

Identification and Quantification of Andrographolide from *Andrographis paniculata* (Burm. f.) Wall. ex Nees by RP-HPLC Method and Standardization of its Market Preparations

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ABSTRACT: *Andrographis paniculata* (Burm. f.) Wall. ex Nees, commonly known as Kalmegh, is widely used as antimalarial drug in herbal and traditional systems. Andrographolide is the major triterpenoid present in the plant and responsible for its therapeutic activities. The identification and quantification of andrographolide were ascertained by various spectrophotometric and chromatographic analyses. The plant sample was extracted with methanol. The amorphous residue obtained from extraction was analyzed for identification of andrographolide by chemical method, TLC, UV-Vis, FT-IR and LCMS/MS analyses. The quantitative estimation of andrographolide content in plant sample was carried out by simple reversed phase HPLC method with C₁₈ column using a mixture of water and methanol (35:65) as mobile phase at a flow rate of 0.7 mL/min, and the estimated concentration level of andrographolide was found as 38.36±0.42 µg/mL in the solution of amorphous residue (50 µg/mL). After quantitative determination of andrographolide, standardization of its market preparations was accomplished using the same RP-HPLC method. The estimated % potencies of andrographolide compared to reference standard in six market preparations were found to be 97.56, 98.37, 98.21, 95.60, 98.91 and 96.40.

Key words: *Andrographis paniculata* (Burm. f.) Wall. ex Nees, Kalmegh, Andrographolide, RP-HPLC, spectrophotometry

INTRODUCTION

Plants have been the basis for medical treatments through much of human history and such traditional medicines are still widely practiced today. The World Health Organization (WHO) estimates that three fourth of the world population presently use herbal medicines for some aspect of primary health care.¹ Approximately 120 active compounds currently isolated from the higher plants are widely used in modern medicine today.² Beyond the pharmaceutical approaches of plants, there is a wide tendency to utilize herbal products as dietary supplements with the intention of improving the quality of life and

preventing diseases of elderly people.³ *Andrographis paniculata* (Burm. f.) Wall. ex Nees (family: Acanthaceae) is a herbaceous plant and most commonly used as medicinal plant in the traditional system.⁴ It grows in many South Asian countries and also used as a traditional medicine in China, Hong Kong, Philippines, Malaysia, Indonesia and Thailand.⁴ *A. paniculata* is the main source of the bitter principle 'andrographolide'. Because of extremely bitter and characteristic taste of *A. paniculata*, it is known as "king of bitters". Beside andrographolide, this plant contains 14-deoxy-11, 12-didehydroandrographolide-2, 14-deoxyandrographolide-3, 3, 14-deoxyandrographolide-4, 14-deoxy-11-oxoandrographolide-5, neoandrographolide-7, 14-deoxyandrographolide-9⁵ in significant quantities.

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The aqueous extract of *A. paniculata* shows antimicrobial activity due to combined effect of arabinogalactan proteins and andrographolide.⁶ It has also been used traditionally for sluggish liver as antidote in case of colic dysentery and dyspepsia.⁷ The fresh leaf juice is a domestic remedy in the treatment of colic pain, loss of appetite, irregular stools and diarrhea.⁸ The active principle, andrographolide is also attributed with some other activities like liver protection⁹, anticancer¹⁰ and antimalarial activities.¹¹ Beside these, *A. paniculata* shows a broad range of pharmacological effects such as inhibition of replication of the HIV virus, antibacterial activity, antihyperglycemic effects, suppression of various cancer cells, anti-inflammatory properties¹² and many more diseases.

Now-a-days, different analytical methods have been developed for isolation, identification and quantification of andrographolide from indigenous plant sources, and for standardization of its marketed products. These analytical methods are highly selective for evaluating the therapeutic potential of the purified bioactive principles in order to achieve the optimal outcomes. The objectives of the present study were to identify and quantify andrographolide in *A. paniculata* and standardize its market preparations so that the herbal and traditional manufacturers can produce their products by maintaining quality requirements. In the present study, a simple, precise and reproducible RP-HPLC method has been developed, through which andrographolide in *A. paniculata* was determined and quantified.

