivatized Colour bright yellow at 366 nm |

Table 1: Validation parameter for estimation of apigenin and luteolin in extract and formulation.

| Analyte   | Apigenin Concentration Obtained | Luteolin concentration Obtained |
|---|---------------------------------|---------------------------------|
| PI-ET   | 12.8 % w/w                      | 4.7 % w/w                       |
| Powder of <i>P. integrifolia</i>  | 19.84 mg/gm %                   | 7.433 mg/gm%                    |
| <b>Dashmul Arishtha</b>   |                                 |                                 |
| <b>Manufacturer 1</b>   |                                 |                                 |
| Bottle size: 225 ml,<br>Label Claim: Each 100ml contains 0.52 gm of 'Dashmula' each.  |                                 |                                 |
| DF1*  | 0.294 mg/ml %                   | 0.542 mg/ml %                   |
| DF2*  | 0.429 mg/ml %                   | 0.368 mg/ml %                   |
| DF3*  | 0.314 mg/ml %                   | 0.509 mg/ml %                   |
| <b>Dashmul Arishtha</b>   |                                 |                                 |
| <b>Manufacturer 2</b>   |                                 |                                 |
| Bottle size : 220 ml,<br>Label Claim: Each 10 ml contains 0.50 gm of 'Dashmula kwath' |                                 |                                 |
| BF4**   | 0.077 mg/ml %                   | 0.343 mg/ml%                    |
| BF5**   | 0.071 mg/ml %                   | 0.311 mg/ml%                    |
| BF6**   | 0.145 mg/ml %                   | 0.607 mg/ml%                    |
| <b>Dashmul Kadha</b>  |                                 |                                 |
| <b>Manufacturer 3</b>   |                                 |                                 |
| Bottle size: 227 ml,<br>Label Claim: Each 100ml contains 418.91 mg of 'Dashmula' each |                                 |                                 |
| KF7***  | 0.176 mg/ml%                    | 0.812 mg/ml%                    |
| KF8***  | 0.242 mg/ml%                    | 0.828 mg/ml%                    |
| KF9***  | 0.098 mg/ml%                    | 0.439 mg/ml                     |

PI-ET- *Premna integrifolia* ethyl acetate extract; \*Manufacturer -1 -Three Batches Coded as DF1, DF2, DF3; \*\*Manufacturer -2- Three Batches Coded as BF4, BF5, BF6; \*\*\*Manufacturer-3 -Three Batches Coded as KF7, KF8, KF9

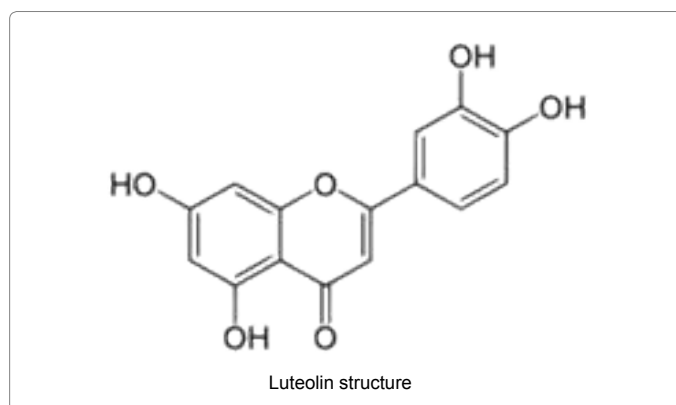
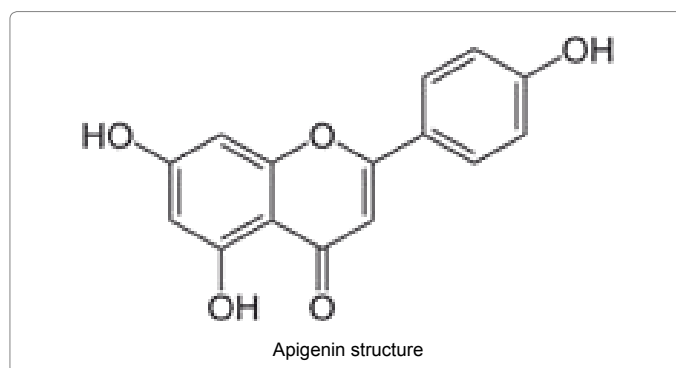
Table 2: Estimation of apigenin and luteolin in PI-ET extract and selected marketed *Dashmula* formulations.

prostate and certain hematological malignancies and even protective for cardiovascular and neurological disorders [4].

Luteolin 3',4',5,7-tetrahydroxyflavone, bioflavonoid used in Chinese traditional medicine for treating various diseases such as hypertension, inflammatory disorders, and cancer [5].

*P. integrifolia*, therefore selected here for biomarking using sophisticated analytical techniques HPTLC using bioactive markers like Apigenin and Luteolin.

This research is first attempt for standardisation of 'dashmul' using two flavonoids simultaneously, along with raw plant extract.



## Materials and Methods

### Equipment

HPTLC Instrument: CAMAG Linomet Syringe V, CAMAG TLC scanner V, CAMAG Digistore- Reprostar 3, CAMAG Twin Trough Chamber, Win CATS version 1.4.2. Software (CAMAG, Switzerland), UV chamber (CAMAG, Switzerland), Pre-coated silica gel 60 F<sub>254</sub> alumiFtablenium plates (0.2 mm thick, Merck, Germany) [19].

