Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(6): 337-344
Received: 21-09-2016
Accepted: 22-10-2016

Chanchal Garg
Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak, Haryana, India

Pratibha Sharma
Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak, Haryana, India

Saurabh Satija
Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak, Haryana, India

Munish Garg
Prof. Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak, Haryana, India

Correspondence
Munish Garg
Prof. Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak, Haryana, India

Stability indicating studies of *Andrographis paniculata* extract by validate HPTLC protocol

Chanchal Garg, Pratibha Sharma, Saurabh Satija and Munish Garg

Abstract
The present study was aimed to develop and validate stability indicating studies *Andrographis paniculata* by HPTLC. The separation was attained by using precoated TLC plates with silica gel $60F_{254}$. The mobile phase used was chloroform-toluene-methanol (7:1.5:1.5, v/v/v). Densitometric analysis was carried out at 254 nm. The linear regression analysis data obtained for the calibration plots displayed good linear relationship with respect to peak height and peak area, in the concentration range 100-500 ng/spot. The limits of detection (LOD) and quantification (LOQ) were found to be 0.52 ng/spot and 1.59 ng/spot, respectively. Stability indicating studies exhibited the well resolved peak of the standard andrographolide and a complete degradation in case of extract of *Andrographis paniculata*. The present study describes the degradation of standard andrographolide and the extract of *Andrographis paniculata* under different stress conditions according to the ICH guidelines i.e. acid, alkali, dry heat, oxidation and photolytic degradation.

Keywords: *Andrographis paniculata*, HPTLC, validation, stability indicating studies, forced degradation studies.

Introduction
Andrographis paniculata belonging to family Acanthaceae is extensively available in Sri Lanka, Pakistan and Indonesia. In India, it is especially found in Tamilnadu, Karnataka, Maharashtra, Orissa, Uttar Pradesh and Uttarakhand [1, 2]. *Andrographis paniculata* is official in Indian Pharmacopoeia as a chief constituent of many Ayurvedic formulations used in the management of liver disorders. It is used for treating neoplasms also [3, 4]. *Andrographis paniculata* proclaims its use in getting rid of body heat and expelling toxins and for this reasons it is also documented as a cold property herb in Traditional Chinese medicine (TCM) [5]. Andrographolide is the major bitter principle present in *Andrographis paniculata*, thus, referred to as 'king of bitters'. The aqueous extract of *Andrographis paniculata* exhibits antimicrobial activity. *Andrographis paniculata* or Kalmegh is widely used in ayurvedic formulations [5]. Andrographolide, a diterpenoid lactone, found in *Andrographis paniculata* is although distributed in the whole plant, but major concentrations are present in leaves and stem which can be isolated crystalline solid [7, 8]. Andrographolide is used to treat a variety of diseases like dysentery, diarrhea, inflammation, fever, sore throat etc. It is also used in the treatment of AIDS and various symptoms associated with immune disorders [9]. *Andrographis paniculata* is widely used in the form of fresh leaf juice as a home remedy for the treatment of colic pain, loss of appetite, irregular stools and diarrhea [10]. Andrographolide shows many other activities like liver protection [11], anti-diabetic [12] and anti-malarial [13]. Andrographolide ($C_{20}H_{30}O_5$) is easily soluble in organic solvents like methanol, ethanol, pyridine, acetic acid and acetone whereas it is slightly soluble in ether and water. Andrographolide can be best extracted from *Andrographis paniculata* using ethanol and methanol as solvents. The melting point of andrographolide is 228–230˚C and the ultraviolet spectrum in ethanol, λmax is 223 nm [14]. Andrographolide, neo-andrographolide and deoxyandrographolide are the three main diterpenoid lactones present in the leaves of *Andrographis paniculata*. The melting point of this compound is

Materials and methods
Plant material
The whole plant *Andrographis paniculata* was collected from Medicinal garden, Maharshi Dayanand University, Rohtak and authenticated from Department of Botany, Maharshi Dayanand University, Rohtak. Voucher specimen no. VS/Phcog/215 was kept in the Department of Pharmacy for future reference. Plant parts were dried at room temperature.

The dried sample was powdered using a mixer grinder and stored in air tight container. Marker andrographolide was procured from Sigma chemicals.

Chemicals
The reagents used in the present study methanol, toluene, chloroform, distilled water, HCl, NaOH, hydrogen peroxide (3% w/v) were of analytical grade from Merck, India.

Preparation of extracts
Methanol was used as solvent for the extraction of andrographolide from *Andrographis paniculata.* Methanol extract was prepared by using the soxhlet method for 3-4 hours at 40-50˚c. The Extract was concentrated on a water bath at 50˚C and solid extract was stored for further studies

Preparation of standard
10 mg marker andrographolide was transferred to a 10ml volumetric flask to make a solution of concentration 1mg/ml. The solution was further diluted with methanol to yield a solution containing 100 µg/ml.

Method validation
Precision
The precision of the method was ascertained by verifying the instrumental precision, intra-day precision and inter-day precision. Instrumental precision was verified by replicate (n=6) applications of same andrographoplide solution. Intra-day precision and inter- day precision was assessed by replicate (n = 6) applications of freshly prepared standard solution of same concentration (200-700 ng/spot), on the same day and on six different days respectively. The repeatability of sample application and measurement of peak area are expressed in terms of % CV.

