4.1 SIMULTANEOUS SPECTROFLUORIMETRIC DETERMINATION

Instruments that measure the intensity of fluorescence are called fluorimeters. Those that measure the fluorescence intensity at variable wavelengths of excitation and emission and are able to produce fluorescence spectra are called spectrofluorimeters (Beckett & Stanlake, 2005). A typical spectrofluorimeter essentially consists of an excitation source, usually a mercury or xenon lamp for illumination of the sample at 90°angle. The source is dispersed by a prism or gratting blazed for high efficiency at shorter wavelengths and another prism or gratting blazed at somewhat greater wavelengths to disperse the emitted fluorescence. The fluorescent intensity is measured with a photo tube attached to a micrometer and a recorder or an oscilloscope.

Two types of informations may be made with the help of spectrofluorimeter

(1.) The wavelength of best excitation.

(2.) Wavelength of the strongest emission.

Advantages

- The method is very sensitive, about one part in 10^8 or 1.0μg/mL in many determinations.
- Fluorimetric methods possess greater specificity than spectrophotometric methods, because there is a choice of wavelength not only for the radiation emitted, but also for the light, which excites it.
- It is one of the newer methods and its potentialities are still largely unexplored.
- The fluorimeter characterstics affect not only the sensitivity, but also the precision. An analytical precision of one part per 100 is a reasonable goal in a careful work with a good instrument, except for the lower concentration ranges (Sharma, 1999).

4.1.1.4 Determination of scopoletin and mangiferin concentration in methanolic extracts Shankhpushpi botanicals

10 mg of methanolic extracts of various Shankhpushpi botanicals were weighed accurately and dissolved in 10 ml of methanol with vigorous shaking. These were filtered and volume made to 100 ml in methanol. These solutions were analyzed in the spectrofluorimeter at different excitation and emission spectra for mangiferin and scopoletin as shown in preliminary analysis and intensity of fluorescence were recorded. Further, concentration of both scopoletin and mangiferin in the extract samples were determined from the standard curves of the same.

4.1.1.5 Spectrofluorimetric Method Development for estimation of Scopoletin and mangiferin concentration in *Shankhpushpi botanicals*

After the success of spectrofluorimeteric analysis in determining the concentration of scopoletin and mangiferin simultaneously in methanolic extracts of *C. decussata* Schult. and Scopoletin in EA CP and CT, it was thought worthwhile to develop a method for the estimation of scopoletin and mangiferin concentration in crude drug samples.

For this purpose, 1 gm shade dried powdered crude drug sample of four botanicals of Shankhpushpi were taken. These were subjected to hot methanolic extraction. The methanolic extracts were obtained after filtration. The methanolic extracts were taken in a volumetric flask and volume was made up to 100 ml with methanol. The intensity of these diluted samples was determined by spectrofluorimeter. Whole procedure was repeated thrice to get triplicate readings.

After the spectrofluorimeteric analysis by crude methanolic extract, the concentration of scopoletin and mangiferin in all the samples were found out by extrapolating from the standard curves (Fig 4.1 and Fig 4.2). Mean concentration of scopoletin and mangiferin present in 1 g of crude drug was, thus determined.

After determining the concentration of scopoletin and mangiferin per ml of the methanolic extract, the mean concentration per gm of crude drug (% w/w) was calculated. The % yield per gram of scopoletin and mangiferin in crude drug powder of Shankhpushpi botanicals were found to be as shown in the table 4.1.

Thus a simple spectrofluorimeteric method for analysis of scopoletin and mangiferin in shankhpushpi was developed. Further recovery studies were performed for the validation of this novel analytical method.

Table 4.1: Percent yields of scopoletin and mangiferin (% w/w) in methanolic extract of *Shankhpushpi botanicals*

Drug sample	Method of extraction	Mean concentration (mg/ml.)		% Yield (g/100g of crude drug)	
		Scopoletin	Mangiferin	Scopoletin	Mangiferin
EA	Hot Percolation	0.020±0.021	-	0.0020	-
CP	Hot Percolation	0.023±0.004	-	0.0023	-
CT	Hot Percolation	0.004±0.003	-	0.0003	-
CD	Hot Percolation	0.008±0.12	0.0014±0.24	0.0004	0.0007

4.1.2 ANALYTICAL METHOD VALIDATION

4.1.2.1 Validation of Linearity

Standard solutions (5 µg/ml to 30 µg/ml) were prepared in methanol and intensity of fluorescence was recorded in the spectrofluorimeter. The standard curves were prepared by plotting concentration as abscissa versus intensity of fluorescence as ordinate. Linear dependence of intensity on concentration was observed throughout the concentration range tested (Table 4.2 and Table 4.3).

4.1.2.2 Validation of Precision and Accuracy

The precision of the method was checked for standard solutions of the methanolic extract at a concentration of 0.1µg/ml, 0.2µg/ml, 0.5µg/ml and 1.0 µg/ml, prepared by appropriate dilutions with methanol. The solutions were analyzed in a spectrofluorimeter at 430-460 nm for scopoletin and 248-520 nm for mangiferin and then intensity was recorded. The corresponding concentration was extrapolated from the standard curve. The whole procedure was repeated thrice for each dilution and the readings were expressed as Mean± S.E.M. (n=3). Then 0.1 µg/ml solutions of scopoletin and mangiferin were prepared by appropriate dilutions and analyzed spectrofluorimetrically. The concentration of scopoletin and mangiferin were calculated for the sample.

These samples of known concentration were added in equal volume (1 ml) to all the previous dilutions, and analyzed to see whether the practical concentration obtained is in correspondence with the theoretical or hypothetical concentration from the standard curve. Percentage recoveries were calculated on the basis of determination of analyte added to a sample containing a known amount of scopoletin and mangiferin (Table 4.4 and 4.5).

