Plant Archives Vol. 20, Supplement 2, 2020 pp. 4337-4342 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

QUANTITATIVE ESTIMATION OF MARKER COMPOUND (LUPEOL AND DIOSGENIN) IN POLYHERBAL FORMULATION BY HPTLC METHOD

Naveen K. Choudhary*, Karan Gupta, Amit K. Jain and Arti Pal
*B R Nahata College of Pharmacy, Mandsaur University, Mandsaur (M.P.) 458001, India
Address for correspondence: Dr. Naveen Choudhary
*Associate Professor : B R Nahata College of Pharmacy, Mandsaur University, Mandsaur (M.P.) 458001, India
Email: pharmanaveen@yahoo.co.in

Abstract

The objective of current study was to develop new simple and precise HPTLC method for standardization of biomarker compound lupeol and diosgenin in Mutrakrichantak churna. Mutrakrichantak churna is ayurvedic formulations devouring eight herbs used for relieve urinary tract infections, kidney stones, high urea, uric acid and creatinine levels. *Crateva nurvala*L. (Varuna) is also used as herb in the churna. The chief constituents of varuna are lupeol and diosgenin. Accordingly it is essential to carry out chemical standardization of active bioactive marker present in the Polyherbal ayurvedic formulation Mutrakrichantak churna. The method was developed using precoated silica gel 60, F_{254} as stationary phase and Isopropyl alcohol: n-butanol (5:5v/v) as a mobile Phase for lupeol and Toluene: Ethyl acetate: Formic acid (5:4:1v/v/v) as a mobile phase for diosgenin. The R_f value of two markers compound was found to be 0.76 (Lupeol) and 0.81 (Diosgenin). Camag TLC Scanner was used as a densitometric scanner. The method was validated according to ICH guidelines. Correlation coefficient (r) was calculated from the standard graph of linearity and it was found to be 0.999 for lupeol and 0.997 for diosgenin. The in-house and marketed formulations were found to be simple, accurate, precise and robust.

***Keywords*:** Mutrakrichantak churna, lupeol, diosgenin, HPTLC, Stationary phase, *Crateva nurvala*

Background

Throughout the past years, community attention in herbal medicine has enhanced exponentially (De Smet, 1997). Interpretation to the World Health Organization, bulk inhabitants (65%–80%) in developing countries depends principally on plants for main health care needs owed to deficiency and lack of admittance to modern medicine (Oliveira *et al.,* 2006; Calixto 2000). The World Health Organization has developed precise guiding principles to maintenance the associated countries to prompt nationalized policies on plant based drugs and to learning their forthcoming safety, efficiency and excellence, as a precondition for global synchronization (pattanayak *et al.,* 2011; Atmakumari & Dathi 2010; Pillai & Pandita 2016). Medicinal plant has biomarkers which have pharmacological potential to fight against diseases.

"Mutrakrichantak churna" as the name choose means conclusion of endeavor in urination. Mutrakrichantak is made up of three words mutra means urine, krich means difficulty and antak means the end. The churna is useful in releasing numerous problems related to kidney such as chronic and serious renal failure, urinary tract infection, kidney stones, increased urea and creatinine, kidney failure etc. Herbs used to make Mutrakrichantak churna are varuna, punarnava, goshur, kaasni, bhumiamla, shirish, shigru and apamarg (Bopana & Saxena 2008).

The roots of *Crateva nurvala* L. Capparaceae, are generally known as Varuna roots (Bhattachargee *et al.,* 2012). The dried roots are used raw drug in traditional systems of medicine in India such as Ayurveda, Siddha, etc. The root is used to treatment diseases like urinary tract infections, kidney stones, high urea, high uric acid and creatinine levels, renal calculi, dysuria, diuretic (Parvin *et al.,* 2011). The Varuna roots contain Lupeol as a chief constituent along with diosgenin (Calixto, 2000). These complexes have been exposed to have various pharmacological activities like anti-inflammatory which show reasonable effects on human health. Ground breaking movements which transpire in the procedures of disentanglement, sanitization and supplementary amplification of common mixes have made it believable to produce fitting approaches for the exploration of value and institutionalization of plant based instructions (Di *et al.,* 2003). High performance thin-layer chromatography (HPTLC) is an enhanced method of thin layer chromatography (TLC). A number of augmentations can be made to the basic method of thin-layer chromatography to systematize the different steps, to upsurge the resolution accomplished and to permit more accurate quantitative measurements. HPTLC has been comprehensively used for the individuality and distinction of the botanicals because of its adaptability, dependability, high-throughput and cost efficiency (Larsen *et al.,* 2004; ICH, 1996).