MATERIALS AND METHODS

Plant materials. The green leaves and stems of *A. paniculata* were collected from the Medicinal Plant Garden, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh and Square Herbal & Nutraceuticals Limited, Pabna, Bangladesh in the month of September, 2012. The taxonomical identification of the plant was confirmed by National Herbarium, Bangladesh (accession number: DACB-37879). The whole plant material was dried under

shade, powdered using blender and stored in air tight container.

Chemicals and instruments. The solvents and reagents for analyses were of analytical grade and used without further purification. Sixty andrographolide capsules, each containing 200 mg of andrographolide, were purchased from six different local herbal and traditional manufacturers. Reference standard of andrographolide with potency 99.06% (Article No. 89821, Batch No. 1000003992, Germany) was supplied by Square Herbal and Nutraceuticals Ltd, Pabna, Bangladesh. HPLC grade methanol (Sigma Aldrich, Germany) and water purified by Millipore purified system were used for analysis. TLC plate (Aluminium foil precoated with Silica 60F₂₅₄), UV-Vis Spectrophotometer (Shimadzu, Japan), FT-IR (Shimadzu, Japan) and LCMS/MS (Shimadzu, Japan) were used. HPLC (Shimadzu, Japan) equipped with a manual injector, a vacuum degasser, a multiple-wavelengths UV/Visible detector (Shimadzu SPD 20A, Japan) and an ODS column namely Capcell Pak (150 mm×4.6 mm I.D., 5 µm particle size) were used for chromatographic analyses.

Extraction of plant material. About 250 g of *A. paniculata* plant powder was weighed and moisture content of the sample was determined by Karl-Fischer method. The plant powder (moisture content 0.132%) was placed in a round bottom flask and methanol (450 mL) was poured in it with uniform mixing. The mixture was then gently warmed in water bath for 60 min and decanted. The process was repeated for three times. The crude extracts were combined, allowed to cool and filtered to remove the coarse particles and the solvent was evaporated under vacuum to get oily residue. The residue was then extracted with chloroform and the organic layer was collected. An amorphous residue (about 10.6 g) was obtained after drying the organic layer which was then stored in air tight container and subjected to further analyses for identification and quantification of andrographolide.

Identification of andrographolide in plant sample

Chemical identification. About 0.5 mg of amorphous residue was dissolved in 5 mL of methanol, then treated with 1 mL of 2,4-dinitrophenylhydrazine and 100 mL of 2M HCl was added. A yellow orange color indicating the presence of andrographolide.^{13,14}

About 8 drops of alcoholic potassium hydroxide (10%) was added to 0.5 mg of amorphous residue and a red color was appeared which kept for 14 min. The red color changed to yellow indicating the presence of andrographolide.¹⁵

TLC analyses. The amorphous residue was subjected to TLC analyses along with reference standard of andrographolide where a mixture of chloroform, methanol and ethyl acetate at ratio of 7:2:1 was used as the mobile phase. The analyzed TLC plates were then sprayed with 3, 5-dinitrobenzoic acid in ethanol (2% w/v solution)¹⁶ and KOH in ethanol (6% w/v solution)¹⁶ for visualizing spot position.

Spectral analyses. The residue and standard andrographolide were both scanned for absorption maxima (λ_{\max}) using UV-Vis Spectrophotometer (methanol as blank) within the range of 200 to 400 nm. The characteristic absorption bands for the functional groups of andrographolide were ascertained through FT-IR analyses (KBr disc) within the range of 400 to 4000 cm^{-1} and the molecular weight was determined by LCMS/MS.

Quantitative analyses of andrographolide

Preparation of sample solutions. The sample solution of 50 $\mu\text{g}/\text{mL}$ concentration was made by dissolving the required quantity of amorphous residue in HPLC grade methanol. The solution was thoroughly shaken and sonicated until complete dissolution of the coarse and visible particles. Then the solution was allowed to cool at room temperature and the volume was adjusted to the mark with methanol, and was filtered through 0.45 μm filter paper. A total of seven sample solutions of 50 $\mu\text{g}/\text{mL}$ concentration were prepared and stored in clean vials bellow 10 $^{\circ}\text{C}$.

Preparation of standard solutions. The standard stock solution of 160 $\mu\text{g}/\text{mL}$ concentration was prepared with reference andrographolide in HPLC grade methanol with shaking and sonication. The standard solutions with the concentrations of 140, 120, 100, 60, 40 and 20 $\mu\text{g}/\text{mL}$ were then prepared by suitable dilution of stock solution with methanol. Finally the prepared standard solutions were filtered separately through 0.45 μm filter paper and stored bellow 10 $^{\circ}\text{C}$.