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection and limit of quantification is the lowest concentration of analyte in a sample which can be detected and quantified. LOD and LOQ were estimated on the basis of signal to noise ratio. 200-700 ng/spot concentrations of the standard andrographolide were applied along with methanol, which was used as blank.

Specificity
The specificity of the method was confirmed by analyzing standard andrographolide and extracts. The spot for andrographolide in the sample was confirmed by comparing the R_f and spectra of the spot with that of sample. The peak purity of andrographolide was examined by comparing the spectra at peak start, peak middle and peak end positions of the spot/ bands.

Linearity
The linearity of any analytical procedure describes its ability within a given range to get test results that are directly proportional to the concentration of analyte in the sample. The content of andrographolide was determined by using a calibration curve established with a standard concentration range from 100 to 500 ng/spot. A stock solution (10 µg/ml) of standard andrographolide was prepared in methanol. The different volumes of stock solution 0.1, 0.2, 0.3, 0.4 and 0.5µl were spotted on the HPTLC plate to attain the concentration of 100, 200, 300, 400 and 500 ng/spot, respectively.

Forced degradation studies
According to ICH guideline, the stress testing aims at the identification of the probable degradation products which may further assist in determination of the intrinsic stability of the molecule and validate the stability indicating procedures adopted. The specificity of the method can be determined through forced degradation studies carried on the sample using acid, alkaline, oxidative, dry heat, photolytic degradations [15].

Alkali hydrolysis
For alkali degradation study, 2 ml of stock solutions of standard andrographolide and extract of *Andrographis paniculata* were taken in two separate 10ml volumetric flasks and 2 ml of 0.1N sodium hydroxide was added. Both the mixtures were heated on a water bath at 80˚C for 2 hours and cooled at room temperature. Then they were neutralized with 0.1N hydrochloric acid and diluted with methanol to make up the volume.

Acid hydrolysis
To perform acid degradation study, 2 ml of stock solutions of standard andrographolide and extract of *Andrographis paniculata* were taken in two separate 10 ml volumetric flasks and 2 ml of 0.1N hydrochloric acid was added. Both the mixtures were heated on a water bath at 80˚C for 2 hours and cooled at room temperature. These were then neutralized with 0.1N sodium hydroxide and diluted to make up the volume with methanol.

Oxidative stress degradation
For carrying out oxidative stress degradation, 2 ml of stock solutions of standard andrographolide and extract of *Andrographis paniculata* were taken in two separate 10 ml volumetric flasks and 2 ml of 3% hydrogen peroxide (H_2O_2) was added. All the mixtures were heated at 80˚C on a water bath for 2 hours and then cooled at room temperature. Finally, they were diluted with methanol to make up the volume.

Dry heat degradation
Analytically pure samples of standard andrographolide and extract of *Andrographis paniculata* were laid open in oven at 80˚C for 2 hours. The solids were then allowed to cool. 10 mg each of standard andrographolide and extract of *Andrographis paniculata* were accurately weighed and dissolved in few ml of methanol in two separate volumetric flasks (10 ml). Volumes of the volumetric flasks were made with the methanol. Aliquots from the stock solutions of standard andrographolide and extract of *Andrographis paniculata* were diluted with methanol to attain the working standards of 100 µg/ml concentration.

Photolytic degradation
The samples of standard andrographolide and extract of *Andrographis paniculata* were exposed to UV light for 6 hours. The solid samples were allowed to cool. 10 mg each of standard andrographolide and extract of *Andrographis paniculata* were accurately weighed and dissolved in few ml of methanol in two separate volumetric flasks (10 ml). Volumes of the volumetric flasks were made with the methanol. Aliquots from the stock solutions of standard andrographolide and extract of *Andrographis paniculata* were diluted with methanol to attain the working standards of 100 µg/ml concentration.

All the reaction solutions were applied on TLC plate and chromatograms were recorded using HPTLC. Chromatography was accomplished on pre-coated TLC plates with Silica gel 60 F_{254}. Aliquots of each of the degraded samples of standard andrographolide and extract were separately applied to the plate with an automatic TLC applicator Linomat-V with N_2 flow, 8 mm from the bottom. Densitometric scanning was performed at 490 nm on CAMAG scanner III. Linear ascending development was done in twin glass chamber previously saturated with the mobile phase chloroform: toluene: methanol (7: 1.5: 1.5 v/v/v).

Results and discussion

Extractive value

The Extractive value of *Andrographis paniculata* extract was 10.33 % w/w.

Development of the optimum mobile phase

Various mobile phases were tried for analysis of Andrographolide and *Andrographis paniculata* extract. HPTLC method was developed by hit and trials methods using different solvent systems according to their polarity. The solvent system the exhibits good resolution and gives good chromatogram with sharp and stable peak was selected. The solvent system chloroform: toluene: methanol (7:1.5:1.5 v/v/v) was found optimum for the studies and the R_f value of andrographolide was found to be 0.38.