Best owing to the ICH guidelines (ICH, 2005; Pillai & Pandita 2016) in our current study the validation parameters established were correctness, accuracy, specificity and Sturdiness for Lupeol and Diosgeninin house formulations and marketed formulations of Mutrakrichantak Churna.

Matrials and Methods

Standards & Chemicals

The analytical grade of organic solvents was acquired from Merck Specialties Pvt. Ltd (Mumbai). Markers Lupeol (≥ 99% purity) and Diosgenin (≥ 98% purity) were procured from Yucca chemicals Mumbai.

Plant materials and formulations

All herbs used as ingredients in the groundwork of Mutrakrichantak churna were collected from Local market of

Mandsaur and authentified by Dr. S.N. Mishra (Botany Scientist) from K.N.K. Agriculture College, Mandsaur. The particular voucher numbers were given for each herb as shown in Table 1. The marketed formulation of Mutrakrichantak churna was procured from local market of Mandsaur.

Preparation of Mutrakrichantak Churna

The Mutrakrichantak Churna was organized by the method as given in Ayurvedic Formulary of India, Part I. Recognition of all the different-different plant material was done as per Ayurvedic Pharmacopoeia of India. All the herbs were dried under shadow. These dried materials were unconsciously powdered mix and finally sheaved using 100 meshes. Now in-house lab organized sample and marketed formulation were ready for HPTLC analysis.

Preparation of Test sample

100 mg marketed and in-house sample of churna were liquefied in 10 ml of methanol (10mg/ml) and was shaken well. Then the solution was sonicated for 15 minute and filtered by using whatman filter paper. 2 µl of each formulation (Marketed and In-house) were applied on TLC plates for HPTLC analysis.

Preparation of stock solution of Lupeol and Diosgenin

A common stock solution (10mg/ml) of Lupeol and Diosgenin was prepared by dissolving 100 mg of each in methanol up to 10ml. Then the solution was sonicated for 15 minute and filtered using whatman filter paper. Working standard solution of 100µg/mlwas prepared for each by diluting 100 times the stock solution. The aliquots 1-5 ml each of lupeol and diosgenin were relocated to 100ml volumetric flask and makeup volume with methanol and applied to TLC.

HPTLC Instrumentation

TLC plates with a dimension of 20 cm × 10 cm Pre-coated with 0.20 mm layers of silica gel 60 F_{254} (Merck, Darmstadt, Germany) were used for chromatography. Samples were applied as 6mm wide bands and 75 mm was the detachment kept between the two bands by use of sample applicator Camag Linomat V equipped with a syringe of 100 µl volume. A continuous application rate of 150 nLs^{-1} was used. CAMAG TLC Scanner with WINCAT software for scanning and certification was used as a densitometric scanner. The slit measurements were 6 × 0.45 mm and the scanning speed 100 mm/s. The radiation cause used was a deuterium lamp at a wavelength of less than 270 nm, 447 nm and under day light.

Chromatographic condition

The mobile phase selected was a mixture of Isopropyl alcohol: n-butanol (5:5v/v) for Lupeol and Toluene: Ethyl acetate: Formic acid (5:4:1v/v) for Diosgenin. Before hand insertion of the plate, the chamber was inundated with Mobile phase vapour for 5 minutes room temperature (25±2°C), with the solvent front (development distance) being7cm. After the TLC plates were developed and dried by using an air dryer, densitometry scanning was achieved at a wavelength of 270 nm for Lupeol and 447nm for Diosgenin.

Calibration curves of Lupeol and Diosgenin and their analysis in formulations

To determine the linearity, standardization curves were plotted. A 1µl of each concentration ranging from 1-5µg/ml was applied on TLC plates to get final concentration of 1-5µg /spot for lupeol as well as diosgenin. The densitometry scanning was achieved for each average and the existence of lupeol and Diosgenin existing in the in-house and marketed formulations were quantified by means of calibration plot.