Chromatographic condition. The andrographolide content in amorphous residue was determined by reversed phase HPLC with C_{18} column. The elution was carried out with a binary solvent system of water and methanol (35:65) as mobile phase at a flow rate of 0.7 mL/min maintaining ambient temperature. The sample injection volume was 20 μL , and the analyses were monitored with the UV-Vis detector at 223 nm.

Quantification of andrographolide in plant sample. The standard solutions of different concentrations were analyzed with the RP-HPLC method and peak areas were recorded. A calibration curve was prepared by using peak areas and concentrations of standard solutions. Sample solutions (50 $\mu\text{g}/\text{mL}$) were analyzed and the peak areas were recorded and concentration of andrographolide in sample solutions were determined by using calibration curve.

Standardization of market preparations

Preparation of market samples. Ten capsules were taken for each market preparation (200 mg andrographolide capsule) and average weight of the contents was determined. The powdered sample equivalent to 200 mg of andrographolide was taken in a clean and dry 100 ml volumetric flask. Methanol (10 mL) was added, and shaken thoroughly and then sonicated for 5min for complete dissolution of drug. The solution was allowed to cool at room temperature and the volume was made up to the mark with methanol. The solution was filtered through Whatman filter paper (No. 42) and then finally filtered through 0.45 μm disk filter. It was kept in air

tight containers and stored below 10 °C. After suitable dilution, the solutions of 50 µg/mL concentration were prepared with HPLC grade methanol.

Estimation of andrographolide in market preparations. The solutions of market samples were analyzed through RP-HPLC method maintaining similar chromatographic conditions mentioned. The sample solutions (50 µg/mL) were injected and the peak areas were recorded at defined retention time. Andrographolide concentration in market samples were determined using the calibration curve for reference standard. All determinations were conducted in triplicate.

RESULTS AND DISCUSSION

TLC analyses. The R_f value of residue (0.38) and standard andrographolide (0.37) matched completely, which indicated the presence of andrographolide in amorphous residue (Figure 1).

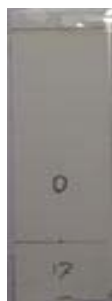


Figure 1. TLC analysis of *A. paniculata* amorphous residue.

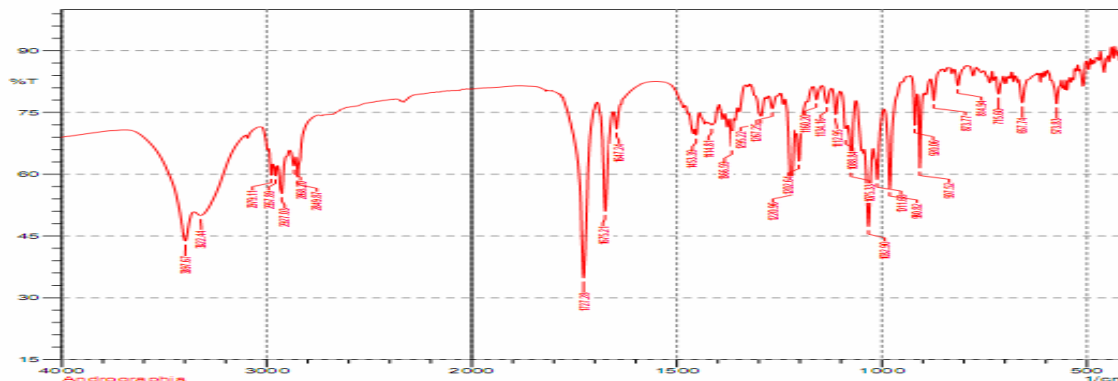


Figure 2. FT-IR spectrum of *A. paniculata* amorphous residue.