Table 4.2 Standard curve of scopoletin in methanol ($\lambda_{max\ excitation}$ 430; $\lambda_{max\ emission}$ 460)

S.No.	Concentration (µg/ml)	Absorbance	Regressed values	Statistical parameters
1.	5	162.375	132.062	
2.	10	278.18	282.522	**Equation of line:**
3.	15	398.542	432.982	$y = 51.57x - 114.06$
4.	20	573.664	583.442	$R^2 = 0.9919$
5.	25	722.558	733.902	
6.	30	913.94	884.362	

Table 4.3 Standard curve of mangiferin in methanol ($\lambda_{max\ excitation}$ 430; $\lambda_{max\ emission}$ 460)

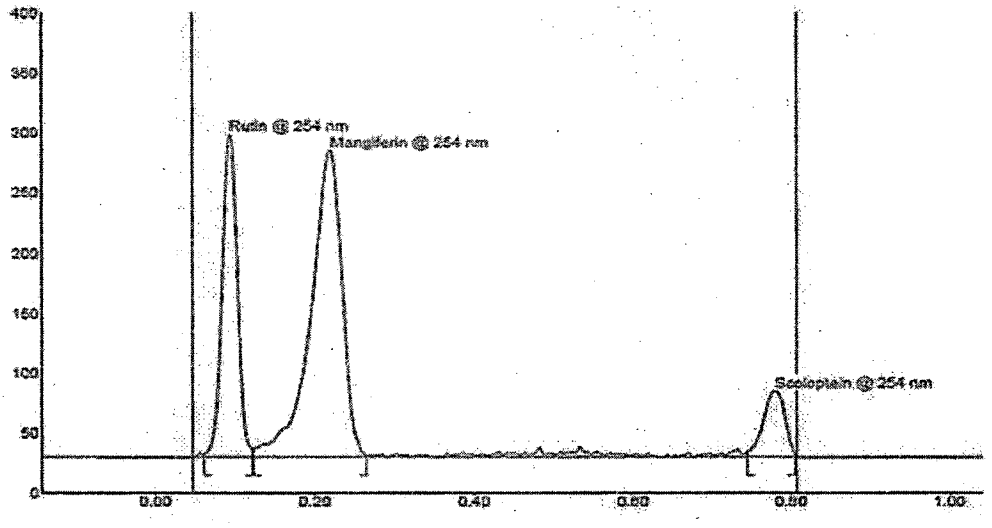
S.No.	Concentration (µg/ml)	Absorbance	Regressed values	Statistical parameters
1.	5	133.229	143.79	
2.	10	373.09	401.64	**Equation of line:**
3.	15	669.758	659.49	$y = 30.092x - 18.398$
4.	20	973.564	917.34	$R^2 = 0.9937$
5.	25	1198.861	1175.19	
6.	30	1381.953	1433.04	

with methanol and 5 g of dried concentrated syrup was taken in 50 ml of Methanol for extraction. All chemical standard markers, extracts and marketed formulation were prepared in the form of stock solution (10mg/10 ml).

4.2.4 Simultaneous estimation and validation of HPTLC method for scopoletin, mangiferin, and rutin for the identification and differentiation on four commercialized shankhpushpi ethanobotanicals in herbal formulation and extracts.

4.2.4.1 Chromatographic Studies

Thin layer chromatographic (TLC) studies were performed using various solvent systems, and finally Ethyl acetate: Acetic acid: Formic acid: Water (10:0.5:0.5: 1.5, v/v) was found to be suitable mobile phase for the proper separation of scopoletin mangiferin and rutin in a single track (Figure 4.3). Chemical structures of these markers are given in figure 4.4. These markers were further fingerprint with various samples of shankhpushpi and its marketed formulation, to ascertain their presence (Figure 4.5, 4.6 & 4.7).



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.06	2.8	0.09	269.1	46.41	0.12	7.0	4217.3	30.92	Rutin
2	0.12	7.3	0.22	255.6	44.09	0.27	1.1	8177.5	59.95	Mangiferin
3	0.75	4.3	0.78	55.1	9.51	0.81	1.9	1246.1	9.14	Scoloptein