Method validation

Specificity

The expression specificity is frequently used interchangeably; the stage specific generally refers to a technique that produces a reaction for a single analyte. The specificity was determined by analyzing orientation standard, test sample, diluents and mobile phase. The spot of each standard in the sample was confirmed by the R_f values of the separate bands of the standard peaks. The peak purity of lupeol and diosgenin were restrained by comparing the spectra at 3 different levels i.e. peak begin, peak summit and peak end of the spot.

Precision

The instrumental precision was determined using six replicates of same concentration of lupeol (2µg/spot) and diosgenin (2µg/spot). Intra-day and inter-day precision were used for approximating repeatability of the method at three dissimilar concentration levels 2, 3 and 5 µg /spot for both lupeol and diosgenin.

Accuracy

Accuracy was determined using standard calculation method by calculating retrieval of lupeol and diosgenin. It was determined using proportion retrieval studies. The in-house and marketed Mutrakrichantak Churna formulations were spiked with 80, 100 and 120% of lupeol and diosgenin standard and then the mixture were analysed for percentage recovery using response curve. Regular of three was taken as each response.

Robustness

Robustness of the method was checkered by slightly changing the arrangement of mobile phase. For lupeol, Isopropyl alcohol: n-butanol (5.5:4.0, v/v) whereas Toluene: Ethyl acetate: Formic acid (5.5:4:1 v/v) for diosgenin were selected and run for chromatogram. Temperature and period of spotting, extension of plate and scanning were also varied with +5%. Result of robustness was checked at three different concentration levels 2, 3 and 5 µg /spot for both lupeol and diosgenin.

Limit of detection and quantification

Blank methanol was spotted six times in a similar way to that of the calibration curve and signal to noise ratio was determined for estimating limit of detection (LOD) and limit of quantification (LOQ). The ratio between standard deviation (SD) of the response and the slope (S) of the calibration curve is used to determine LOD and LOQ. 3:1 (SD/S) was considered to be best for LOD whereas 10:1 (SD/S) for LOQ (Hamidi *et al.,* 2017).

Results and Discussion

Optimization of Mobile Phase

An optimum mobile phase plays an important role in the development of chromatographic methods (Darekar *et al.,* 2008). To optimize mobile phase different ratios of Isopropyl alcohol and n-butanol was studied. Isopropyl alcohol: n-butanol (5:5) resulted in sharp, well defined peak of lupeol at R_f 0.76. While solvent system Toluene: ethyl acetate: formic acid (5:4:1v/v) (Jurado *et al.,* 2017) resulted in sharp, well defined peaks of diosgenin at R_f 0.81. The three dimensional HPTLC overlay of Lupeol and Diosgenin are shown in Fig. 1.

Calibration curve of lupeol and diosgenin, their analysis in formulation

Linearity is an important performance characteristic of any instrumental method. Numerous graphical and mathematical methods are applied to calculate linearity (Bridwell *et al*., 2010). A good linear association was obtained for lupeol and diosgenin between response and concentration over the range of 1-5 µg/band at 270 nm and 1-5 µg/band at 447 nm correspondingly. Correlation coefficient (r) was found to be 0.999 for lupeol and 0.997 for diosgenin. (Fig. 2, Table 2)

Precision

The precision of an analytical modus operandi expresses the proximity of conformity (degree of scatter) between a successions of measurements obtained from multiple sampling of the similar consistent sample (Armbruster & Pry 2008). In order to obtain closeness in a series of experiments, instrument precision was checked by repeated scanning of the same spot of lupeol (2 µg/ spot) and diosgenin (2 µg/ spot). The results were expressed as percentage relative standard deviation (%RSD) and were found to be less than 1% as shown in Table 2.

Limit of detection and quantification

The minimum amount of analyte that could be detected in experimental conditions is known as limit of detection (LOD), whereas the lowest amount of analyte that could be quantified is known as limit of Quantification (Betz *et al.,* 2011).