Spectral analyses. The TLC analyses showed the presence of andrographolide in amorphous residue of *A. paniculata* which was further confirmed through UV-Vis, FT-IR and LCMS/MS analyses. The residue and standard andrographolide were subjected to UV-Vis spectrophotometry and λ_{max} was observed at 223 nm for both. The FT-IR spectrum of the extracted plant residue showed (Figure 2) the characteristic absorption bands at 1454 cm^{-1} (stretching vibration of C=C bond); 1648, 1675 cm^{-1} (stretching vibration of C=O); 2927, 2958, 2980 cm^{-1} (stretching vibration of C-H) and 3322, 3397 cm^{-1} (stretching vibration of O-H). Similar characteristics were observed in case of standard andrographolide.¹⁷

The molecular weight of compound in amorphous residue was determined through LCMS/MS method and the observed value was at m/z 349.1 $[M-H]^+$ (Figure 3), which was found to be similar with the reported literature.¹⁸

Quantification of andrographolide in plant sample. Quantification of andrographolide in amorphous residue was carried out through RP-HPLC method. A standard solution of reference andrographolide was injected and the retention time was found to be 7.557 min (Figure 4).

A calibration curve was prepared by using peak areas and concentration (Table 1) of standard solutions, and the content of andrographolide in residue was quantified by using linear regression equation of the calibration curve.

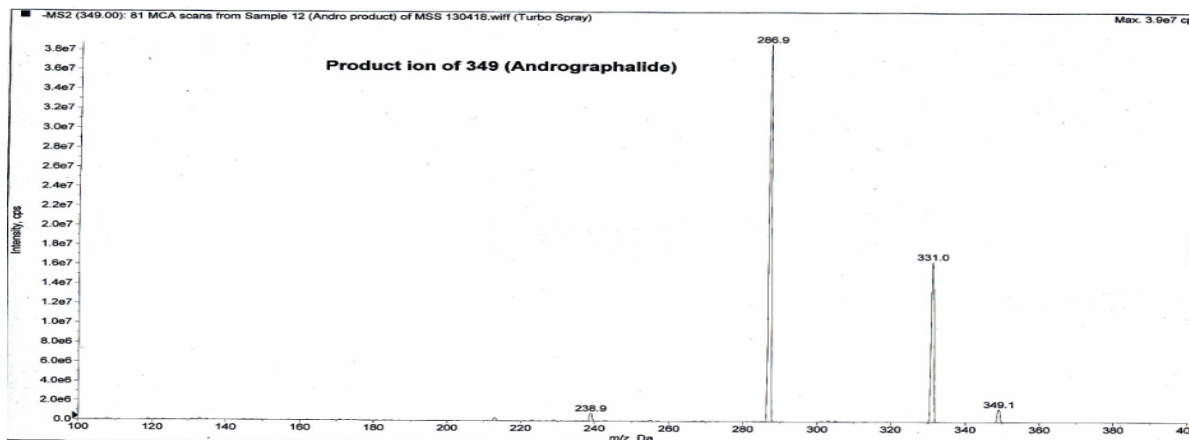


Figure 3. LCMS/MS spectrum of *A. paniculata* amorphous residue.

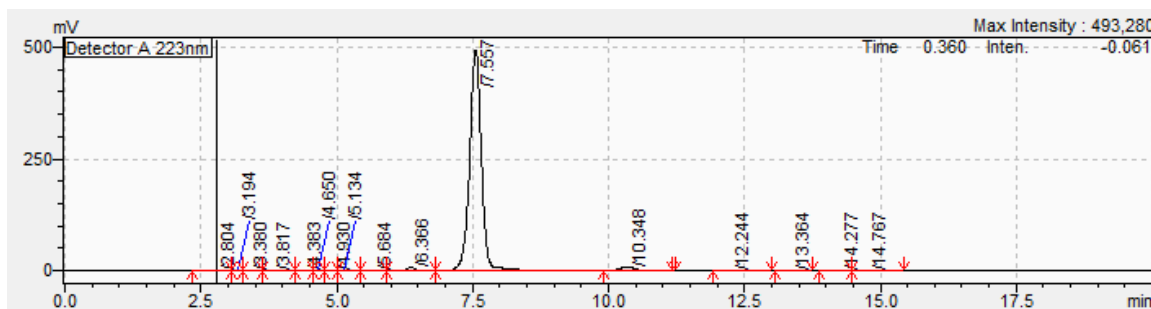


Figure 4. Chromatogram for reference standard of andrographolide observed by RP-HPLC.

Table 1. Peak areas of RP-HPLC chromatogram for various concentrations of reference standard of andrographolide.

