International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.5, No.4, pp 1554-1560, Oct-Dec 2013

Simultaneous Estimation of Chebulagic acid and Chebulinic acid in Marketed Polyherbal Formulations by HPTLC

KVVS Krishna*, K Gouri Sankar, M Syam Vardhan, T Tamizhmani

Department Of Pharmacognosy, Bharathi College Of Pharmacy, Bharathi Nagara, Maddur Tq., Mandya Dt., Karnataka, India-571422.

***Corres. Author: kvvskrishna@rediffmail.com,**
Phone: +91-8019899825.

Abstract: A simple, rapid and reliable HPTLC method has been developed for simultaneous estimation of Chebulagic acid and Chebulinic acid in marketed polyherbal formulations. Identification and quantification were performed on 10cm x 10cm, layer thickness 0.2mm, aluminum- backed silica gel 60 F_{254} HPTLC plates previously washed with methanol. The solvent system consisting of Ethyl Acetate: Toluene: Formic Acid: Methanol (6:1:1:2%v/v) was used as a mobile phase. This system gave compact spots for Chebulagic acid and Chebulinic acid. The spots were scanned at λ=254nm. Calibration plots were established showing the dependence of response on the amount chromatographed. The linear regression analysis data for the calibration plots showed good linear relation with R^2 = 0.998 and 0.9991 with respect to peak area for Chebulagic acid and Chebulinic acid respectively, in concentration range of 420-500ng/spot. The method was validated for precision, recovery, Limit of Detection and Limit of Quantification. The proposed HPTLC method was found to be simple, precise and accurate and can be used for the quality control of the raw materials as well as formulations.
Key Words: Chebulagic acid, Chebulinic acid, HPTLC, Polyherbal formulations.

Introduction:

Standardization and analysis of the chemical marker of the Ayurvedic and other poly herbal formulations is always very big problem. Quantitative estimation of chemical markers of each ingredient in the polyherbal preparation requires ideal separation technique by which these markers are separated with highest purity and with least inferences from each other [1]. For botanicals and herbal preparations, there is a requirement for scientific proof and clinical validation with chemical standardization, biological assays, animal models and clinical trials [2]. Chebulagic acid is a benzopyran derivative found in the fruits of *Terminalia chebula*. It has been found to be immunosuppressive, hepatoprotective and a potent alpha-glucosidase inhibitor, a human gut enzyme useful in diabetic studies. It has been shown to be active against *Staphylococcus aureus* and *Candida albicans* [3]. Chebulinic acid is one of the ellagitannins in the fruits of *Terminalia chebula*, which has many potential uses in medicine. Chebulinic acid showed many bioactivities including inhibition of cancer cell growth, anti-*neisseria gonorrhoeae* activity, inhibiting the contractile responses of cardiovascular muscles and blocking the binding of HIV rgp120 to CD4 [4]. *Terminalia chebula* is rich in tannin (30-32%). The chief phytoconstituents of tannin are chebulic acid, chebulagic acid, chebulinic acid, corilagin, gallic acid and ellagic acid [5]. Therefore, the simultaneous estimation of chebulagic acid and chebulinc acid would be an important

parameter for the quality control of polyherbal formulations containing *Terminalia chebula* as one of the ingredient.

Extensive literature survey reveals that, a very few analytical methods have been reported for the quantification of Chebulagic acid and Chebulinic acid which includes High performance capillary electrophoresis (HPCE), RP- HPLC, TLC-UV Densitometry and High speed counter current chromatography [6-9].

In the present study, we report the development of a simple, optimized and validated HPTLC method for the simultaneous estimation of Chebulagic acid and Chebulinic acid in marketed polyherbal formulations. The method was validated on the basis of its linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) according to ICH requirements.