### Analytical reference compound and chemicals

Apigenin, (A3145-5 mg, Sigma Aldrich), Luteolin (L9283-10 mg, Sigma Aldrich), Analytical grade solvents: Pet. Ether (60-80°C),

chloroform, ethanol, ethyl acetate, Toluene, formic acid (Merck), Natural product reagent (2-aminoethyl diphenylborinate) (D9754- 5 gm, Sigma Aldrich).

### Plant material and marketed formulation

**Stem powder of *P. integrifolia*:** Agnimanth are distributed through flora of Trimbakeshwar forest area of Nashik District. Few of them identified, spotted stem branches were collected and herbarium was prepared. These 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 cut into small pieces, dried under shade for about 15 days, milled, shifted through sieve 25/30 no.-600  $\mu$ . Dried powder packed in air tight container and stored to cool, dry condition.

**Preparation ethyl acetate extract of *P. integrifolia*:** Accurately weighed 200 gm of stem powder of *P. integrifolia* charged in Soxhlet assembly and extracted exhaustively with alcohol. Alcoholic extract conc. over rotary evaporator, dried, made hydro alcoholic and fractionated with pet. ether (60-80°C), chloroform and ethyl acetate consecutively. Ethyl acetate extract concentrated over rotary evaporator, dried, yield noted and designated as PI-ET.

**Marketed formulation:** 'Dashmula arishtha': Manufacturer 1;3 batches, Manufacturer 2;3 batches, Dashmula Kadha: Manufacturer 3;3 batches, selected, batch no., volume, label claim noted and designated with codes.

**Treatment to selected marketed formulation:** Above Selected and coded batches were concentrated, made hydro alcoholic and fractionated with ethyl acetate extraction. All ethyl acetate extracts were concentrated, dried, yield noted and designated as for Manufacturer 1, 2, 3; 3 batches as DF1, DF2, DF3, BF4, BF5, BF6, KF7, KF8 and KF9 respectively.

**Standard apigenin solution:** 5 mg of standard apigenin was dissolved in 5 ml of alcohol in volumetric flask (1000 ppm), sonicated, and marked as Stock solution. Working stock prepared to obtain 20 ppm. (20 ng/ $\mu$ l) from it, designated as- AS.

**Standard luteolin solution:** 5 mg of standard Luteolin was dissolved in 5 ml of alcohol in volumetric flask (1000 ppm), sonicated and marked as Stock solution- Working stock prepared to obtain 20 ppm. (20 ng/ $\mu$ l) from it, designated as - LS.

**Ethyl acetate extract of *P. integrifolia* solution:** 10 mg of ethyl acetate extract was dissolved in 10 ml of volumetric flask (1000 ppm), sonicated, filtered through whatman filter paper No.1 and marked as stock solution. Working stock prepared as 0.5 mg/ml, designated as PI-ET.

**Chromatographic condition:** It was as follow-

Stationary phase: Pre-coated Silica Gel G 60 F<sub>254</sub> HPTLC Plates (0.2 mm Thick, Size-20 × 10 cm),

Mobile Phase: Toluene: Ethyl acetate: Formic Acid (6:4:0.15)

Saturation Time: 10 min

Wavelength: 347 nm (Iso-absorptive Point between Apigenin and Luteolin)

Lamp: Deuterium, Scanning slit width 6 × 0.45 mm, Scanning speed 20 mm/s.

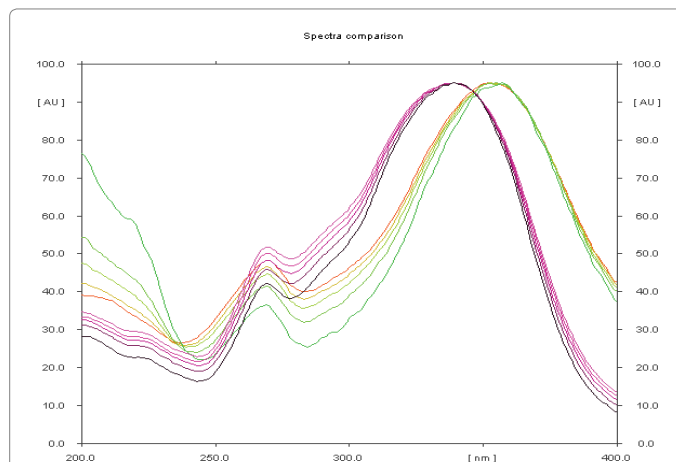


Figure 1a: Overlay spectra of apigenin and luteolin with PI-ET, DF1, BF4 and KF7.

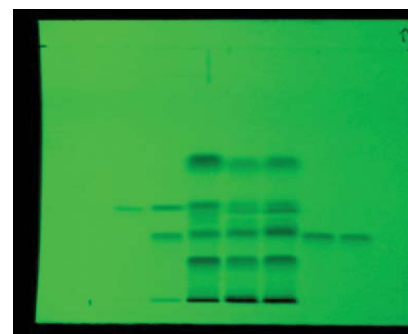


Figure 1b: Finger printing HPTLC chromatogram of standard apigenin, PI-ET, DF1, BF4, KF7 and standard luteolin at 254 nm.