HPTLC study of standard andrographolide with extract of Andrographis paniculata

HPTLC study was carried out an extracts of *Andrographis paniculata*. Standard was quantified accurately using pre-coated HPTLC plates with silica gel F_{254} and the mobile phase used was chloroform: toluene: methanol (7:1.5:1.5 v/v/v) (Figure 1). The R_f value was found to be 0.38. The chromatograms of andrographolide and methanolic extract of *Andrographis painculata* are shown in Figure 2 and 3. The R_f value of andrographolide was compared with the R_f value of extract i.e 0.38.

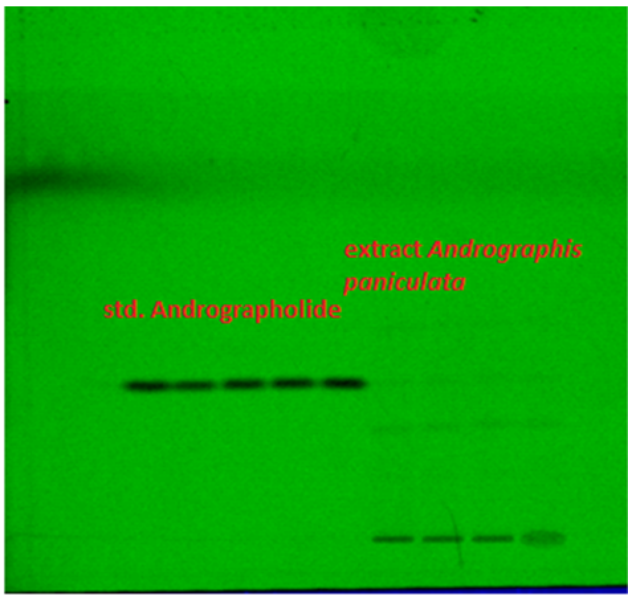


Fig 1: HPTLC plate of andrographolide and extract of *Andrographis paniculata.*

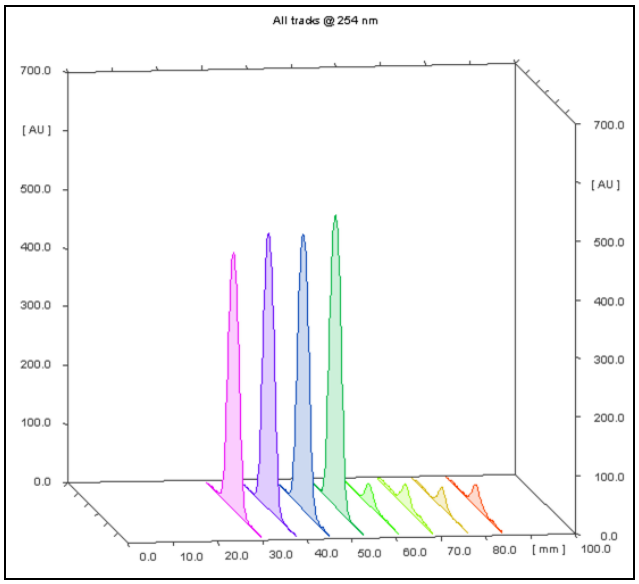


Fig 2: Three dimensional peak of andrographolide and their respective peak in *Andrographis paniculata.*

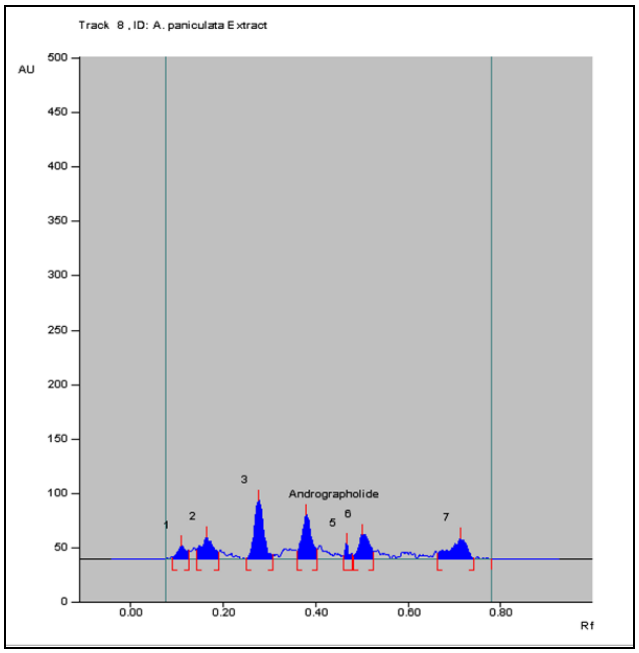


Fig 3: HPTLC chromatogram of *Andrographis paniculata* extract showing presence of andrographolide.

Validation of the method

Linearity

The linear regression data for the calibration curves (n= 5) as shown in Table 1. Results showed a good linear relationship over the concentration range 100-500 ng per spot with respect to peak height and peak area, slope 0.993, Y= 3.534x + 3510 (Figure 4). No significant difference was noticed in the slopes of standard curves. Figure 5 shows chromatogram of standard andrographolide.

Table 1: Linearity regression data for calibration curve.