Figure 4.3 Simultaneous Chromatogram of scopoletin, mangiferin, and rutin

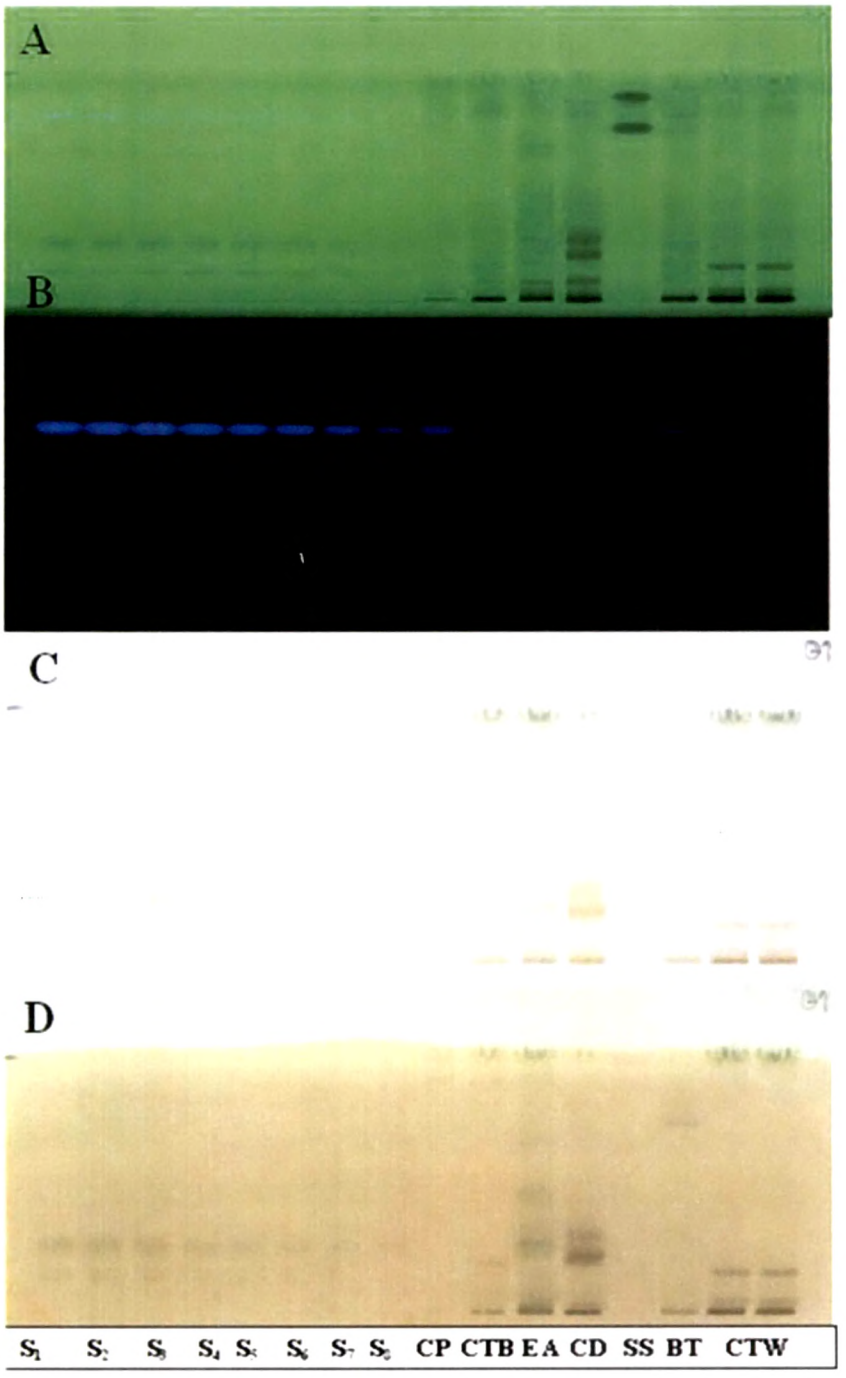


Figure 4.6 TLC plates (A) 254 nm (UV); (B) 366 nm (UV); (C) Visible mode (D) After $FeCl_3$ treatment

Where S_1-S_8 – Equimixing Standard of Scopoletin, Mangiferin and rutin in the concentration range 200-1600 ng/µL.

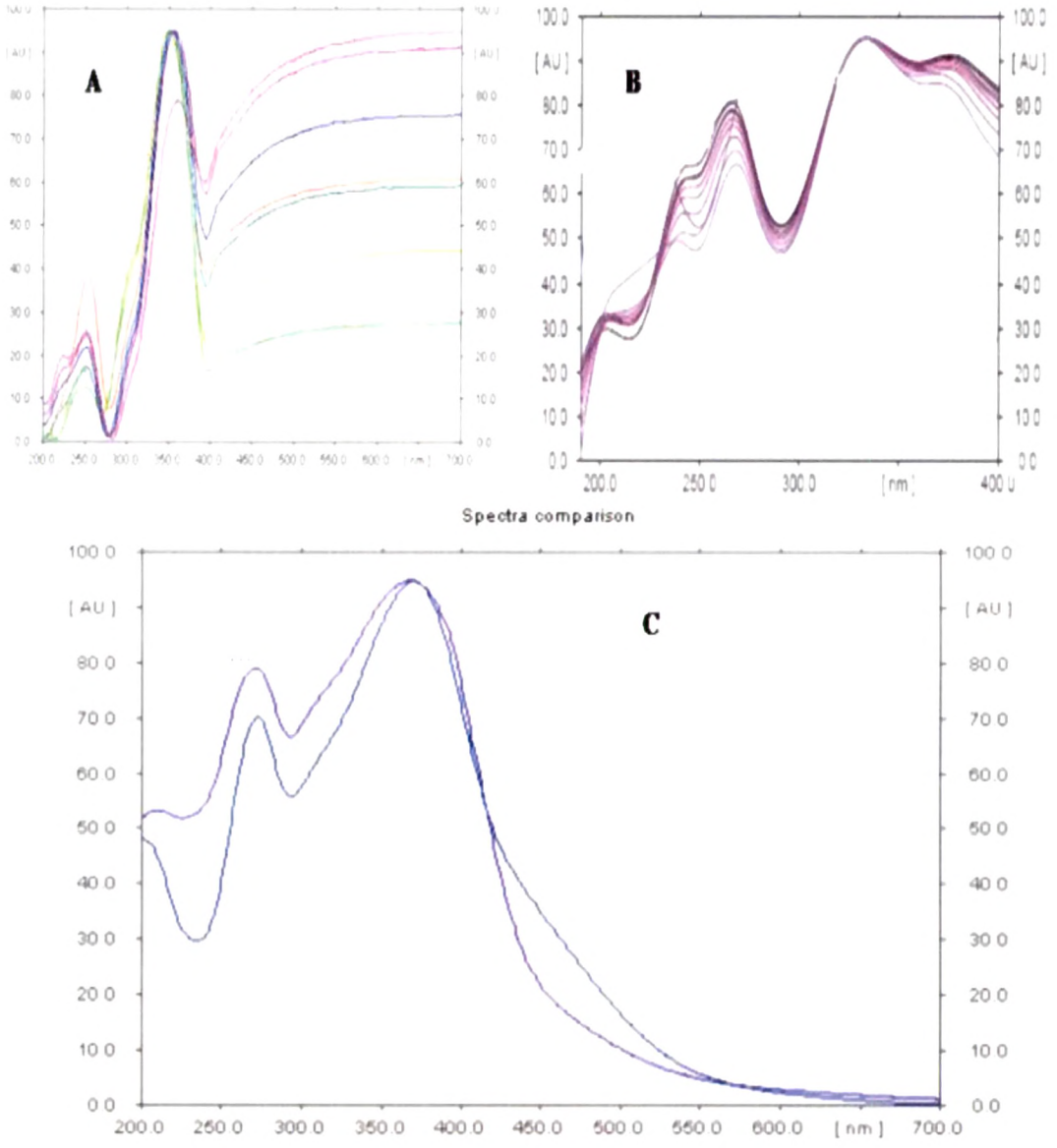


Figure 4.7 UV spectral overlain (A) Scopoletin versus all samples (B) Mangiferin versus CD and BT (C) Rutin versus CT.