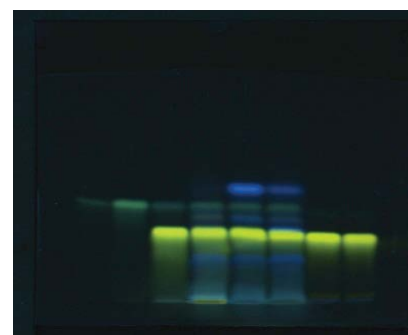


Figure 1c: After derivatization with NP-PEG reagent at 366 nm.

Sample Band length-8 mm, Solvent Run -90 mm, Area Temperature-22 ± 2°C.

Applicator Syringe-100  $\mu$ l, X-8 mm, Y-15 mm, Spray gas- Nitrogen Inert gas,

Sample Application speed 0.2  $\mu$ l/s.

Scanner- TLC scanner 5 (1.14.26), Photo documentation- Digistore- Reprostar 3

The HPTLC analysis was performed using above chromatographic condition, using Linomat 5 syringe 5  $\mu$ l, 10  $\mu$ l of AS and 5  $\mu$ l, 10  $\mu$ l of LS applied on pre coated TLC Silica gel 60 F<sub>254</sub> plates as band length of 8 mm, sample were air dried, mobile phase Toluene: Ethyl acetate: Formic acid (6:4:0.3) poured to CAMAG twin trough chamber,

saturation allowed for 10 min., spotted plates were developed till 90 mm, dried with dryer, scanned over TLC scanner 5 (1.14.26) with absorption/remission mode at scan speed 20 mm/s, spectral scan done in between 200-400 nm with 100 nm/s speed, spectra of apigenin and luteolin overlaid and iso-absorptive wavelength noted, again plates were rescanned at 347 nm in detection scanner mode. Retention Factor (Rf), AUC for both standard AS and LS noted, used the data for further detection.

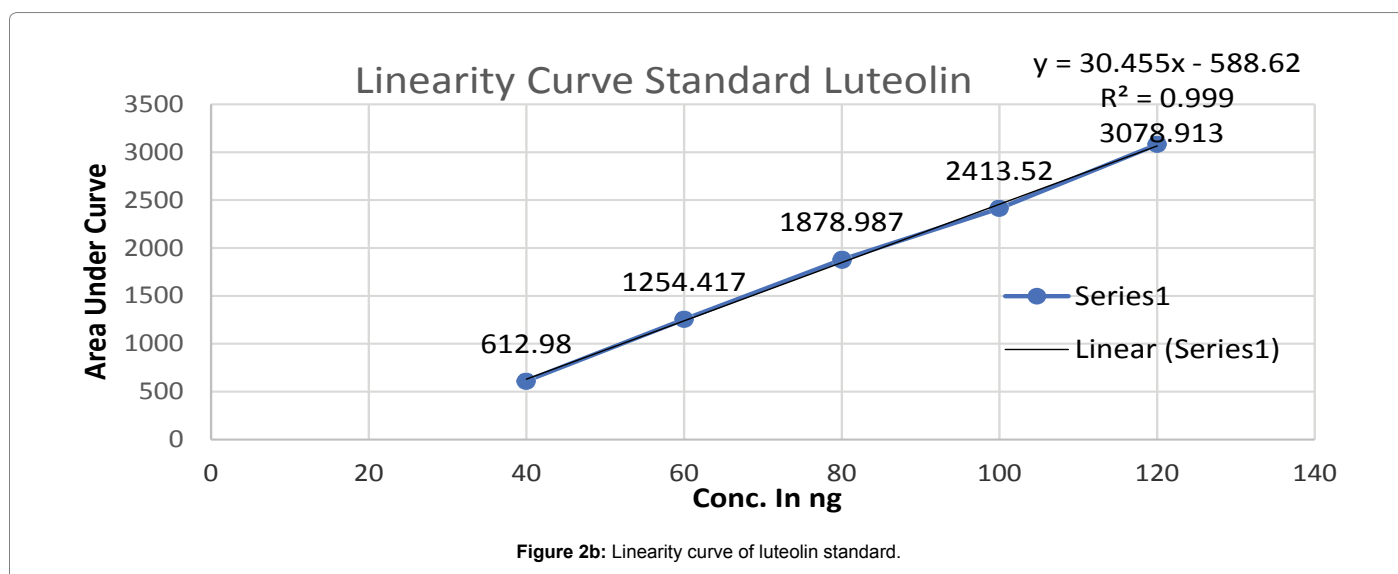
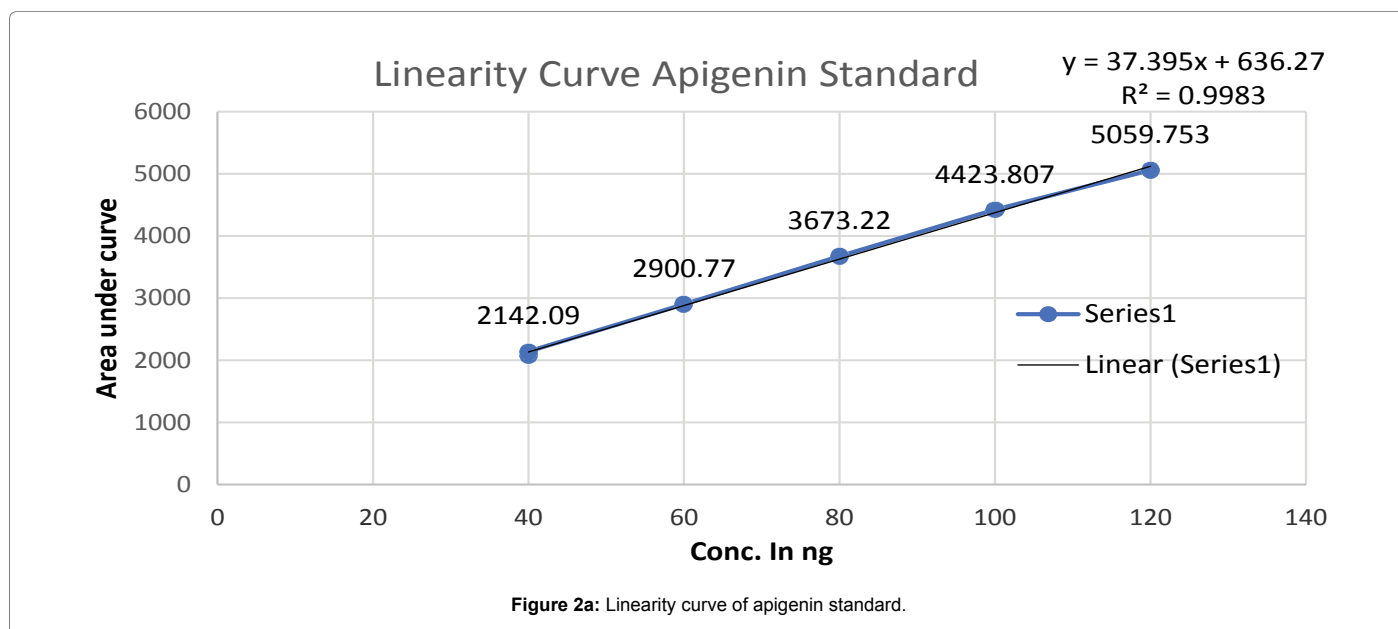
#### Fingerprinting and specificity of apigenin and luteolin standard in PI-ET and in DF1, BF4, KF7