Parameters	Value
Linearity range	100-500 ng
Correlation of Coefficient (r^2)	0.993
Slope	3510

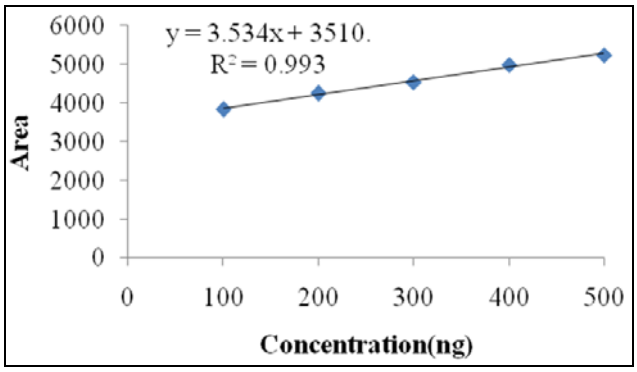


Fig 4: Calibration curve of standard andrographolide (n=6).

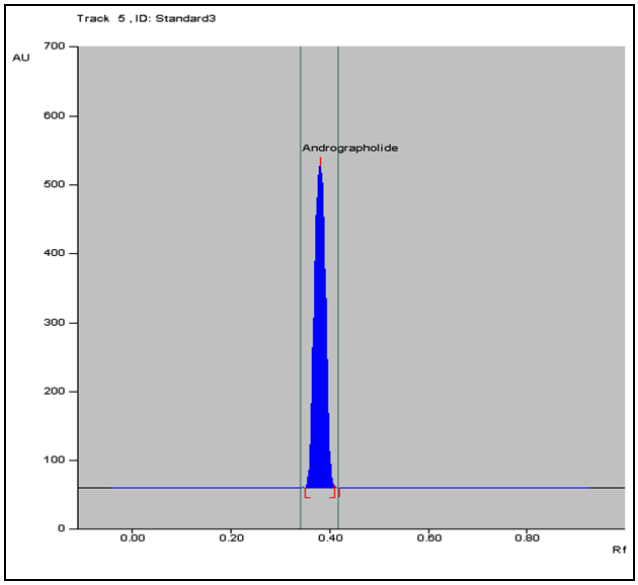


Fig 5: Chromatogram of standard Andrographolide

Precision

The inter and intra- day precision of HPTLC method (n= 6) is shown in Table 2 and the repeatability study results expressed in terms of %RSD are depicted in Table 3, which describes intra- and inter-day variation of andrographolide at different concentration levels of 200- 700 ng per spot (n= 6). Table 2 shows

Table 2: Inter and intra- day precision of HPTLC method (n=6).

Sr. no	Amount ng/spot	Inter-day precision SD	% RSD	Intra- day precision SD	% RSD
1	200	110.39	1.39	80.960	1.02
2	300	83.951	1.058	121.929	1.534
3	400	89.674	1.12	44.984	0.56
4	500	128.63	1.619	115.59	1.465
5	600	67.392	0.858	137.978	1.74
6	700	103.581	1.294	84.526	1.06

Table 3: Results of repeatability (n=3).

Sr. no	Amount(ng)	SD	%RSD
1	200	69.354	0.868
2	300	123.08	1.55
3	400	137.978	1.74

Limit of Detection (LOD) and limit of quantification (LOQ)

Detection limit and quantification limit were calculated using the formula

LOD = 3.3 x σ/S, LOQ = 10 x σ/S

Where, σ = standard deviation of the response, S = slope of the calibration curve

LOD and LOQ were found to be 0.527 and 1.59 respectively, which shows the sufficient sensitivity of the method.

Specificity

The specificity of the method was confirmed by analyzing standard andrographolide and extracts of *Andrographis paniculata*. The spot for andrographolide in the sample was validated by comparing the R_f and spectra of the spot with that of sample. The peak purity of andrographolide was verified by comparing the spectra at peak start, peak middle and peak end position of the spot/ bands (Figure 6).

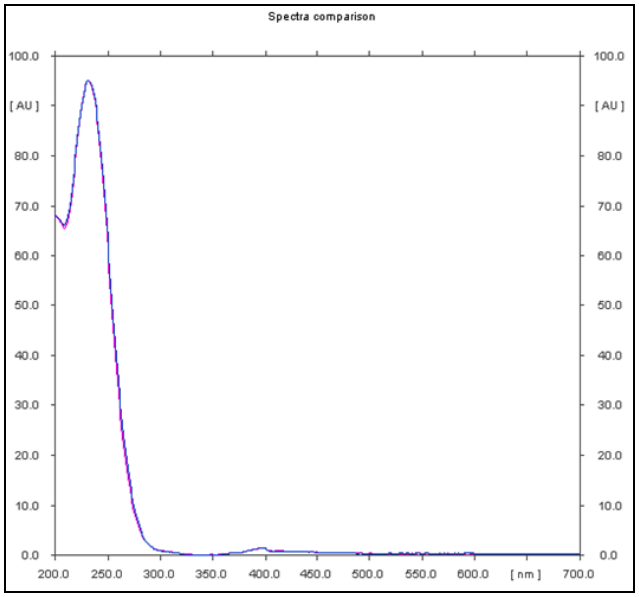


Fig 6: Overlay spectrum of standard andrographolide over extract.