Accuracy and Recovery studies

Accuracy is defined as closeness of experimental value to actual value (Cesar & Pianetti 2009). It was determined using percentage recovery studies. Percentage recovery of lupeol and diosgenin was found to be 98.1 and 96.98% respectively in in-house formulation whereas 99.43 and 98.27 % respectively in marketed formulation. (Table 4)

Robustness

Robustness reflects the capacity of analytical method to remain unaffected in slightly varied conditions (Veaaman, 1996) Results revealed low values of % RSD i. e. less than 1% after introducing small deliberate changes in the developed HPTLC method.

Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in presence of other component that may expected to be present in sample matrix. The peak purity was calculated as per regression (r^2). The values for lupeol were r2 (Start, middle) = 0.9984 and r^2 (middle, end) = 0.9979, values for diosgenin were r^2 (Start, middle) = 0.9963and r^2 (middle, end) = 0.9958.

The results of correlation coefficient reveal a good linear relationship between response and concentration and obeying Beer's law. Lupeol was found to be 1.769 and 1.534 % whereas diosgenin was found to be 1.324 and 1.213 % in in-house and marketed formulation respectively. In the precision section the results revealed repeatability of developed method as well as proper functioning of the HPTLC methods. The method was also evaluated for intra-day and inter-day precision and results was found to be precise (Table 3). Limit of detection and quantification the results here revealed LOD for lupeol and for diosgenin to be 1µg /spot. LOQ for lupeol to be 6µg /spot and for diosgenin to be 7µg /spot as shown in Table 2 which indicates the adequate sensitivity of the method and results revealed accuracy of method in a desired range. Chromatographic specificity was investigated by comparing the R_f value of standards and samples and it was found to be identical. No impurities or degradation products were found along with the peaks of standard drug solutions, hence making the method specific.

Conclusion

From the experimental data and results obtained, it can be concluded that the UV Spectroscopy and HPTLC method was found to be simple, precise, specific, sensitive and accurate for the Quantitative estimation of the Lupeol and Diosgenin in the polyherbal formulation, Mutrakrichantak Churna. The method was validated according to ICH guidelines. The marker compound was identified in the product by the spectral scanning. The selected mobile phase gave good resolution of the compound in the product. This method can be used for identification of lupeol and diosgenin in other ayurvedic or herbal products.

Table 1: Authentification of Herbs Present In Mutrakrichantak Churna

Herbs	Code	Part used	Quantity	Voucher No.
Crateva nurvala	CN	Root	30 gm.	KNK/2018/212
Boerhavia diffusa	BD	Root	30 gm.	KNK/2018/213
Tribulus terrestris	TT	Whole	30 gm.	KNK/2018/214
Chicorium intybus	CI	Leaves	30 gm.	KNK/2018/215
Phyllanthus niruri	PI	Whole	20 gm.	KNK/2018/216
Albezzia lebbok	AL	Bark	20 gm.	KNK/2018/217
Moringa oleifera	MO	Bark	20 gm.	KNK/2018/218
Achyranthes aspera	AA	Fruit/ Seeds	10 gm.	KNK/2018/219

Table 2: Method Validation Parameters for the Quantification of Lupeol and Diosgenin