The HPTLC analysis for fingerprinting and specificity of Apigenin and Luteolin standard done using developed parameter as above, applying on track- AS, PI-ET, DF1, BF4, and KF7, LS, plates developed as above and scanned in detection (347 nm) and spectral mode (200-400 nm). Rf obtained for AS and LS, identified and marked in PI-ET, DF1, BF4, KF7. The identified bands spectral scan over laid for confirmation

of specificity. The purity of the bands was confirmed at start, middle and end position of chromatogram. The plate was derivatized with NP-PEG reagent and chromatogram visualized at 366 nm in fluorescence mode as shown in Figures 1a-1c.

#### Linearity calibration curve for standard apigenin and standard luteolin and analysis of PI-ET

For simultaneous estimation, over same plate samples applied as AS-2, 2, 3, 4, 5, 6  $\mu$ l (40 to 120 ng), PI-ET- 1, 1.5, 3  $\mu$ l (0.5 mg/ml), LS-2, 3, 4, 5, 6, 6  $\mu$ l (40 to 120 ng) over track 1 to 15 with band length 8 mm. Chromatogram developed till 90 mm in above mobile phase, scanned at 347 nm and recorded as per above developed condition at Rf- 0.39 for Apigenin and Rf-0.29 for Luteolin. Such procedure repeated thrice and average Area Under Curve(AUC) noted. Standard linearity calibration curve for both standard prepared separately by plotting AUC vs conc. in ng, using linearity calibration curve of apigenin and luteolin concentration in PI-ET calculated as per Figures 2a and 2b.



| Concentration <sup>T</sup> (ng/band) | Average Concentration <sup>a</sup> (n=3) Intraday (ng/band) | Intra day % RSD | Average Concentration <sup>a</sup> (n=3) Inter day (ng/band) | Inter day % RSD |
|--------------------------------------|---|-----------------|--|-----------------|
| 40                                   | 37.85   | 1.3403          | 37.38  | 1.8197          |
| 80                                   | 77.37   | 0.7785          | 77.12  | 0.4437          |
| 120                                  | 115.51  | 0.5200          | 114.85   | 0.7487          |

T- Theroretical, a- Obtained, %RSD- Relative Standard Deviation

**Table 3a:** Intermediate precision study for standard apigenin.

| Concentration <sup>T</sup> (ng/band) | Average Concentration <sup>a</sup> (n=3) Intraday (ng/band) | Intra day % RSD | Average Concentration <sup>a</sup> (n=3) Inter day (ng/band) | Inter day % RSD |
|--------------------------------------|---|-----------------|--|-----------------|
| 40                                   | 39.11   | 0.5920          | 38.96  | 0.3632          |
| 80                                   | 77.68   | 0.5661          | 77.04  | 1.0246          |
| 120                                  | 119.95  | 1.3306          | 119.24   | 0.5218          |