No.	Concentration (µg/mL)	Peak area
1	20	1317009
2	40	2792160
3	60	3986327
4	100	5959159
5	120	7443306
6	140	8245356

The linear regression equation of calibration curve was: $Y = 57250X - 37719$

Where, X = concentration and Y = peak area with correlation coefficient (R^2), 0.995 (Figure 5).

The solution of amorphous residue (50 µg/mL) was injected maintaining the similar chromatographic condition of reference standard and the retention time

for andrographolide was observed at 7.570 min (Figure 6). The analyses of residue were replicated for seven times with the prepared sample solutions and the peak areas were obtained. The estimated quantity of andrographolide was 38.36 ± 0.42 µg/mL in the solution of amorphous residue (Table 2).

Standardization of market preparations

Solutions of six market preparations (50 µg/mL) were analyzed and each chromatogram showed different peak areas at defined retention time (Figure 7).

The concentration of andrographolide in each market preparation was determined by using the linear regression equation of calibration curve for reference standard. The amount of andrographolide in capsules were found to be 195.121 ± 1.545 mg, 196.743 ± 1.926 mg, 196.420 ± 0.856 mg, 191.199 ± 0.735 mg, 197.825 ± 1.169 mg and 192.803 ± 0.184 mg with the estimated % potency of 97.56, 98.37, 98.21, 95.60, 98.91 and 96.40, respectively (Table 3).

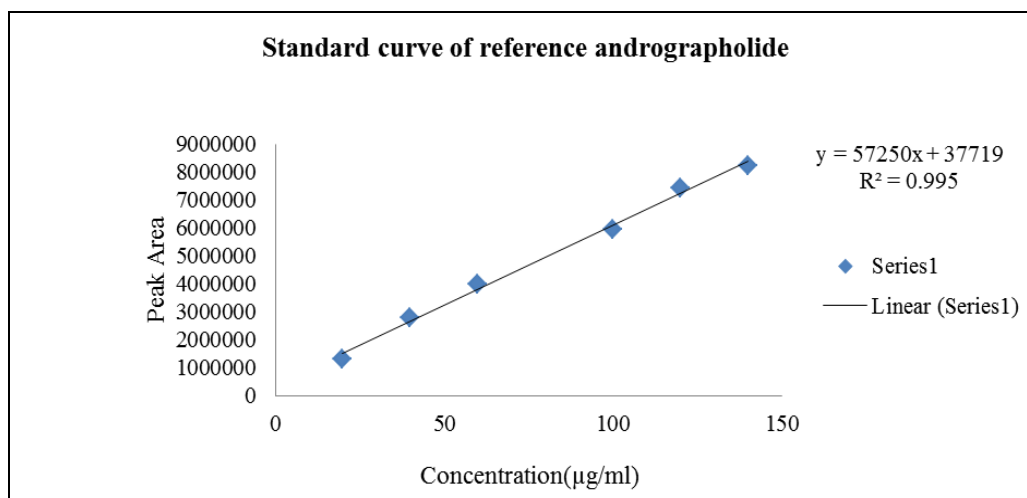


Figure 5. Calibration curve for reference standard of andrographolide.

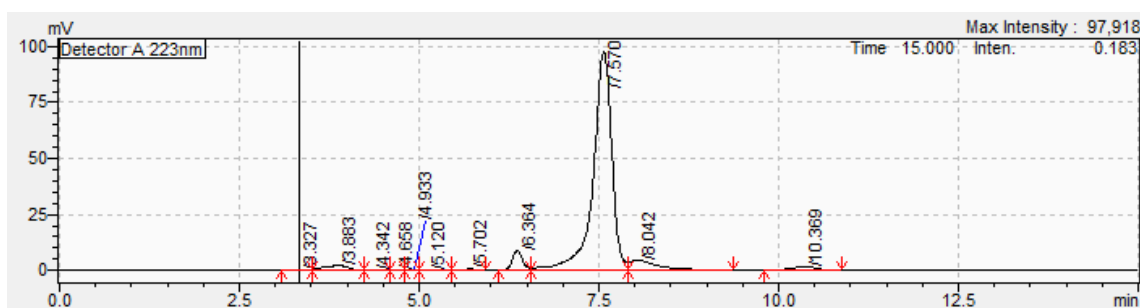
Figure 6. RP-HPLC profiles of *A. paniculata* amorphous residue.