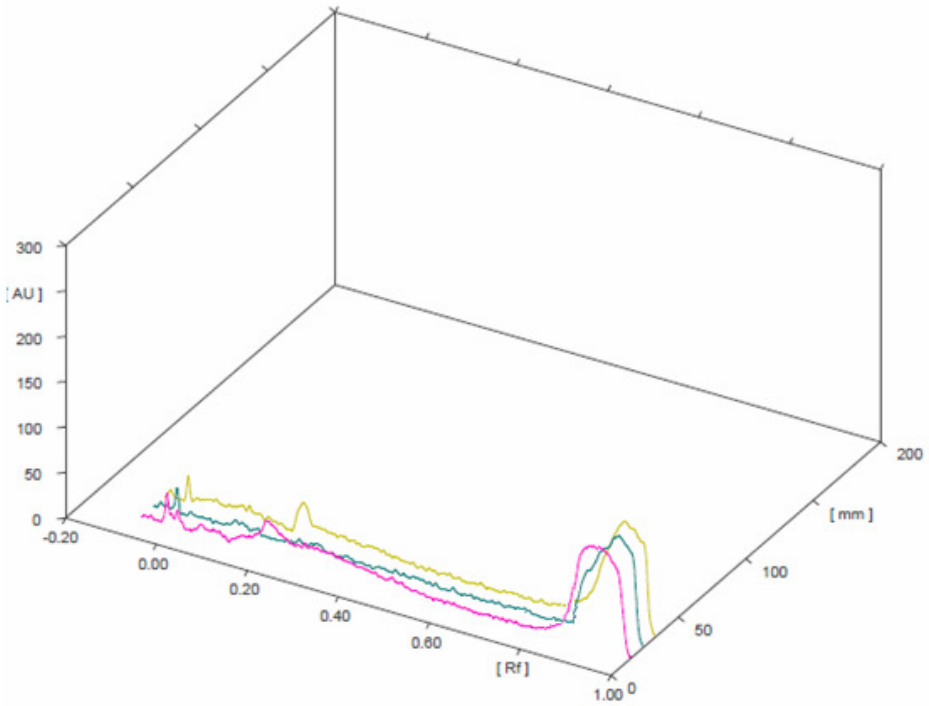
Method Property	Lupeol	Diosgenin
R_f	0.76	0.81
Instrumental precision (RSD [%] n=6)	0.9	0.8
Intra assay precison (RSD [%] n=6)	0.7	0.9
Intermediate precision (RSD [%] n=6)	0.6	0.8
Correlation coefficient, r	0.9997	0.9971
Calibration range (µg)	1-5	1-5
LOD	1	1
LOQ	5	6
Specificity	Specific	Specific
Robustness	Robust	Robust

Table 3: Intra-day and Inter-Day Precision of HPTLC (n=6)

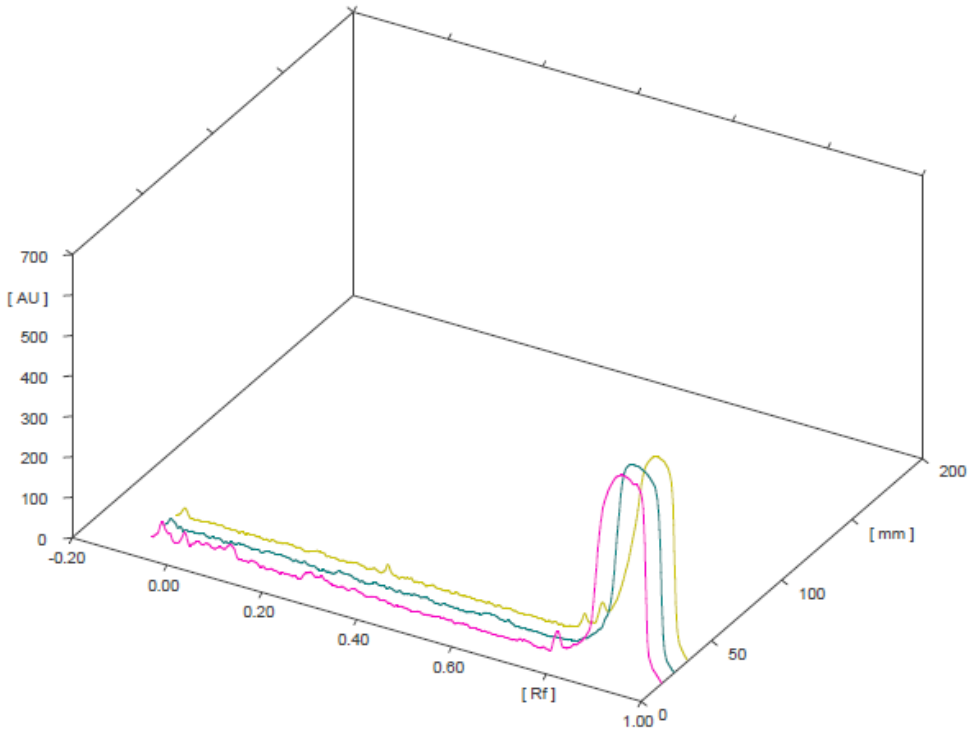
Amount	Intra-day precision			Inter-day Precision		
(µg/spot)	Mean area	SD	%RSD	Mean area	SD	%RSD
Lupeol						
2	99.12	0.10	0.11	97.16	1.04	1.07
3	99.46	0.35	0.35	97.17	0.07	0.08
5	98.59	0.45	0.46	98.56	0.59	0.60
Diosgenin						
2	98.36	0.11	0.12	97.53	0.25	0.26
3	98.57	0.41	0.43	98.51	0.44	0.45
5	98.49	0.14	0.14	99.12	0.58	0.5