T- Theroretical, a- Obtained, % RSD- Relative Standard Deviation

**Table 3b:** Intermediate precision study for standard luteolin.

|                 | Level of % Recovery | Amount In PI-ET (ng) | Amount of Standard Added (ng) | Total amount of Standard taken (ng) | Total amount of Standard obtained (ng) | % recovery ± SD (n=3) |
|-----------------|---------------------|----------------------|-------------------------------|-------------------------------------|--|-----------------------|
| <b>In PI-ET</b> |                     |                      |                               |                                     |  |                       |
| <b>AS</b>       | 80%                 | 44.83                | 35.86                         | 80.69                               | 88.18                                  | 109.29 ± 0.345        |
|                 | 100%                | 44.83                | 44.83                         | 89.66                               | 90.64                                  | 101.10 ± 0.567        |
|                 | 120%                | 44.83                | 53.79                         | 98.62                               | 102.81                                 | 107.25 ± 0.234        |
| <b>LS</b>       | 80%                 | 43.16                | 34.52                         | 77.68                               | 82.42                                  | 106.11 ± 0.385        |
|                 | 100%                | 43.16                | 43.16                         | 86.12                               | 81.03                                  | 94.09 ± 0.583         |
|                 | 120%                | 43.16                | 51.79                         | 94.95                               | 92.65                                  | 97.58 ± 0.241         |
| <b>In DF1</b>   |                     |                      |                               |                                     |  |                       |
| <b>AS</b>       | 80%                 | 39.27                | 31.42                         | 70.69                               | 68.87                                  | 97.43 ± 0.365         |
|                 | 100%                | 39.27                | 39.27                         | 78.54                               | 81.52                                  | 103.80 ± 0.765        |
|                 | 120%                | 39.27                | 47.12                         | 86.39                               | 88.12                                  | 102.01 ± 0.376        |
| <b>LS</b>       | 80%                 | 36.21                | 28.96                         | 65.17                               | 64.98                                  | 99.72 ± 0.287         |
|                 | 100%                | 36.21                | 36.21                         | 72.42                               | 72.65                                  | 100.33 ± 0.456        |
|                 | 120%                | 36.21                | 43.45                         | 79.66                               | 76.76                                  | 96.37 ± 0.345         |

PI-ET- *Premna integrifolia* ethyl acetate extract, DF1- Manufacture 1 -coded Batch, AS- Apigenin Standard, LS- Luteolin Standard.

**Table 4a:** Accuracy (% Recovery) for PI-ET extract and DF1 extract with spike of standard apigenin and luteolin at 80%, 100% and 120% level [18].

| (ng) Amount     | (n=3)      | Amount of Standard Obtained in ng |        |        | WMS      | BMS      | F value         |
|-----------------|------------|-----------------------------------|--------|--------|----------|----------|-----------------|
|                 |            | Day 1                             | Day 2  | Day 3  |          |          |                 |
| <b>PI-ET+AS</b> |            |                                   |        |        |          |          |                 |
| <b>80 %</b>     | Mean       | 91.54                             | 90.34  | 91.02  | 0.994011 | 1.086944 | <b>1.093493</b> |
| <b>(80.69)</b>  | %RSD       | 0.493                             | 1.428  | 1.159  |          |          |                 |
| <b>100%</b>     | Mean       | 95.63                             | 96.69  | 97.89  | 1.800944 | 0.732044 | <b>0.406478</b> |
| <b>(89.66)</b>  | %RSD       | 1.176                             | 1.613  | 1.279  |          |          |                 |
| <b>120%</b>     | Mean       | 104.31                            | 103.39 | 105.16 | 2.706011 | 2.341944 | <b>0.86546</b>  |
| <b>(98.62)</b>  | %RSD       | 1.465                             | 1.649  | 1.168  |          |          |                 |
| <b>PI-ET+LS</b> |            |                                   |        |        |          |          |                 |
| <b>80%</b>      | Mean       | 81.153                            | 81.08  | 81.33  | 0.341867 | 0.0499   | <b>0.145963</b> |
| <b>(77.68)</b>  | %RSD       | 0.878                             | 1.034  | 0.429  |          |          |                 |
| <b>100%</b>     | Mean       | 83.23                             | 82.85  | 82.88  | 0.36     | 0.132844 | <b>0.369012</b> |
| <b>(86.12)</b>  | %RSD       | 1.102                             | 0.864  | 0.244  |          |          |                 |
| <b>120%</b>     | Mean (n=3) | 90.16                             | 90.02  | 89.26  | 1.081278 | 0.707078 | <b>0.653928</b> |
| <b>(94.95)</b>  | %RSD       | 0.477                             | 1.190  | 1.548  |          |          |                 |

PI-ET- *Premna integrifolia* ethyl acetate extract, AS- Apigenin Standard, LS- Luteolin standard, WMS- Within Mean Square, BMS- Between Mean Square, F Value=BMS/WMS

**Table 4b:** Precision study for PI-ET with spike of standard apigenin and luteolin at 80%, 100% and 120% level (One Way ANOVA) [18].