4.2.4.2 Calibration of scopoletin, mangiferin and rutin and their analysis in different shankhpushpi extract and formulation

Different concentrations (200-1200 ng/spot) of scopoletin, mangiferin, and rutin were plotted against peak area to obtain a calibration plot. 20 µL aliquots of the extract solution and extracts of marketed formulation (1 mg/mL) were applied. After chromatography, the amount of scopoletin, mangiferin and rutin present in the respective extracts and formulation were determined by means of their calibration plot (Figure 4.8).

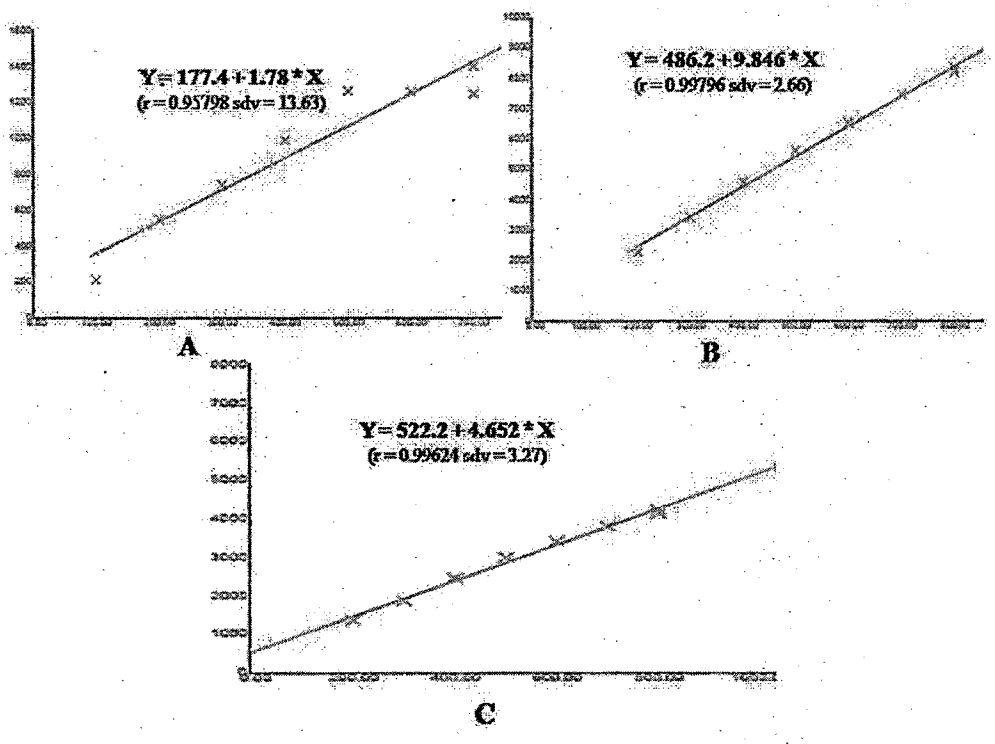


Figure 4.8 Calibration plot for (A) Scopoletin (B) Mangiferin (C) Rutin

4.2.5 Method Validation

4.2.5.1 Accuracy and precision

Repeatability of the sample application and measurement of peak area were carried out using six replicates of the same spot (500 ng/spot) of scopoletin, mangiferin and rutin, which were expressed in terms of percentage relative standard deviation (% RSD) and standard error (SE). The intra-day precision were determined at three difference concentration levels of different marker 300, 500 and 900 ng/spot,

three times on the same day and inter-day precision were determined at three difference concentrations of markers 300, 500 and 900 ng/spot, three times on five different interval days over a period of one week (Kumar et al., 2008).

4.2.5.2 Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition like Ethyl acetate: Acetic acid: Formic acid: Water (10:0.5:0.5: 1.5, v/v), Ethyl acetate: Acetic acid: Formic acid: Water (9: 0.8:0.5: 1, v/v) were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity were varied in the range of ± 5%. The plates were prewashed by methanol and activated at 60^{O}C ± 5 for 2, 5, 7 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. Robustness of the method was done at three different concentration levels 300, 500 and 900 ng/spot (Agrawal et al., 2004).

4.2.5.3 Limits of detection and quantification

Three different levels (100, 300 and 500 ng/mL) of the different standard stock solution (1 mg /mL) of scopoletin, mangiferin and rutin were prepared. Blank methanol were spotted six times following the same method as explained in instrument and chromatographic conditions and the signal-to-noise ratio was determined. The compounds were identified on the basis of its R_f values and UV-Vis spectral overlaying of the standard compounds. Standards were diluted and applied on TLC plate to plot the calibration curves. LOD was determined based on the lowest concentration detected by the instrument from the standard while the LOQ was determined based on the lowest concentration quantified in the sample (Kumar et al., 2008).

4.2.5.4 Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spots for scopoletin, mangiferin and rutin in sample were confirmed by comparing the R_f and spectra of the spot with that of standard. The peak purity of scopoletin, mangiferin and rutin were assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot (Agrawal et al., 2004).