All the results of the method performance parameters for validation of HPTLC protocol are summarized in Table 4.

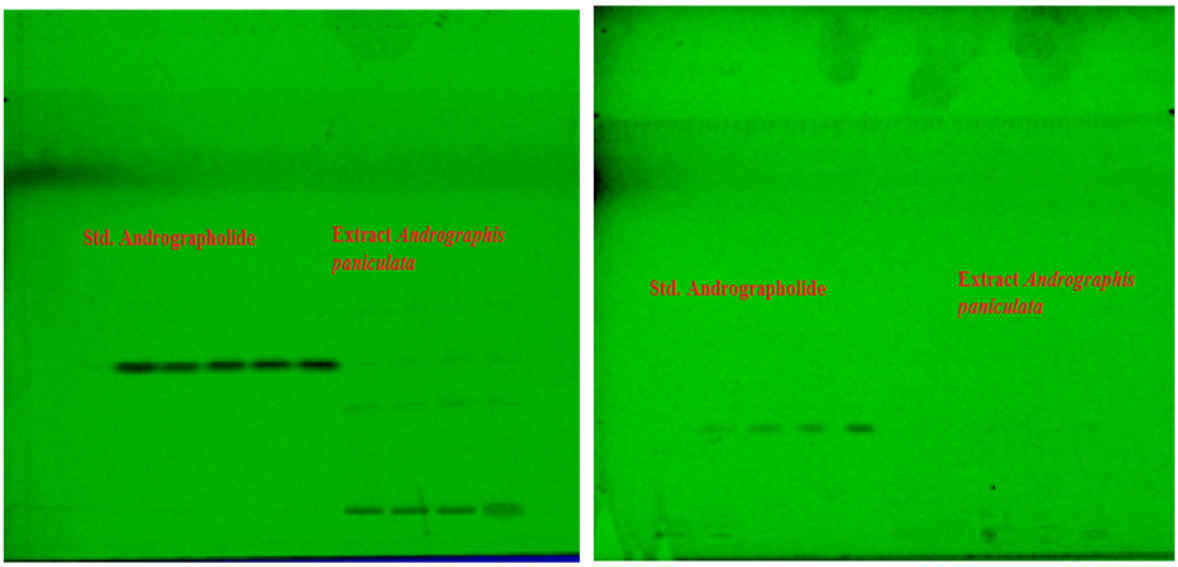
Table 4: Method performance parameters for validation of HPTLC protocol.

Parameters	Method
Selectivity	Selective
Specificity	Specific
Linear range (ng/spot)	100-500
Correlation coefficient (r)	0.993
Linear regression equation *Y= mx + c*	Y= 3.534x + 3510
LOD (ng/spot)	0.527
LOQ (ng/spot)	1.59
Repeatability (%RSD, n=6)	0.868-1.74
Inter day (n=6)	67.392-128.63
Intra day (n=6)	0.56-1.74

Forced degradation studies

Well separated spots were seen for the degraded samples for acid, base, hydrogen peroxide and light for pure andrographolide whereas in case of methanolic extract of

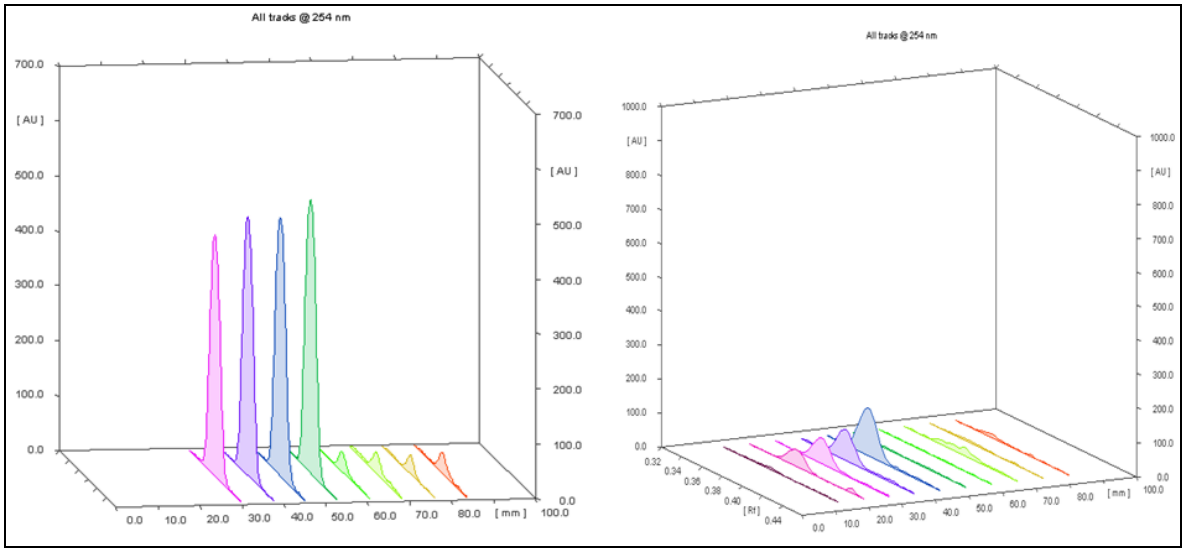
Andrographis paniculata the spots were seen slightly. The HPTLC plates of standard andrographolide and exract of *Andrographis paniculata* at 254 nm before degradation and after degradation studies are shown in Figure 7.