| (ng) Amount    | (n=3) | Amount of Standard Obtained in ng |        |        | WMS      | BMS      | F value         |
|----------------|-------|-----------------------------------|--------|--------|----------|----------|-----------------|
|                |       | Day 1                             | Day 2  | Day 3  |          |          |                 |
| <b>DF1+AS</b>  |       |                                   |        |        |          |          |                 |
| <b>80%</b>     | Mean  | 63.21                             | 63.11  | 64.65  | 0.641533 | 2.217678 | <b>3.45684</b>  |
| <b>(70.69)</b> | %RSD  | 1.2026                            | 0.7697 | 1.6299 |          |          |                 |
| <b>100%</b>    | Mean  | 78.94                             | 80.75  | 79.34  | 0.665444 | 2.697411 | <b>4.053548</b> |
| <b>(78.54)</b> | %RSD  | 0.8183                            | 1.0619 | 1.1576 |          |          |                 |
| <b>120%</b>    | Mean  | 85.16                             | 85.56  | 87.31  | 3.287878 | 3.935511 | <b>1.196976</b> |
| <b>(86.39)</b> | %RSD  | 1.86                              | 1.27   | 1.84   |          |          |                 |
| <b>DF1+LS</b>  |       |                                   |        |        |          |          |                 |
| <b>80%</b>     | Mean  | 65.59                             | 65.99  | 65.44  | 0.265833 | 0.2425   | <b>0.912226</b> |
| <b>(65.17)</b> | %RSD  | 0.5755                            | 1.003  | 0.7116 |          |          |                 |
| <b>100%</b>    | Mean  | 70.08                             | 70.00  | 69.80  | 0.120867 | 0.064011 | <b>0.529601</b> |
| <b>(72.42)</b> | %RSD  | 0.6060                            | 0.5103 | 0.3345 |          |          |                 |
| <b>120%</b>    | Mean  | 75.27                             | 75.38  | 75.20  | 0.513667 | 0.024844 | <b>0.048367</b> |
| <b>(79.66)</b> | %RSD  | 0.9726                            | 0.8613 | 1.015  |          |          |                 |

WMS- Within Mean Square, BMS- Between Mean Square, F Value=BMS/WMS, DF1- Manufacture-1-coded Batch, AS- Apigenin Standard, LS- Luteolin standard

**Table 4c:** Precision study for DF1 with spike of standard apigenin and luteolin at 80% ,100% and 120% level (One Way ANOVA) [19].

**HPTLC analysis of marketed formulation:** Samples were applied over TLC plates as AS-2, 4, 6 µl, DF1-4.2 µl, DF2-4.2 µl, DF3-4.2 µl, BF4-4.0 µl, BF5-4.0 µl, KF7-3.5 µl, KF8- 3.5 µl, KF9- 3.5 µl, LS-2,4,6 µl on track 1 to 15 tracks respectively. Chromatogram developed till 90 mm in above mobile phase, scanned at 347 nm and recorded as per above developed condition at Rf 0.39 for Apigenin and Rf-0.29 for Luteolin. Such procedure repeated thrice and average AUC noted. As per standard linearity calibration curve for both standard apigenin and luteolin concentration in DF1, DF2, DF3, BF4, BF5, BF6, KF7, KF8 and KF9 calculated as Figures 2a and 2b.

**Validation of HPTLC method:** The method was validated as per International Conference on Harmonization Guidelines- Q2 (R1), 2005. Linearity for Apigenin and luteolin standard were studied in range of 40-120 ng /band for both. The calibration curve developed by plotting Peak area vs Conc. in ng, regression equation with slope, intercept and coefficient of correlation was calculated Table 3.

The Limit of detection (LOD) and limit of quantification (LOQ) were calculated using equation:

$$LOD = \frac{3.3 \times \text{Standard deviation of the Y - intercept}}{\text{Slope of the calibration curve}}$$

$$LOQ = \frac{10 \times \text{Standard deviation of the Y - intercept}}{\text{Slope of the calibration curve}}$$

**Accuracy (% recovery):** The accuracy of the method was established by performing recovery experiments over known concentration on Extract -PI-ET and formulation DF1 at 80%, 100% and 120% spike of AS and LS separately in triplicate for each experiment and analyzing it with %RSD. As per Table 4a.

**Intermediate precision:** Precision study for Standard AS and LS done for three concentration as 40, 80, 120 ng/band, done in triplicate for intraday and inter day precision.

Precision study over recovery method for PI-ET and DF1 done separately in triplicate for 80%, 100% and 120% spike of AS and LS 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 (Tables 4b and 4c).

**Repeatability precision:** The repeatability of the method was assessed by three concentration 40,80,120 ng/band in triplicate for each AS and LS done. The percentage relative standard deviation was expressed as Tables 3a and 3b.

**Robustness:** Mid concentration of 80 ng/band in triplicate of both standards subjected for robustness study using variability like wavelength 347-5 nm, slit width change as 6 × 0.3 mm, scan speed change as 40 mm/s.