Experimental:

All chemicals and reagents including Ethyl Acetate, Formic Acid, Methanol, Toluene and Alcoholic $FeCl_3$ were of analytical grade and were used throughout the experiment. Analytically pure samples of Chebulagic acid and Chebulinic acid were procured as gift sample from M/s Natural Remedies Pvt. Ltd., (Bangalore, India). The polyherbal formulations i.e., Formulation I (Agnitundi Bati) and Formulation II (Gokshuradi Guggulu), used for present study were purchased from Gururaja Pharmacy, Bangalore.

Instrumentation

Analysis was performed on 10cm x 10cm plates cut from 20cm x 20cm aluminum-backed silica gel 60 F_{254} plates (E. Merck). Samples were applied to the plates by means of a Linomat-V automatic spotter with the aid of Hamilton 100µl syringe. TLC plates were developed in flat bottom twin trough chamber. Densitometry was performed with a TLC scanner-3 with Win-CATS 4 software resident in a Pentium IV computer.

Preparation of Standard solution

Accurately weighed 10mg of Chebulagic acid standard was dissolved in 10ml of methanol in a volumetric flask (A). 1ml of this solution was diluted to 10ml with methanol (B). Accurately weighed 10mg of Chebulinic acid standard was dissolved in 10ml of methanol in a volumetric flask (C). 1ml of this solution was diluted to 10 ml with methanol (D). Working standard solutions B and D were used for the HPTLC analysis.

A stock solution containing 100mcg/ml Chebulagic acid (B) and Chebulinic acid (D) were prepared in methanol. Calibration solutions were prepared by diluting the stock solution so that application of 4.2-5µl volumes gave a series of spots covering the range 420 to 500ng of Chebulagic acid and Chebulinic acid respectively.

Preparation of Sample solution

Amount equivalent to the contents of the formulation was extracted twice with 10 ml of methanol by boiling for 10 minutes. Extract obtained was filtered using Whatman filter paper, concentrated to less than 10ml and transferred to a 10ml volumetric flask and volume was made up with methanol. Agnitundi Bati and Gokshuradi Guggulu were weighed in amounts of 1gm each respectively.

Simultaneous estimation of markers in polyherbal formulations

From the sample solutions, 5.0µl was applied on the precoated silica gel plate and process was repeated to develop and the plate was scanned at λ=254nm. The amount of Chebulagic acid and Chebulinic acid in the samples was calculated from the calibration equation generated and by using the average area of triplicate sample aliquots.

Validation of the method

After the development of HPTLC method for the simultaneous estimation of the poly herbal formulations, validation of the method was carried out according to the ICH guidelines with respect to Linearity, Accuracy, Precision, Limit of Detection and Limit of Quantification [10-13]. Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be

expected to be present in the sample matrix. Test and standard solution of Chebulagic acid and Chebulinic acid were spotted on the TLC plate, developed and scanned. The test chromatogram was compared with the standard. Linearity of the method was performed by analyzing standard solution of Chebulagic acid and Chebulinic acid by the proposed method in concentration range of 420 to 500ng/spot. Accuracy of the proposed method was determined by recovery study. Recovery study was carried out by adding three different quantities of Chebulagic acid and Chebulinic acid (230, 460 and 690ng/spot) to preanalyzed samples of polyherbal formulations. The procedure was repeated for five times as discussed above in sample preparation. From the linear regression, percentage recovery of Chebulagic acid and Chebulinic acid were determined. Precision was determined by repeatability, intraday and inter day reproducibility experiment of the proposed method. The Intraday repeatability was evaluated by preparing and analyzing the standard solution of the drug six times in a day. The inter day reproducibility was determined by analyzing freshly prepared solution once a day over six consecutive days under same operative condition. Limit of detection and Limit of Quantification of Chebulagic acid and Chebulinic acid were calculated and reported in **Table2.**

Results And Discussion:

Quantification of markers in polyherbal formulations

The two markers were detected in the marketed polyherbal formulations. After sprayed with alcoholic $Fecl_3$, Chebulagic acid and Chebulinic acid appeared at Rf 0.48 (pale green) and 0.58 (dark green) respectively. Under UV light at 254nm, Chebulagic acid and Chebulinic acid appeared at Rf 0.48 (pale blue) and 0.58 (dark blue) respectively. The mobile phase comprised of Ethyl Acetate: Toluene: Formic acid: Methanol (6:1:1:2) v/v was used for the development of TLC plate which gave better resolution of all the compounds among the mobile phase tried. The chromatograms of polyherbal formulations were quantified with respect to Chebulagic acid and Chebulinic acid. The chromatograms of Chebulagic acid and Chebulinic acid (Figure 1 and 2) and marketed polyherbal formulations (Figure 3 and 4) were shown complete separation of two markers from other constituents. The content of Chebulagic acid and Chebulinic acid in polyherbal formulations were reported in Table 1.

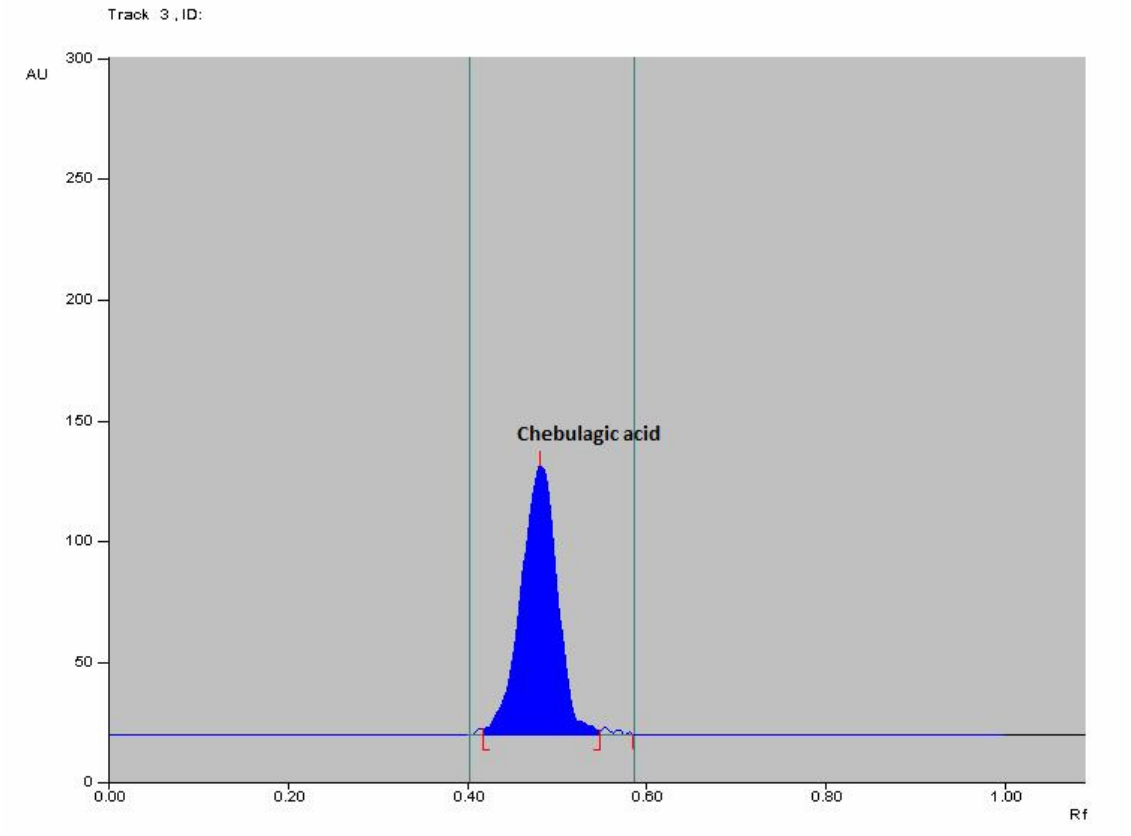

Figure 1: Typical HPTLC Chromatogram of Chebulagic acid

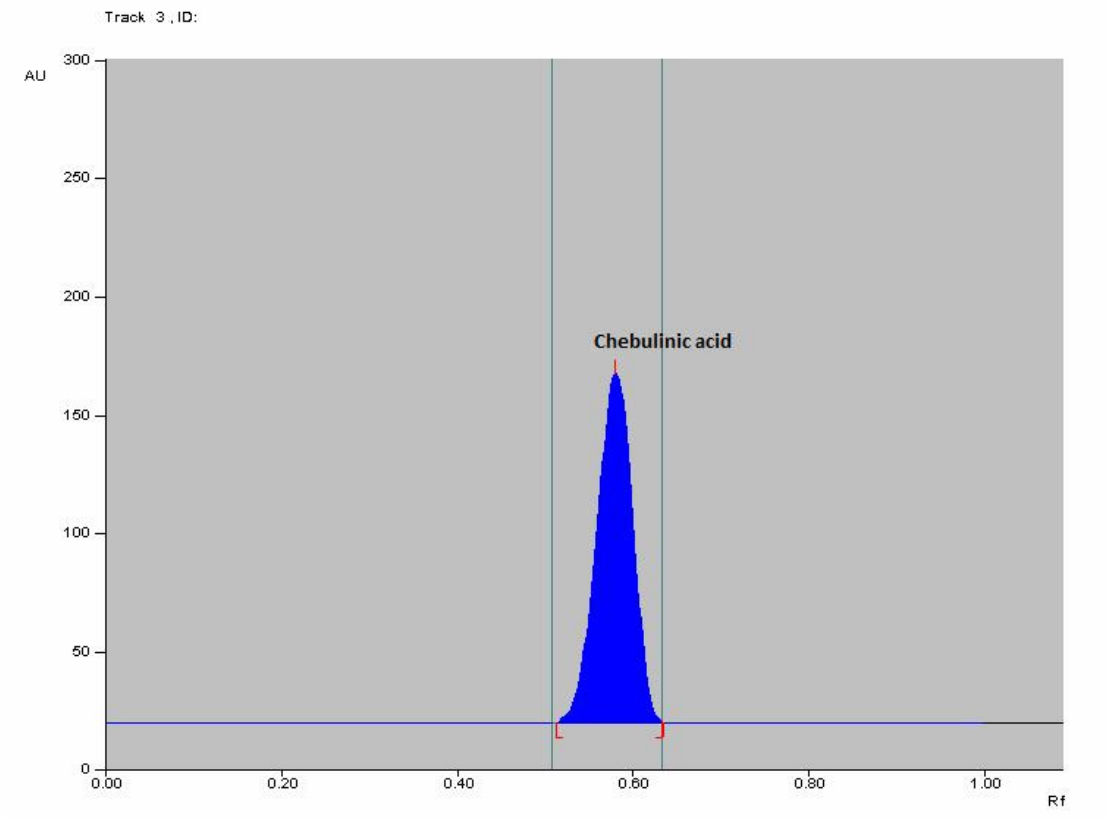

Figure 2: Typical HPTLC Chromatogram of Chebulinic acid

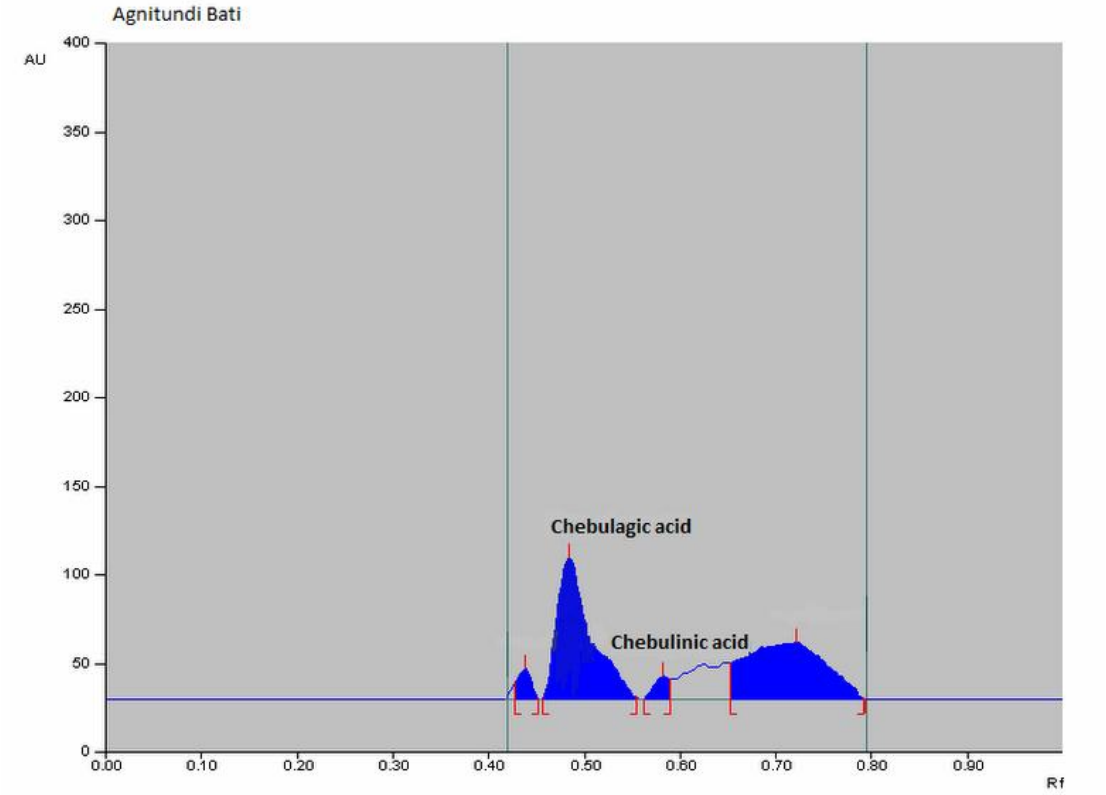

Figure 3: Typical HPTLC Chromatogram of Formulation I

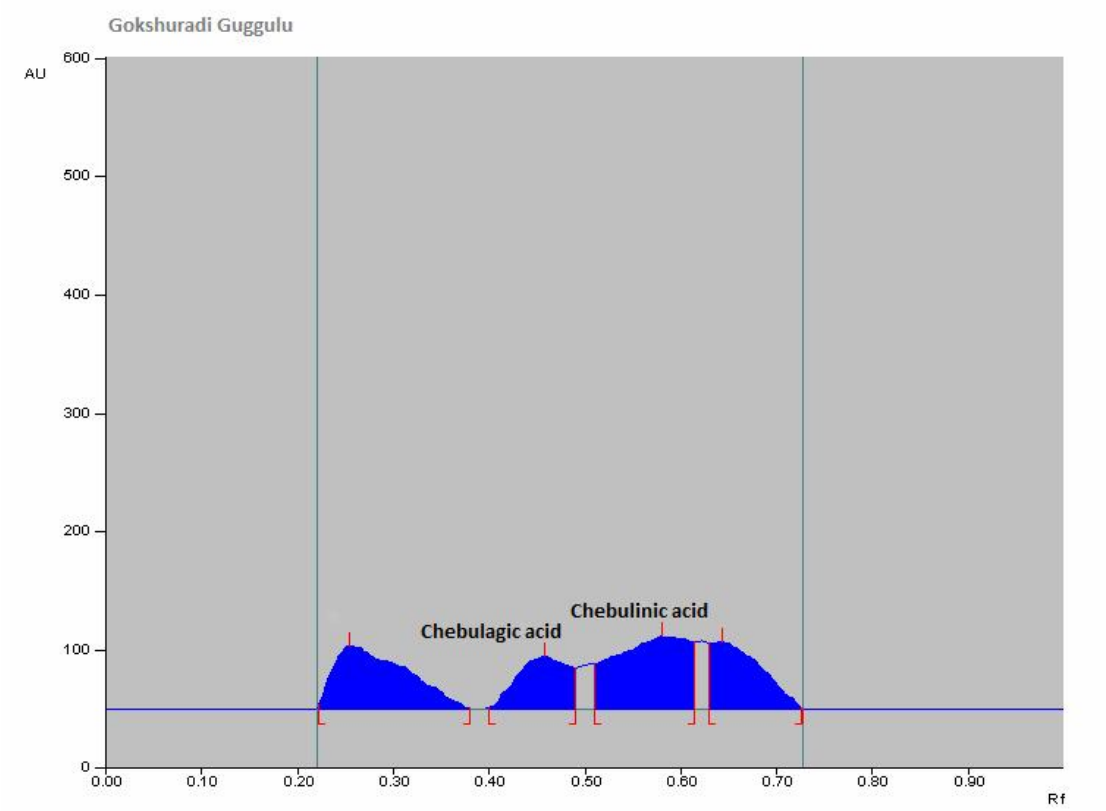

Figure 4: Typical HPTLC Chromatogram of Formulation II

Table 1: Content of Chebulagic acid and Chebulinic acid in polyherbal formulations

Samples	Amount of Chebulagic acid (%w/w)	Amount of Chebulinic acid (%w/w)
Agnitundi Bati	0.050	0.0025
Gokshuradi Guggulu	0.042	0.064

Method validation for HPTLC fingerprinting method