Table 4: Percentage Recovery of Lupeol and Diosgenin

Preparation	Compound	Amount present in sample (µg)	Amount added (µg)	Amount found (µg)	Recovery (%)	Average recovery (%)
In-house	Lupeol	0.876	0.700	1.534	97.3	98.1
		0.876	0.876	1.734	98.97	
		0.876	1.051	1.890	98.04	
	Diosgenin	0.692	0.553	1.214	97.51	96.98
		0.692	0.692	1.346	97.39	
		0.692	0.830	1.462	96.05	
Marketed	Lupeol	0.724	0.579	1.299	99.69	99.43
		0.724	0.724	1.401	98.10	
		0.724	0.868	1.601	100.5	
	Diosgenin	0.596	0.476	1.049	97.85	98.27
		0.596	0.596	1.168	97.98	
		0.596	0.715	1.298	99.00	

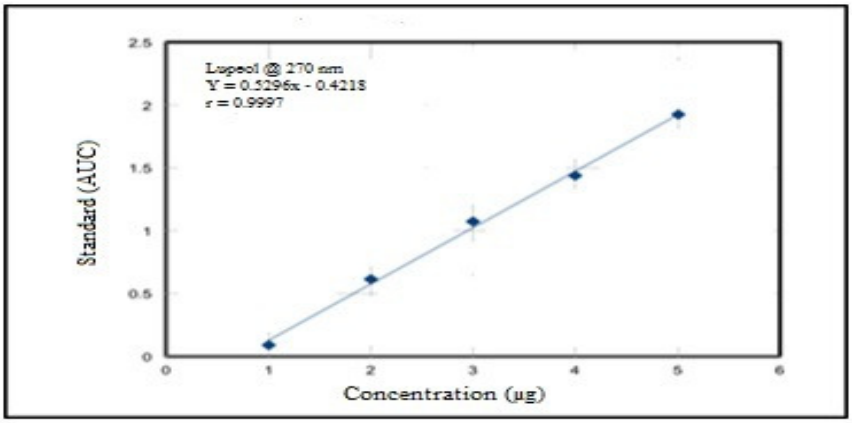


(a) Chromatograph of Lupeol R_f-0.76

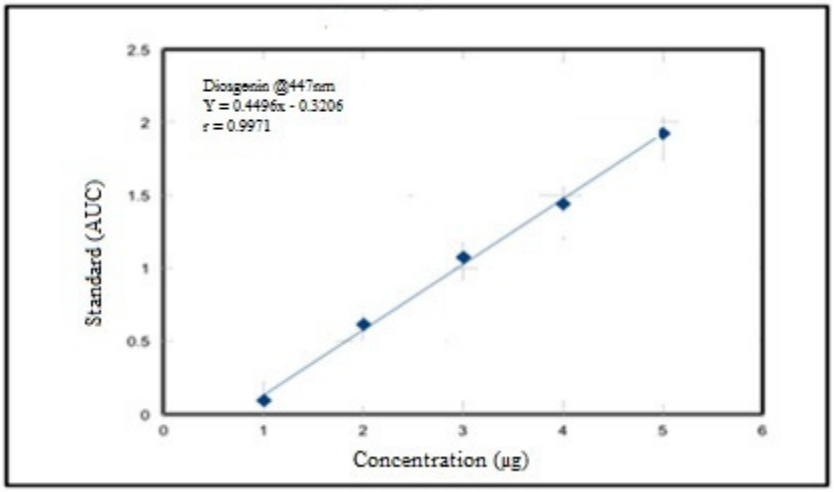


(b) Chromatograph of Diosgenin R_f-0.81

Fig. 1: The three dimensional HPTLC overlay of Lupeol and Diosgenin in in-house, marketed formulation and standard lupeol and diosgenin



(a) Lupeol



(b) Diosgenin

Fig. 2: Calibration plot

References

Armbruster, D.A. and Pry, T. (2008). Limit of blank, limit of detection and limit of quantification. Clin Biochem Rev; 29 (Suppl 1): S49–S52.