Before degradation After degradation

Fig 7: HPTLC plate of standard andrographolide and methanolic extract of *Andrographis paniculata* extract at 254 nm before degradation and after degradation studies.

The three dimensional representation of peaks of standard andrographolide and extract of *Andrographis paniculata* at 254 nm before degradation and after degradation is given in figure 8. It displays that the sharp and well resolved peaks were obtained for the standard andrographolide and extract of *Andrographis paniculata* with almost same R_f value, but after degradation studies, standard andrographolide showed the well resolved peak, but in case of extract of *Andrographis paniculata*, complete degradation took place and hence single peak was observed.



Before degradation After degradation

Fig 8: Three dimensional peak of andrographolide and their respective peak in *Andrographis paniculata* before degradation and after degradation.

Alkali hydrolysis

Chromatogram of alkaline hydrolysis showed complete degradation of standard andrographolide and extract of *Andrographis paniculata* therefore, no peak was observed.

Acid hydrolysis

Chromatogram of acid degraded samples showed degradation product peaks at R_f 0.35 for standard andrographolide but the extract of *Andrographis paniculata* was completely degraded, hence no peaks were observed.

Oxidative hydrolysis

Chromatogram of oxidative stress degraded samples exhibited the degradation product peaks at R_f 0.35 for standard andrographolide and R_f 0.38 for the extract.

Photolytic degradation

Chromatogram of photolytic degraded samples displayed the

degradation product peaks at R_f 0.35 for standard andrographolide and the extract was completely degraded therefore displaying no peak.

Dry heat degradation

Chromatogram of dry heat degraded samples exhibited the degradation products peaks at R_f 0.35 for standard andrographolide and the extract was completely degraded.

This degradation studies indicated that standard andrographolide and *Andrographis paniculata* extract were susceptible to alkali hydrolysis, acid hydrolysis and oxidative stress degradation, photolytic degradation and dry heat degradation. Standard andrographolide showed single sharp peak before degradation, but it showed different contaminated peaks when degraded at different parameters. Figure 9 shows the comparison of chromatograms of standard andrographolide before degradation and after undergoing various degradation parameters. Figure 10 shows the comparison of chromatogram of extract before and after degradation parameters. Table 5 and 6 summarizes the results of the degradation studies for standard andrographolide and the extract of *Andrographis paniculata* respectively.