Table 2. Quantification of andrographolide in amorphous residue.

Concentration of amorphous residue	No. of RP-HPLC analyses	Peak area of HPLC chromatogram	Concentration ($\mu\text{g/mL}$) of andrographolide	Average concentration \pm S.D.
50 $\mu\text{g/mL}$	1	2187589	38.87	38.36 \pm 0.42
	2	2136636	37.98	
	3	2130911	37.88	
	4	2169841	38.56	
	5	2193886	38.98	
	6	2135491	37.96	
	7	2152666	38.26	

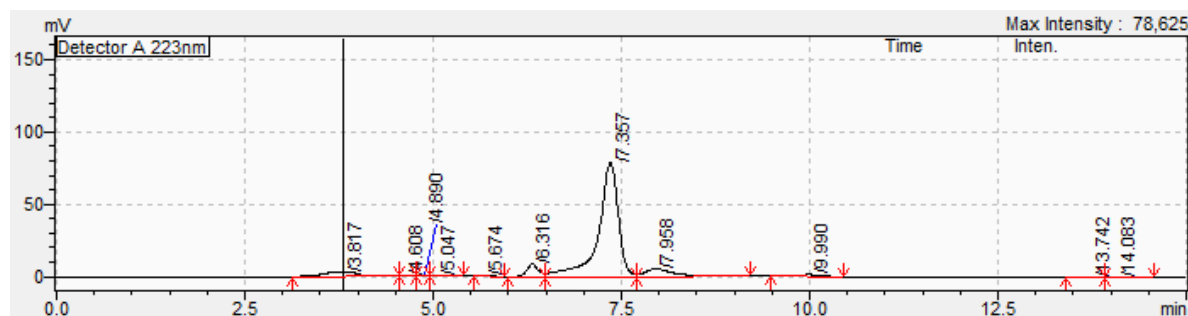
Figure 7. Chromatogram of *A. paniculata* market preparations obtained during RP-HPLC.

Table 3. Estimation of andrographolide in market preparations (50 µg/mL).

	Replicate	Peak area	Concentration (µg/mL)	Andrographolide content found in capsule (mg)	Average ± SD	Claimed andrographolide content in capsule (mg)	Potency (%)
Sample-1	1	2035304	36.21	196.7362	195.121± 1.545	200	97.56
	2	2029579	36.11	195.5862			
	3	1993511	35.48	193.0396			
Sample-2	1	2187589	38.87	198.9211	196.743± 1.926	200	98.37
	2	2136636	37.98	194.2373			
	3	2165261	38.48	197.0715			
Sample-3	1	2098851	37.32	196.9152	196.420± 0.856	200	98.21
	2	2105721	37.44	197.1291			
	3	2078814	36.97	195.2164			
Sample-4	1	1980916	35.26	192.1247	191.199± 0.735	200	95.60
	2	1974046	35.14	191.1475			
	3	1964886	34.98	190.3262			
Sample-5	1	1945421	34.64	196.3257	197.825± 1.169	200	98.91
	2	1980916	35.26	199.1767			
	3	1964886	34.98	197.9728			
Sample-6	1	1958016	34.86	192.5701	192.803± 0.184	200	96.40
	2	1965402	34.99	193.0204			
	3	1959095	34.88	192.8173			

Standardization of *A. paniculata* preparations were accomplished through identification, quantification and comparative analyses between standard andrographolide and its market preparations. The results showed that market preparations contained significant quantity of andrographolide compared to reference standard. The described methods have the advantages of simple, precise, less time consuming and more convenient especially for routine analyses, and may be used for evaluating the quality of herbal and traditional medicines by their manufacturers.

CONCLUSION

The developed RP-HPLC method along with TLC, UV-Vis, FT-IR and LCMS/MS are highly efficient for identification and quantification of andrographolide in *A. paniculata*. Now-a-days, most of the herbal and traditional manufacturers are marketing andrographolide preparations for its potential therapeutic activities but due to lack of proper instrumentations and knowledge, manufacturers cannot maintain the desired quality requirements of the preparations. For this reasons, we have developed the prescribed method which is simple, sensitive, statistically validated and ensures the quality requirement of the products. All these

methods will open up a new door for the herbal and traditional manufacturers to identify, quantify the phytomarker and standardize their market preparations by meeting the regulatory requirements imposed by regulatory authorities.