## Results and Discussion

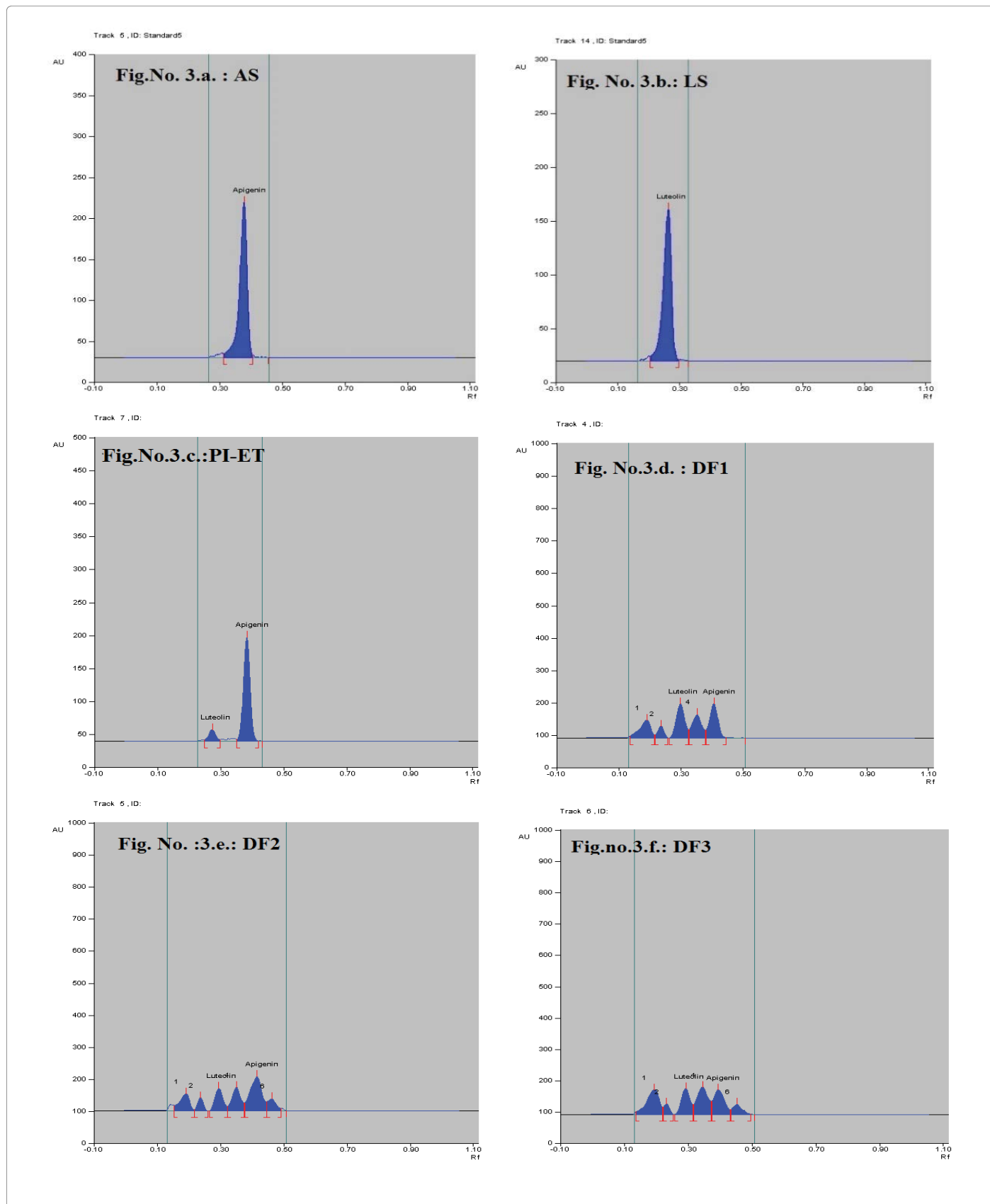
In fingerprinting and specificity study with developed chromatographic condition in mobile phase Toluene: Ethyl Acetate: formic acid (6:4:0.3), Rf- obtained 0.39 and 0.29 for standard apigenin and standard luteolin respectively. There is single spot over track for standard. Track of PI-ET, DF1, BF4, KF7 shows spots with similar Rf for Apigenin and luteolin. Spectral scan of this selected spots gave specific overlay for Apigenin and Luteolin. Upon derivatization with NP-PEG reagent Spot of apigenin and luteolin appears bright yellow visually in day light, parrot green and bright yellow in florescence mode at 366 nm respectively.