4.2.5.5 Recovery studies

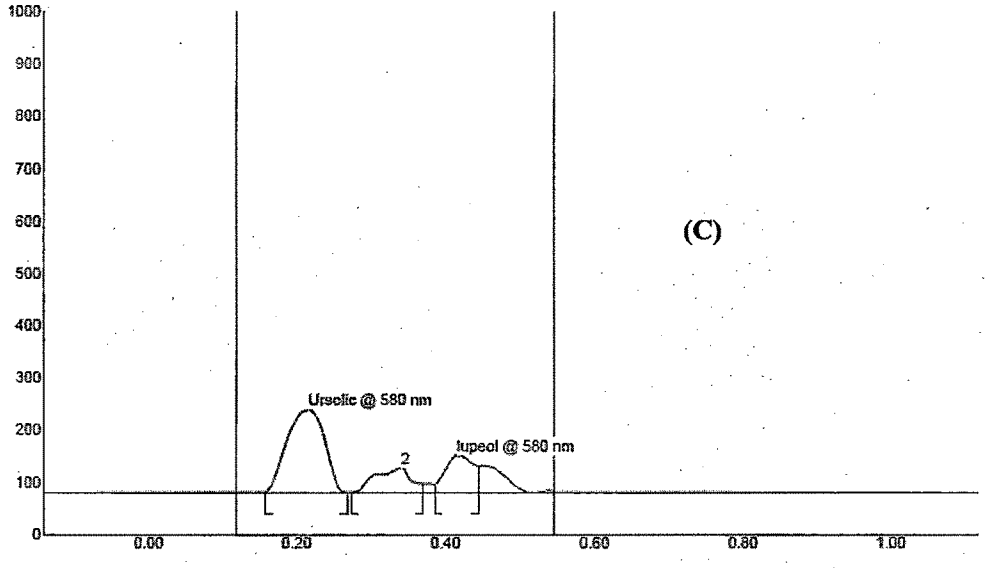
To study the accuracy and precision of the method, recovery studies were performed by the method of standard addition. The recovery of added standard was studied at three different levels, each being analysed in a manner similar to that described for the assay (Kumar et al., 2008; Agrawal et al., 2004; Biringanine et al., 2006; Wagner et al., 2008; Abourashed & Mossa, 2004). The methanolic extract of CD, CT, EA, CP, BT and SS were used for recovery studies. These were preanalysed by the developed method as mentioned in the experimental sections and found to contain 16 mg and 13.1 mg of rutin per gram of CT white flower and CT blue flower extract respectively, 44 mg and 8 mg of mangiferin per gram of CD and BT extract, 18.95, 9.35, 8.8, 4.5, 5.2, and 4.5 mg of scopoletin per gram of CP, EA, SS, BT, CT and CD extract respectively. Thus 20 µg of the same pre-analysed extracts contained 160 and 103 ng/spot of rutin in CTW and CTB respectively, 880 and 180 ng/spot of mangiferin in CD and BT, 379, 187, 176, 90, 104 and 90 ng/spot of scopoletin in CP, EA, SS, BT, CT and CD respectively, which were used for the recovery studies. The pre-analysed extract samples of all were spiked with an extra 1:2 ratio i.e. 100 ng/spot and 200 ng/spot of the respective standard of scopoletin, mangiferin and rutin content of respective sample, which were reanalysed by the proposed method. The experiments were conducted six times to check for the recovery of markers.

4.2.6 Simultaneous estimation and validation of HPTLC method for ursolic acid, betullinic acid, stigmasterol and lupeol for the identification and differentiation on four commercialized shankhpushpi ethanobotanicals in herbal formulation and extracts.

4.2.6.1 Chromatographic Studies

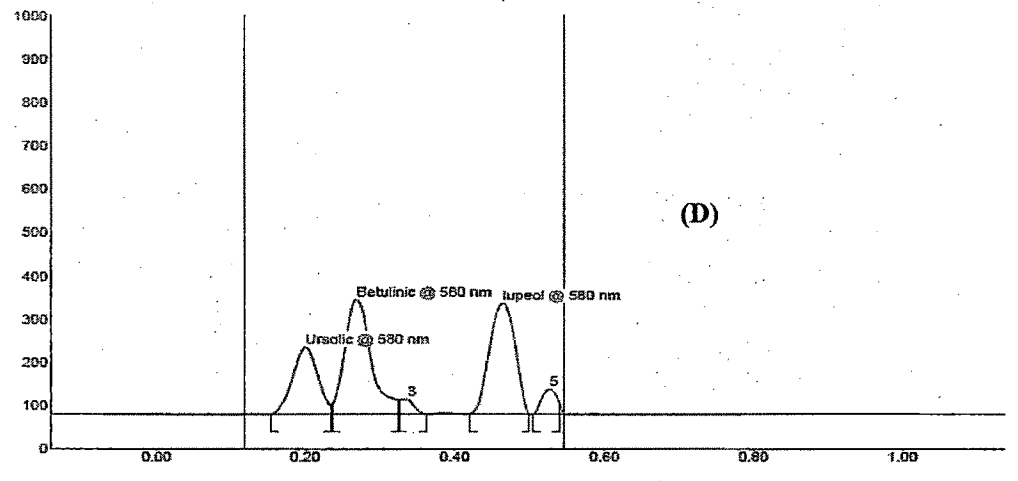
Thin layer chromatographic (TLC) studies were performed using various solvent systems, and finally Petroleum ether: Ethyl acetate: Toluene (7:2:1, v/v/v) was found to be suitable mobile phase for the proper separation of ursolic acid, betullinic acid, stigmasterol and lupeol in a single track (Fig 4.9). The chemical structure of ursolic acid, betullinic acid, stigmasterol and lupeol are given in figure 4.10. These markers were further fingerprint with various samples of shankhpushpi and its marketed formulation, to ascertain their presence (Figure 4.11 & 4.12).

Track 11, ID: Sample 13



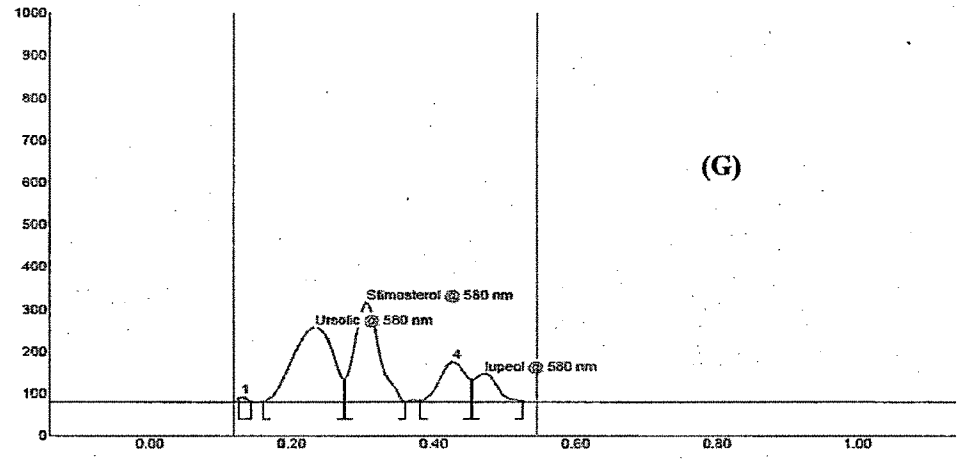
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.16	0.4	0.22	159.1	57.26	0.27	0.0	6520.4	61.62	Ursolic
2	0.27	0.0	0.34	47.0	16.92	0.37	17.1	1841.0	17.40	unknown *
3	0.39	15.2	0.42	71.7	25.82	0.44	50.6	2221.0	20.99	lupeol