Linear correlation was obtained between peak areas and concentrations of Chebulagic acid and Chebulinic acid in the range of 420-500ng/spot. Characteristic parameters for regression equation and correlation were reported in Table 2. The linearity of the calibration graphs was validated by the high value of correlation coefficients of the regression. The percent recovery obtained was 99.55±0.31 to 100.66±0.46% for Chebulagic acid and 99.46±0.66 to 100.79±1.00% for Chebulinic acid. The results of recovery study were reported in Table 3. For both Chebulagic acid and Chebulinic acid, relative standard deviation of all the parameters is less than 2% for the degree of repeatability indicating the high repeatability of the proposed method. The results of precision were reported in Table 4 for Chebulagic acid and Chebulinic acid. Value of % Relative Standard Deviation (RSD) of intra-day and inter-day reveal that the proposed method was precise. The limit of detection (LOD) and Limit of Quantification (LOQ) of the drug was calculated by signal to noise ratio. LOD and LOQ for Chebulagic acid and Chebulinic acid were found to be 0.35ng/spot & 1.07ng/spot and 0.26ng/spot & 0.81ng/spot respectively.

Table 2: Characteristic parameters for the proposed HPTLC method

Parameters	Chebulagic acid	Chebulinic acid
Calibration range (ng/spot)	420-500	420-500
Detection wavelength	254 nm	254 nm
Mobile phase (Ethyl acetate : Toluene: Formic acid : Methanol)	6: 1 : 1:2	6 : 1 : 1: 2
Rf value	0.48	0.58
Regression equation (y*)	Y=6.5879x-3.4749	Y=8.6765x+14.696
Slope (b)	6.5879	8.6765
Intercept (a)	-3.4749	14.696
Correlation coefficient(r^2)	0.998	0.991
Limit of detection (ng/spot)	0.35	0.26
Limit of quantitation (ng/spot)	1.07	0.81

*y = b x + a, where x is the concentration of Chebulagic acid and Chebulinic acid in ng/spot and y is the peak area at respective wavelength.