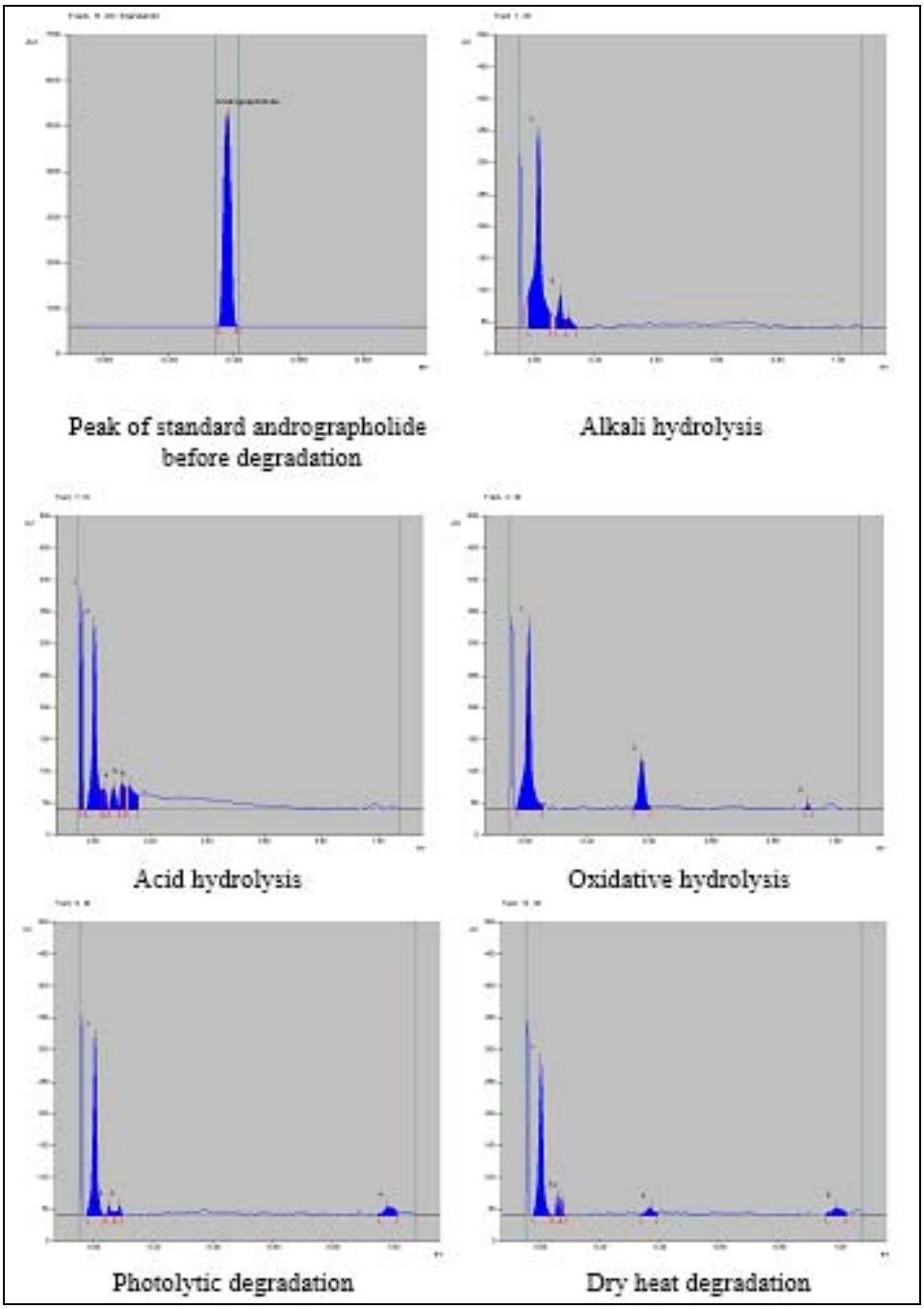


Fig 9: Chromatogram comparison of standard andrographolide before degradation and after degradation parameter.

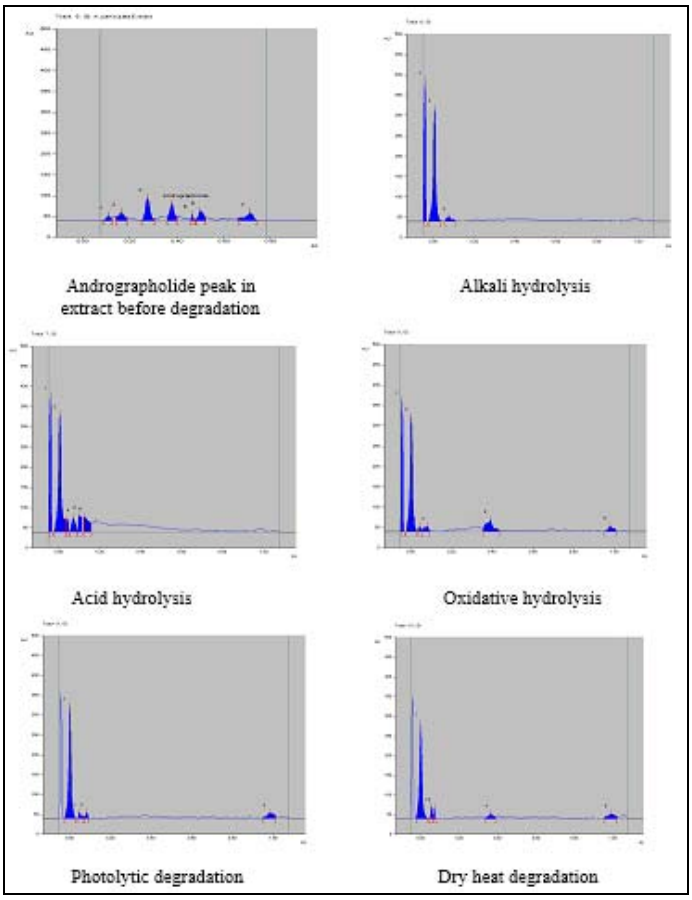


Fig 10: Chromatogram comparison of andrographolide present in extract of *Andrographis paniculata* before degradation and after degradation parameters

Table 5: Degradation study of standard andrographolide at different parameters

Sr. no	Sample	Parameters	Condition	Time	Rf value of standard Andrographolide before degradation	Rf value of standard Andrographolide after degradation
1	Andrographolide standard	Alkali hydrolysis	1 N NAOH at 80˚C in water bath	2 hrs	0.38	No peak found (completely degraded)
2	Andrographolide standard	Acid hydrolysis	1 N HCL at 80˚C in water bath	2 hrs	0.38	0.35
3	Andrographolide standard	Oxidative stress degradation	3% H2O2 at 80˚C in water bath	2 hrs	0.38	0.35
4	Andrographolide standard	Photolytic degradation	Photolytic, expose to UV	6 hrs	0.38	0.35
5	Andrographolide standard	Dry heat degradation	Dry heat, hot air oven at 80˚C	2 hrs	0.38	0.35

Table 6: Degradation study of extract *Andrographis paniculata*

Sr. no	Sample	Parameters	Condition	Time	Rf value of Andrograoholide present in extract of *Andrographis paniculata* before degradation parameters	Rf value of Andrographolide present in extract of *Andrographis paniculata* after degradation Parameters
1	Extract *Andrographis paniculata*	Alkali hydrolysis	1N NAOH at 80˚C in water bath	2 hrs	0.38	No peak found (completely degraded)
2	Extract *Andrographis paniculata*	Acid hydrolysis	1N HCL at 80˚C in water bath	2 hrs	0.38	No peak found (completely degraded)
3	Extract *Andrographis paniculata*	Oxidative stress degradation	3% H_2O_2 at 80˚C in water bath	2 hrs	0.38	0.36
4	Extract *Andrographis paniculata*	Photolytic degradation	Photolytic, expose to UV	6 hrs	0.38	No peak found (completely degraded)
5	Extract *Andrographis paniculata*	Dry heat degradation	Dry heat, hot air oven at 80˚ C	2 hrs	0.38	No peak found (completely degraded)