Track 12, ID: Sample 14



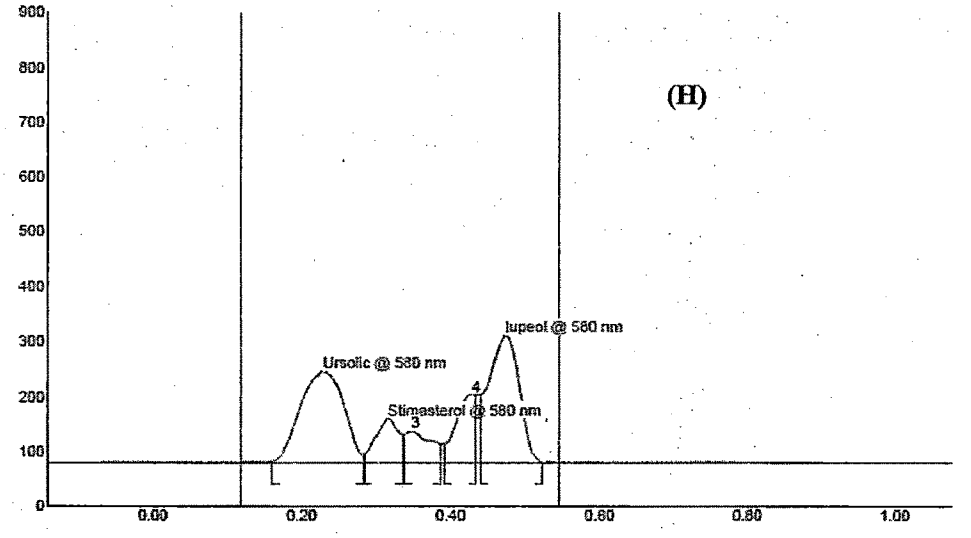
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.15	0.2	0.20	157.1	20.22	0.23	22.9	4277.4	20.72	Ursolic
2	0.24	24.3	0.27	267.1	34.39	0.32	33.8	7721.8	37.41	Betulinic
3	0.33	33.8	0.34	35.9	4.62	0.36	0.1	544.5	2.64	unknown *
4	0.42	1.4	0.46	258.4	33.26	0.50	0.3	7146.3	34.62	lupeol
5	0.51	0.3	0.53	58.4	7.52	0.54	29.0	949.9	4.60	unknown *

Track 14, ID: Sample 16



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.13	10.6	0.13	11.8	2.00	0.14	0.1	100.7	0.51	unknown *
2	0.16	0.8	0.23	178.1	30.15	0.27	54.8	8210.0	41.87	Ursolic
3	0.28	56.6	0.31	236.7	40.07	0.36	0.1	6668.0	34.01	Stimasterol
4	0.38	4.0	0.43	96.0	16.26	0.45	52.8	2828.7	14.43	unknown *
5	0.45	52.9	0.47	68.0	11.52	0.53	2.8	1798.8	9.17	lupeol

Track 15, ID: Sample 17



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.16	2.6	0.23	166.1	25.28	0.28	14.5	7880.9	35.94	Ursolic
2	0.28	15.0	0.32	79.9	12.16	0.34	50.2	1980.2	9.03	Stimasterol
3	0.34	50.5	0.35	56.5	8.60	0.39	33.2	1628.7	7.43	unknown *
4	0.39	34.0	0.43	123.4	18.79	0.43	123.0	2670.6	12.18	unknown *
5	0.44	123.0	0.47	231.0	35.17	0.52	0.2	7768.3	35.43	lupeol

Figure 4.11 Simultaneous chromatogram of various shankhpushpi botanicals (Track 9 Track-15) for identification of ursolic acid, betullinic acid, stigmasterol and lupeol

Where (A) CP, (B) CTB, (C) EA, (D) CD, (E) SS, (F) BT, (G) CTW, (H) CTW

4.2.6.2 Calibration of ursolic acid, betullinic acid, stigmasterol and lupeol, and their analysis in different shankhpushpi extract and formulation

Different concentrations (100-600 ng/spot) of ursolic acid, betullinic acid, stigmasterol and lupeol were plotted against peak area to obtain a calibration plot. 20 µL aliquots of the extract solution and extracts of marketed formulation (1 mg/mL) were applied. After chromatography, the amount of ursolic acid, betullinic acid, stigmasterol and lupeol present in the respective extracts and formulation were determined by means of their calibration plot (Figure 4.13 and Table 4.61).