Table 3: Recovery studies of Chebulagic acid and Chebulinic acid in Polyherbal formulations by HPTLC method

Sample	Initial amount (ng/spot)	Amount added (ng/spot)	Recovery ± SD (%)	
			Chebulagic acid	Chebulinic acid
Agnitundi Bati	460	230(50%)	99.67±0.30	99.46±0.66
		460(100%)	100.48±0.46	100.79±1.00
		690(150%)	99.80±0.18	99.68±0.40
Gokshuradi Guggulu	460	230(50%)	99.55±0.31	99.78±0.68
		460(100%)	100.66±0.46	100.31±1.02
		690(150%)	99.73±0.18	99.87±0.40

Table 4: Precision of the Intra-daily and Inter-daily HPTLC measurement for Chebulagic acid and Chebulinic acid

Compound	Intraday precision[a]		Interday precision[b]	
	Concentration (ng/spot)	RSD (%)	Concentration (ng/spot)	RSD (%)
Chebulagic acid	460	1.323	460	1.730
Chebulinic acid	460	1.016	460	1.33

[a] Samples were analyzed six times a day
[b] Samples were analyzed once a day over six consecutive days

Conclusion:

In the present study, on the simultaneous estimation of Chebulagic acid and Chebulinic acid in marketed polyherbal formulations by HPTLC, wide variations in the content of Chebulagic acid and Chebulinic acid in the formulations to be administered or prescribed by the physicians were observed. This shows that Polyherbal formulations are not properly standardized. This leads to marked differences in the therapeutic efficacy of the formulations when administered. Hence, the newly developed method for the simultaneous estimation of Chebulagic acid and Chebulinic acid in Polyherbal formulations can be adapted to standardize the formulations. This would also minimize or avoid the batch-to-batch variations in the therapeutic efficacy of such Polyherbal formulations.

References:

1. Mukherjee PK. Quality Control of Herbal Drugs. New Delhi: Business Horizons, India; 2005. P.741.
2. Ong ES. Extraction methods and chemical standardization of botanicals and herbal preparations 2004. J Chromatogr B. 812:23-33.
3. Hamada S, Kataoka T, Woo JT, Yamada A, Yoshida T, Nishimura T, Otake N, Nagai K. Immunosuppressive effects of gallic acid and chebulagic acid on CTL-mediated cytotoxicity. Biol Pharm Bull. 1997 Sep; 20(9):1017-9.
4. Weaver JL, Pine PS, Dutschman G, Cheng YC, Lee KH, Aszalos A. Prevention of binding of rgp120 by anti-HIV active tannins. Biochem Pharmacol. 1992 Jun 9; 43(11):2479-80.
5. Ansari SH. Text Book of Essentials of Pharmacognosy, Birla Publications, 2005-2006; 1:351-53.
6. Ding G, Lu YR, Ji CR and Liu YZ. Analysis of tannins in *Fructus chebulae* and its confusion varieties by HPCE. J Sep Sci, 2008; 26:612-25.
7. Ratnayake S and Jan SZ. Uptake of *Terminalia chebula* tannin components by Hide:Analysis using TLC-UV Densitometry. J Nath Sci, Coun Srilanka, 1984; 12(1):92-103.
8. Han Q, Song J, Qiao C, Wong L, Wong L and Xu H. Preparative isolation of hydrolysable tannins (chebulagic acid and chebulinc acid) from *Terminalia chebula* by High-Speed Counter-Current Chromatography. J Sep Sci, July 2006; 29(11):1653-57.
9. Pawar V, Lahorkar P and Ananth Narayana DB. Development of a RP-HPLC Method for analysis of *Triphala curna* and its applicability to test variations in *Triphala curna* preparations. Indian J Pharm Sci, Jul-Aug 2009; 71(4):382-86.
10. International Conference on Harmonization (ICH), Validation of Analytical Procedures: Text on Validation of Analytical Procedures Q2A, 1994.
11. International Conference on Harmonization (ICH), Validation of Analytical Procedures: Methodology Q2B, 1996.
12. International Conference on Harmonization (ICH), Validation of Analytical Procedures: Text and Methodology Q2 (R1), 2005.
13. Guidelines on Validation of Analytical Procedure Methodology. Geneva; International Conference on Harmonization; 1996.

*****