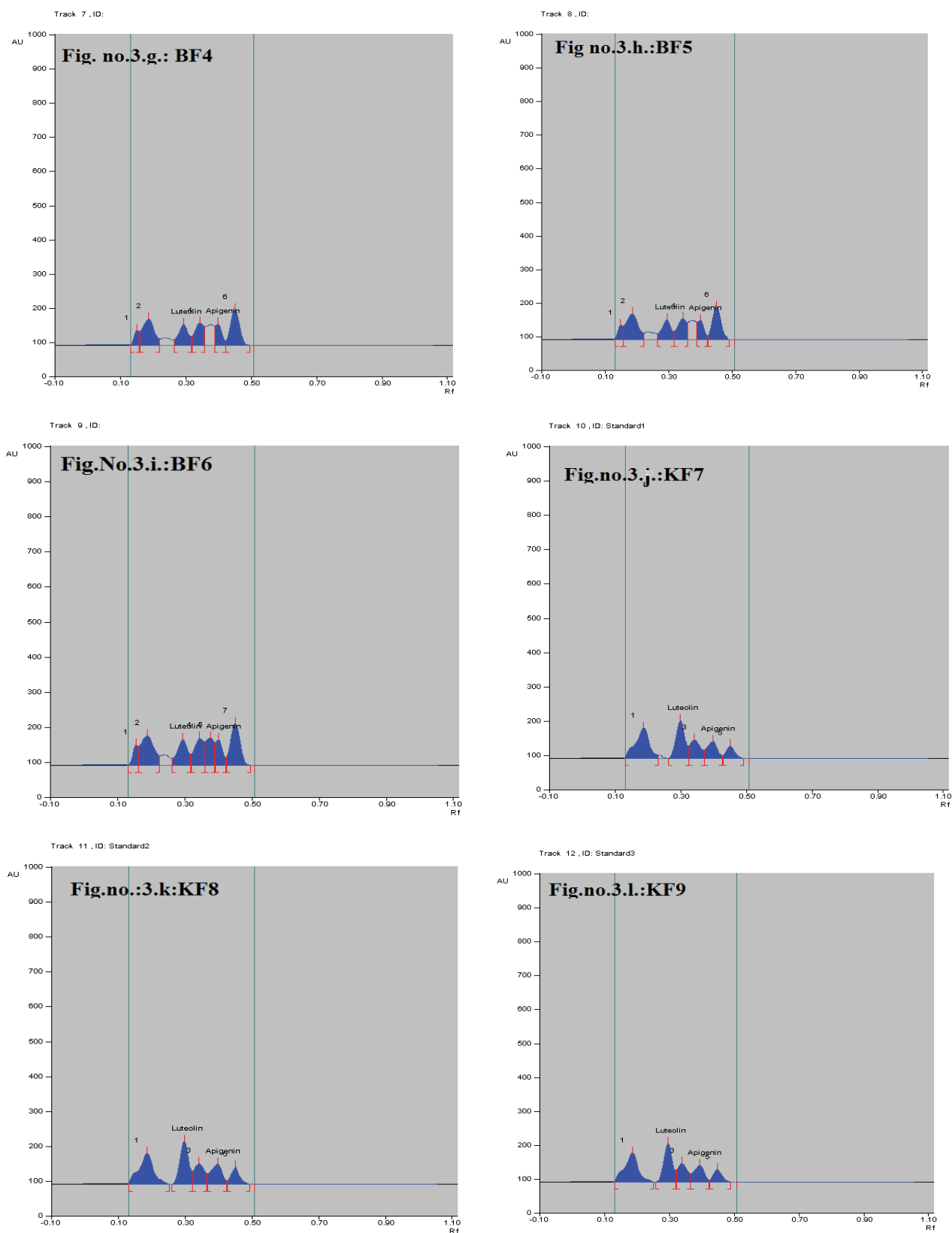
That confirms presences and helped for fingerprinting of Apigenin and luteolin bioactive flavonoids in PI-ET and in selected formulations.

The calibration curve of Standard Apigenin and Standard Luteolin was found to be linear in range of 40-120 ng/band for both, with good regression coefficient 0.9983, 0.9997 for Apigenin and luteolin respectively. Table 1 summaries the validation parameter.

Estimation analysis for Extract PI-ET and formulation as shown in Table 2 indicates high %value for PI-ET extract due to highly purified flavonoid fraction from extract. Variability observed between manufactures and even within batches.

Resolution of extract and formulations are better while estimating apigenin and luteolin simultaneously as shown in Figures 3a-3l.





Figures 3a-3l: HPTLC resolution chromatogram of standard apigenin, standard luteolin, PI-ET extract, DF1, DF2, DF3, DF4, DF4, DF5, DF6, KF7, KF8, and KF9 marketed formulation respectively.



Accuracy study with %recovery at three concentration level performed in PI-ET and in one of formulation DF1 shows result as per Table 4a Precision study with repeatability performed over low, mid high concentration over both standard expressed as %relative standard deviation inters and intraday as per Tables 3a and 3b.

Accuracy with% recovery in PI-ET and DF1 at 80, 100, 120 %level with spike of standard and luteolin 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 Tables 4b and 4c. 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, 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 standard Apigenin and luteolin shows %RSD value as 1.4370, 1.3355, 0.7955, 1.2563, 1.1729, 0.6875 respectively.

## Conclusion

Hence with regards to the method development, validation and simultaneous estimation, it can be concluded that,

- HPTLC method is simple, precise, rapid and selective for simultaneous estimation of bioflavonoid Apigenin and Luteolin in Dashmul- *P. integrifolia* stem and ethyl acetate extract and marketed formulations.
- PI-ET shows high %of Apigenin and luteolin due to highly purified fraction of extract.
- The result of this study though shows great variance in %content of this bioflavonoids between manufactures and even in batches but method is precise and accurate through validation therefore can be method of analysis qualitative and quantitative for quality control parameter for herbals.
- HPTLC method development for standardization of Ayurvedic 'Dashmula formulation' gives high throughput results.

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