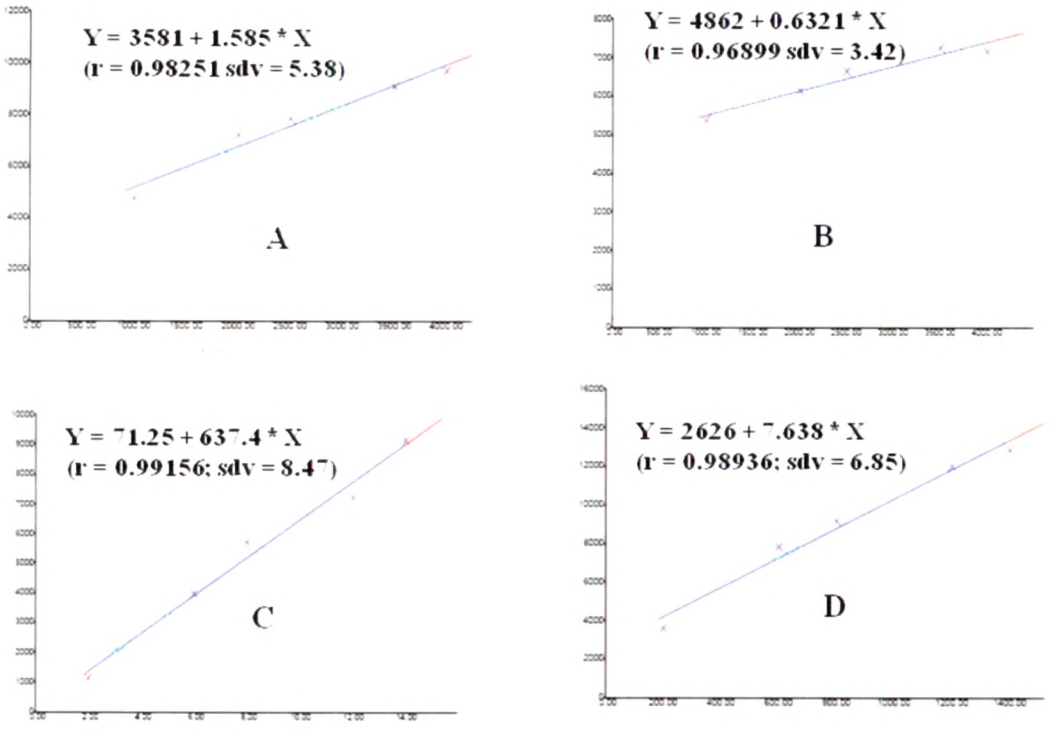


Figure 4.13 Calibration plot for (A) ursolic acid; (B) betullinic acid; (C) stigmasterol; and (D) lupeol.

4.2.7 Method Validation

4.2.7.1 Accuracy and precision

Repeatability of the sample application and measurement of peak area were carried out using six replicates of the same spot (300 ng/spot) of ursolic acid, betullinic acid, stigmasterol and lupeol, which were expressed in terms of percentage

4.2.7.5 Recovery studies

To study the accuracy and precision of the method, recovery studies were performed by the method of standard addition. The recovery of added standard was studied at three different levels, each being analysed in a manner similar to that described for the assay (Kumar et al., 2008; Agrawal et al., 2004; Biringanine et al., 2006; Wagner et al., 2008; Abourashed & Mossa, 2004). The methanolic extract of EA, CP, CTB, CTW, CD, SS and BT were used for recovery studies. These were preanalysed by the developed method as mentioned in the experimental sections and found to contain 150.45, 134.2, 156.15 and 146.1 mg of Ursolic acid per gram of CT blue flower, CT white flower, CD, and BT extract respectively; 121.6 and 110.6 mg of Betullinic acid per gram of EA and SS extract; 92.75, 250.9, 158.6, 154.95, 31.947, 39.21 and 369.95 mg of Stigmasterol per gram of EA, CP, CTB, CTW, CD, SS and BT respectively; 30.12 and 32.73 mg of Lupeol per gram of CTB and CTW extract respectively. Thus 20 µg of the same pre-analysed extracts content is given in table 4.6, which were used for the recovery studies. The pre-analysed extract samples of all were spiked with an extra 1:2 ratio i.e. 200 ng/spot and 400 ng/spot of the respective standard of ursolic acid, betullinic acid, stigmasterol and lupeol content of respective sample, which were reanalysed by the proposed method. The experiments were conducted six times to check for the recovery of markers.

Table 4.6 Content of markers in analysed sample of shankhpushpi botanicals

Sample	Amount of markers present in 20 µL of the sample (Stock 10 mg/ 10 ml)			
	Ursolic acid	**Betullinic acid**	**Stigmasterol**	**Lupeol**
EA	-	2.432 µg	1.855 µg	-
CP	-	-	5.018 µg	-
CTB	3.009 µg	-	3.172 µg	602.47 ng
CTW	2.684 µg	-	3.099 µg	654.79 ng
CD	3.123 µg	-	638.94 ng	-
SS	-	2.212 µg	784.2 ng	-
BT	2.922 µg	-	7.399 µg	-

4.3 RESULTS AND DISCUSSION

4.3.1 Simultaneous Spectrofluorimetric Determination

Once the standard curve for both scopoletin and mangiferin were prepared using series of standard dilutions from 5 µg/ml to 30 µg/ml, the dilutions covering the detection limit of the instrument, it became feasible to estimate scopoletin and mangiferin in the herbal extracts by measuring their fluorescence intensity within the range of excitation and emission wavelengths for scopoletin, i.e. 430 to 460 nm and for mangiferin, i.e. 248 to 520 nm

After the success of spectrofluorimetric analysis in determining the concentration of scopoletin and mangiferin in methanolic extracts of Shankhpushpi botanicals, it was thought worthwhile to develop a method for the determination of scopoletin and mangiferin concentration in crude drug samples.

Thus a simple analytical method was developed which proved to be very crucial in estimating concentration of scopoletin and mangiferin simultaneously in various drug samples. The developed method was validated for specificity, reproducibility and accuracy. The method was found to be specific for scopoletin and mangiferin since both are fluorescent. Linearity range was found to be in the range of 5-30 µg/ml. The correlation coefficients (r) were 0.9919 and 0.9937 indicating good linearity between fluorescence intensity and concentration. Repeated scanning of the samples three times checked precision of the method. Carrying out a recovery study checked reproducibility and accuracy of the method. A known concentration of scopoletin and mangiferin were added to varying concentrations of the aqueous extract i.e. 0.1, 0.2, 0.5 and 1.0 µg/ml. The sample of known concentration was added in equal volume to the various dilutions of the extract and analyzed spectrofluorimetrically to see whether the practical concentration obtained is in correspondence with the theoretical or hypothetical concentration from the standard curve. The percentage recovery for scopoletin and mangiferin was found to be in the range of 98- 102%. Hence this developed spectrofluorimetric method is quick and reliable for simultaneous quantitative monitoring of scopoletin and mangiferin in raw material, processed powder and in herbal preparations containing *said botanicals of shankhpushpi.*

4.3.3.2 Robustness of the method

The standard deviation of peak areas was calculated for each parameter and R.S.D. % was found to be less than 2%. The low values of R.S.D. % as shown in table 4.8, indicated robustness of the method.