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REFERENCES

1. World Health Organization, 2000. Promoting the role of traditional medicine in health systems: a strategy for the African Region 2001-2010 (document reference AFR/ RC50/ Doc.9/R), Harare.
2. Fabricant, D.S. and Farnsworth, N.R. 2001. The value of plants used in traditional medicine for drug discovery. *Environ. Health Perspect.* **109**, 69-75.
3. Massimo, M. 2003. Dietary supplements of plant origin-nutrition and health approach, Taylor & Francis Ltd., New York, Chapter 3, pp. 26-29.

4. Tang, W. and Eisenbrand, G. 1992. Chinese drugs of plant origin: Chemistry, pharmacology, and use in traditional and modern medicine. *Pharmazie unserer Zeit*. **21**, 281-282.
5. Poonam K., Tiwari, U.K., Shukla, A. and Gaur, A.K. 2010. Chemical constituents isolated from *Andrographis paniculata* Nees. *Indian J. Chem.* **49B**, 356-359.
6. Singha, P.K., Roy, S. and Dey, S. 2003. Antimicrobial activity of *Andrographis paniculata* Nees. *Fitoterapia* **74**, 692-694.
7. Handa, S.S. and Sharma, A. 1990. Hepatoprotective activity of andrographolide from *Andrographis paniculata* Nees against carbon tetrachloride. *Indian J. Med. Res.* **92**, 276-283.
8. Saxena, S., Jain, D.C., Bhakuni, R.S. and Sharma, R.P. 1998. Chemistry and pharmacology of *Andrographis* species. *Indian Drugs* **35**, 458-467.
9. Saraswat, B., Visen, P.K.S., Patnaik, G.K. and Dhawan, B.N. 1995. Effect of andrographolide against galactosamine induced hepatotoxicity. *Fitoterapia* **66**, 415-420.
10. Lal, J., Tripathi, H.C. and Tandon, S.K. 1986. Antidiabetic activity of andrographolide. *Indian J. Pharmacol.* **18**, 58-61.
11. Rahman, N.N., Furuta, T., Kojima, S., Takane, K. and Mohd, M.A. 1999. Antimalarial activity of extracts of Malaysian medicinal plants. *Ethnopharmacol. J.* **64**, 249-254.
12. Yang, S., Evens, A.M., Prachand, S., Singh, A.T., Bhalla, S. and David, K. 2010. Mitochondrial mediated apoptosis in lymphoma cells by the diterpenoid lactone andrographolide: the active component of *Andrographis paniculata* Nees. *Clin. Cancer Res.* **16**, 4755-4768.
13. Ali, M. 2009. Pharmacognosy and Phytochemistry. CBS publishers & distributors, Delhi, Chapter 3, pp. 181-182.
14. Mukherjee, P.K. 2002. Quality Control of herbal drugs: An approach to evaluation of botanicals. Business Horizons Pharmaceutical Publisher, New Delhi, pp. 578-580.
15. Medicinal Plant Research Institute, Department of Medical Science, Ministry of Public Health, 1999. Standards of Thai Herbal Medicine: *Andrographis paniculata* Nees. The War Veterans Organization Press, Bangkok, pp. 66-67.
16. Meenu, S. and Sharma, R.G. 2013. Identification, purification and quantification of andrographolide from *Andrographis paniculata* (Burm. f.) Nees by HPTLC at different stages of life cycle of crop. *J. Curr. Chem. Pharm. Sci.* **3**, 23-32.
17. Cuthbertson, D.J., Johnson, S.R., Piljac-Žegarac, J., Kappel, J., Schäfer, S., Wüst, M., Ketchum, R.E.B., Croteau, R.B., Marques, J.V., Davin, L.B., Lewis, N.G., Rolf, M., Kutchan, T.M., Soejarto, D.D. and Lange, B.M. 2013. Accurate mass-time tag library for LC/MS based metabolite profiling of medicinal plants. *Phytochemistry* **91**, 187-197.
18. Dilip, B.J. and Bhaskar, N.T. 2012. Quantitative estimation of andrographolide by reverse phase high liquid chromatography method from *Andrographis paniculata*. *Int. J. Phytopharm.* **2**, 149-153.