The present study confirms the development of validated, precise, specific, accurate and stability indicating HPTLC technique for standard andrographolide and extract of *Andrographis paniculata*. HPTLC is the most commonly used techniques to ensure the development of products with consistent safety and efficacy profiles. It consumes less amount of solvents and therefore can be regarded as more economic and environment friendly. It is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks. This work illustrates a simple, sensitive and robust HPTLC method for the simultaneous determination of the standard andrgrapholide and presence of andrographolide in extract of *Andrographis paniculata*. The proposed method met the ICH validation, acceptance criteria concerning; linearity, ranges, precision and accuracy, specificity [16]. Moreover the method adopted was stability indicating one because well separated andrographolide from its degradation product can be quantified easily. Stability testing performs a significant role in the testing program for drug substances as the instability of the product affects the prime essential requisites, i.e. quality, efficacy and safety of any drug [17]. Study of degradation products or impurity profile of an andrographolide can be future scope of the present research work. Additionally, the applicability of the proposed method to practical life situations was evaluated through the analysis of commercially available andrographolide and the results attained were satisfactory.

Acknowledgement
Financial Assistance to the department from the university Grants Commission is acknowledged.

References

1. Sabu KK, Padmesh P, Seeni S. Intraspecific variation in active principle content isozymes of *Andrographis paniculata* Nees (Kalmegh): a traditional hepatoprotective medicinal herb. Journal of Medicinal and Aromatic Plant Sciences. 2000; 23:637-647.
2. Rao NK. Anti-hyperglycemic and renal protective activities of *Andrographis paniculata* roots chloroform extract. Iranian Journal of Pharmacology and Therapeutics. 2006; 5:47-50.
3. Balachandran P, Govindarajan R. Cancer - an ayurvedic perspective. Pharmacological Research. 2005; 51:19-30.
4. Patarapanich C, Laungcholatan S, Mahaverawat N, Chaichantipayuth C, Pummangura S. HPLC determination of active diterpene lactones from *Andrographis paniculata* Nees planted in various seasons and regions in Thailand. Thai Journal of Pharmaceutical Sciences. 2007; 31:91-99.
5. Mishra SK, Sangwan NS, Sangwan RS. *Andrographis paniculata* (Kalmegh). Pharmacognosy Review. 2007; 1:283-298.
6. Sharma M, Sharma A, Tyagi S. Quantitative HPLC analysis of *Andrographis paniculata* at two different stages of life cycle of plant. Acta Chimica and Pharmceutica Indica. 2012; 2:1-7.
7. Zhang C, Kuroyangi M, Tan BK. Cardiovascular activity of 14-deoxy-11,12- didehydroandrographolide in the anaesthetized rat and isolated right atria. Pharmacological Research. 1998; 38:413-417.
8. Lomlim L, Jirayupong N, Plubrukarn A, Heat accelerated degradation of solid state andrographolide. Chemical and Pharmaceutical Bulletin. 2003; 51:24-26.
9. Calabrese C, Berman SH, Babish, JG. A phase I trial of andrographolide in HIV positive patients and normal volunteers. Phytotherapy Research. 2000; 14:333-338.
10. Saxena S, Jain DC, Bhakuni RC, Sharma RP. Chemistry and pharmacology of Andrographis species. Indian Drugs. 1998; 35:458-467.
11. Saraswat B, Visen PKS, Patnaik GK, Dhawan BN. Effect of andrographolide against galactosamine induced hepatotoxicit. Fitoterapia. 1995; 66:415-420.
12. Lal J, Tripathi HC, Tandon SK. Antidiabetic activity of andrographolide. Indian Journal of Pharmacology. 1986; 18:58-61.
13. Rahman NN, Furuta T, Kojima S, Takane K, Mohd MA. Antimalarial activity of extracts of Malaysian medicinal plants. Journal of Ethnopharmacology. 1999; 64:249-254.
14. Panossian A, Davtyan T, Gukassyan N, Gukasova G, Mamikonyan G, Gabrielian E. Effect of andrographolide and Kan Jang- fixed combination of extract SHA-10 and extract SHE-3 on proliferation of human lymphocytes, production of cytokines and immune activation markers in the whole blood cells culture, Phytomedicine. 2002; 9:598-605.
15. ICH guidelines. Q1A (R2): Stability testing of new drug substances and products (revision2). International Conference on Harmonization. Geneva. 1993.
16. Bimal N, Sekhon SB. High performance thin layer chromatography: Application in pharmaceutical science. Pharmtechmedica. 2013, 2.
17. Kommanaboyina B, Rhodes CT. Trends in stability testing with emphasis on stability during distribution and storage. Drug Development and Industrial Pharmacy. 1999; 25:857-868.