Table 4.8 Robustness Testing (n=6)

Parameter	S.D.[a] of peak area			R.S.D. % [a]		
	Scopoletin	Mangiferin	Rutin	Scopoletin	Mangiferin	Rutin
Mobile phase composition	1.52	1.62	1.58	1.13	1.34	1.24
Amount of mobile phase	1.01	1.21	1.12	1.01	1.21	1.12
Temperature	1.68	1.34	1.26	1.02	1.08	0.98
Relative humidity	1.34	1.54	1.41	1.21	1.28	1.25
Plate pretreatment	0.98	1.12	1.05	0.73	0.89	0.79
Time from spotting to chromatography	0.65	0.96	0.87	0.53	0.63	0.49
Time from scanning to chromatography	0.48	0.78	0.64	0.52	0.57	0.34

[a] Average of three concentration 300, 500, 900 ng/spot

4.3.3.3 Limit of detection and quantification

The LOQ and LOD were calculated from the equations LOD = 3xN/B and LOQ = 10xN/B, where N is the SD of the peak area of the standard (n = 3), taken as a measure of the noise, and B is the slope of the corresponding calibration curve. The LOQ and LOD were shown in table 4.9.

Table 4.12 Robustness Testing (n=6)

Parameter	S.D.[a] of peak area				R.S.D. % [a]			
	Ursolic	Betulinic	Stigmasterol	Lupeol	Ursolic	Betulinic	Stigmasterol	Lupeol
Mobile phase composition	1.62	1.52	1.43	1.58	1.24	1.19	1.34	1.13
Amount of mobile phase	1.21	1.01	0.97	1.12	1.12	1.34	1.21	1.01
Temperature	1.34	1.68	1.12	1.26	0.98	0.95	1.08	1.02
Relative humidity	1.54	1.34	1.26	1.41	1.25	1.17	1.28	1.21
Plate pretreatment	1.12	0.98	0.97	1.05	0.79	0.83	0.89	0.73
Time from spotting to chromatography	0.96	0.65	0.79	0.87	0.49	0.59	0.63	0.53
Time from scanning to chromatography	0.78	0.48	0.67	0.64	0.34	0.43	0.57	0.52

[a] Average of three concentration 300, 500, 900 ng/spot

4.4.2.3 Limit of detection and quantification

The LOQ and LOD were calculated from the equations LOD = 3xN/B and LOQ = 10xN/B, where N is the SD of the peak area of the standard (n = 3), taken as a measure of the noise, and B is the slope of the corresponding calibration curve. The LOQ and LOD were shown in table 4.13.

Table 4.13 R_f, Linear regression, LOD and LOQ for ursolic acid, betullinic acid, stigmasterol and lupeol

Compound	R_f	Regression equation	R*	SD (%)	LOD (ng/ spot)	LOQ (ng/spot)
Ursolic acid	0.24	Y = 3581 + 1.585 x	0.98251	5.38	30	80
Betulinic acid	0.31	Y= 4862 + 0.6321 x	0.96899	3.42	15.24	50.82
Stigmasterol	0.38	Y = 71.25 + 637.4 x	0.99156	8.47	20	60
Lupeol	0.54	Y = 2626 + 7.638 x	0.95121	6.85	50	100

*Correlation coefficient

4.4.2.4 Specificity

The peak purity of individual ursolic acid, betullinic acid, stigmasterol and lupeol were assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e., *r* (start, middle) = 0.9991 and *r* (middle, end) = 0.9993. The peak purity of ursolic acid, betullinic acid, stigmasterol and lupeol were assessed by comparing the spectra of standard at peak start, peak apex and peak end positions of the spots, i.e., *r* (start, middle)= 0.9973 and *r* (middle, end) = 0.9979. Good correlation (*r* = 0.9994) was also obtained between standard and sample.

4.4.2.5 Recovery studies

The proposed method may used for extraction and subsequent estimation of scopoletin, mangiferin and rutin from pharmaceutical dosage form after spiking it with 100 ng/spot and 200 ng/spot of additional standards. The results of content estimation and recovery studies are listed in table 4.14. The recoveries obtained were in the range 99.67–100.95%, showing the reliability and reproducibility of the method.

Compound	Plant extract and formulation	Amount of compound present in plant extract (ng)	Amount of standard added (ng)	Theoretical Amount of standard found in mixture (ng)	Amount of standard found in mixture (ng) [n=3]	Recovery (%)
Stigmasterol	EA	1855	100	1955	1957.00±0.58	100.10±0.02
			200	2055	2056.33±2.91	100.06 ±0.14
	CP	5018	100	5118	5121.00±2.00	100.05±0.03
			200	5218	5219.00±1.15	100.01±0.02
	CTB	3172	100	3272	3274.00± 0.58	100.06±0.01
			200	3372	3373.00±2.52	100.02±0.07
	CTW	3099	100	3199	3194.67±4.70	99.86±0.15
			200	3299	3282.33±13.98	99.49±0.42
	CD	638.94	100	738.94	739.15±0.46	100.02±0.06
			200	838.94	832.28±1.59	99.20±0.19
	SS	784.2	100	884.2	886.30±0.64	100.23±0.07
			200	984.2	984.17±2.29	99.99±0.23
	BT	7399	100	7499	7498.00±3.21	99.98±0.04
			200	7599	7607.67±8.21	100.11±0.11
Lupeol	CTB	602.47	100	703.47	708.51±1.43	100.71±0.20
			200	803.47	804.96±2.22	100.18±0.28
	CTW	654.79	100	754.79	764.75±1.26	101.32±0.17
			200	854.79	853.22±3.97	99.81±0.46

Where CTW- *Clitorea ternatea* white flower; CTB- *Clitorea ternatea* blue flower; CD- *Canscora decussata*; BT- *Brain Tab*; CP- *Convolvulus pluricaulis*; EA- *Evolvulus alsinoids*; SS- *Shankhpushpi Syrup*.

*All values are Mean ± SEM