Atmakuri, L.R. and Dathi, S. (2010). Current trends in herbal medicines. J Pharm Res 3(1): 109-113.

Betz, J.M.; Brown P.N. and Roman, M.C. (2011). Accuracy, Precision and Reliability of Chemical Measurements in Natural Products Research. Fitoterapia 82(1): 44–52.

Bhattacharjee, A.; Shashidhara, S.C. and Narayana, A. (2012). Phytochemical and ethno-pharmacological profile of Crataeva nurvala Buch-Hum (Varuna): A review". Asian Pac J Trop Biomed 2(2): S1162–8.

Bopana, N. and Saxena, S. (2008). *Crataeva nurvala*: A Valuable Medicinal Plant. J Herbs Spices Med Plants, 14(1-2): 107-127.

Bridwell, H.; Dhingra, V.; Peckman, D. and Roark, J. (2010). Perspectives on method validation: importance of adequate method validation. Qual Assur J., 13: 72–77.

Calixto, B.J. (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Braz J Med Biol Res., 33:179-89.

Cesar, I.C. and Pianetti, G.A. (2009). Robustness evaluation of the chromatographic method for the quantitation of lumefantrine using Youden's test 45(2): 235-240.

Darekar, R.S.; Khetre, A.B.; Sinha, P.K.; Jeswani, R.M. and Damle, M.C. (2008). Quantization of lupeol octa cosanoate in *Hemidesmus indicus* R. Br. root powder by HPTLC ; Rasayan J Chem 1(3): 526-531.

De Smet, P.A. (1997). The role of plant-derived drugs and herbal medicines in healthcare. Drugs; 54(6): 801-40.

Di, X.; Chan, K.K.; Leung, H.W. and Huie, C.W. (2003). Fingerprint profiling of acid hydrolyzates of polysaccharides extracted from the fruiting bodies and spores of Lingzhi by high-performance thin-layer chromatography. J Chromatogr A. 1018(1): 85-95.

Hamidi, D.; Aulia, H. and Susanti, M. (2017). High Performance Thin layer Chromatography: Densitometry Method for Determination of Rubraxanthone in the Stem Bark Extract of *Garcinia cowa* Roxb. Pharmacognosy Res; 9(3): 230-233.

https://www.ayurmedinfo.com/2018/03/06/mutrakrichantak-churna/

ICH, Q2A, (2005). Validation of analytical procedures: text and methodology, in: Proceedings of International Conference on Harmonization, Geneva.

ICH, Q2B, (1996). Validation of analytical procedure: methodology, IFPMA, in: Proceedings of International Conference on Harmonization, Geneva.

Jurado, J.M.; Alcazar, A.; Muniz, V.R.; Ceballos, M.S.G. and Raposo, F. (2017). Some practical considerations for linearity assessment of calibration curves as function of concentration levels according to the fitness-for-purpose approach. Talanta, 172(1): 221-229.

Larsen, T.; Axelsen, J.; Ravn, H.W.J. (2004). Simplified and rapid method for extraction of ergosterol from natural samples and detection with quantitative and semi quantitative methods using thin-layer chromatography. J Chromatogr A 1026: 301–304.

Oliveira, W.P.; Bott, R.F. and Souza, C.R. (2006). Manufacture of standardized dried extracts from medicinal Brazilian plants. Dry Technol; 24(4): 523-33.

Parvin, S.; Kader, A.; Muhit, A.; Haque, E.; Mosaddik, A. and Wahed, M.I. (2011). Triterpenoids and phytosteroids from stem bark of *Crataeva nurvala* buch ham. J Appl Pharm Sci 1(9): 47-50.

Pattanayak, P.; Behera, M.; Mohapatra, P. and Panda, S. (2011). Standardization and evaluation of laxative activity of a poly herbal formulation. Der Pharm Lett 3(1): 276-286.

Pillai, D. and Pandita, N. (2016). Validated high performance thin layer chromatography method for the quantification of bioactive marker compounds in Draksharishta, an ayurvedic polyherbal formulation. Revista Brasileira de Farmacognosia volume, 26: 558–563.

Vessman, J. (1996). Selectivity or specificity? Validation of analytical methods from the perspective of an analytical chemist in the pharmaceutical industry. J Pharmaceut Biomed 14 (8-10): 867-869.