



**IDENTIFICATION, PURIFICATION AND QUANTIFICATION OF
ANDROGRAPHOLIDE FROM ANDROGRAPHIS PANICULATA
(BURM. F.) NEES BY HPTLC AT DIFFERENT STAGES
OF LIFE CYCLE OF CROP**

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ABSTRACT

A simple, rapid, selective and quantitative HPTLC method has been developed for determination of Andrographolide in *Andrographis paniculata* at different stages of life cycle of crop. Whole plant material at different stages, from 30 days of plantation up to maturity of the crop was studied. Chromatogram scanned at 250 nm. The results observed under UV light showed a good separation for all compounds. The spots for Andrographolide in samples were confirmed by comparing the R_f and spectra of the spot with that of standard biomarker of Andrographolide. R_f for Andrographolide was found to be 0.31. The average Andrographolide content varied from 0.42% to 2.02% in the sample studied.

Key words: Andrographolide, *Andrographis paniculata*, Kalmegh, HPTLC, King of Bitters.

INTRODUCTION

Over the past twenty years, interest in medicinal plants has grown enormously from the use of herbal products as natural cosmetics and for self-medication by the general public to the scientific investigations of plants for their biological effects in human beings. Beyond this pharmaceutical approach to plants, there is a wide tendency to utilize herbal product to supplement the diet, mainly with the intention of improving the quality of life and preventing the diseases of elderly people¹. India has been identified as a major resourceful area in the traditional and alternative medicines globally. Medicinal plants constitute an important component of flora and are widely distributed in India. *Andrographis paniculata* (Burm. f.) Nees is one of the widely distributed medicinal plants in India and using since ancient times in traditional ayurvedic systems of medicines. Although plants in general show habitat and distribution preferences but there are many species, which are neutral and adapted to other environmental conditions. According to Briggs and Walters², much of the variations in phenotype observed in natural populations of a species were earlier attributed to environmental influences. Variations within and between populations of a species are not uncommon. Even within a medicinal plant species, sometimes one variety was preferred over others. It is also not surprising that curative properties of a plant species change according to seasons or developmental stages and hence “Vaidyas” (Traditional medicines experts) prefer to collect required plants or their parts during certain time period only³. A study of variation in the active principles is often an important criteria in the investigation of variation in such plants. *Andrographis paniculata* (Burm. f.) Nees belongs to the family of Acanthaceae. It is available abundantly in south eastern Asia i.e., India, Sri Lanka, Pakistan and Indonesia. It is found in wild

through out of plains of India especially in Tamil Nadu, Karnataka, Maharashtra, Orissa, Uttar Pradesh and Uttarakhand. *Andrographis paniculata*, “Kalmegh” of Ayurveda is an erect annual herb extremely bitter in taste in each and every part of the plant body. The plant is known in north-eastern India as ‘Maha-tita’, literally ‘King of bitters’ and also known by various other vernacular names (Table 1). It is also known as ‘Bhui-neem’ since the plant, though much smaller in size and has bitter taste as that of Neem (*Azadirachta indica*).

Table 1: List of vernacular names of *Andrographis paniculata* (Burm. f.) Nees

S. No.	Language	Common Name
1	Arabic	Quassabhuva
2	Assamese	Chiorta
3	Bengali	Kalmegh
4	Chinese	Chuan Xin Lian
5	English	The Creat
6	Gujarati	Kariyatu
7	Hindi	Kalmegh, Kirayat
8	Indonesian	Sambiloto
9	Kannada	Nelaberu
10	Malayalam	Nelavepu, Kiriyaattu
11	Marathi	Oli-Kiryata
12	Oriya	Bhuinimba
13	Sanskrit	Kalmegha, Bhunimba
14	Tamil	Siriyangai
15	Telugu	Nilavembu
16	Thai	Fa-Talai-Jorn

Andrographis paniculata or Kalmegh is one of the most widely used plants in ayurvedic formulations⁴. *Andrographis paniculata* is the main source of the bitter principle Andrographolide and called “King of bitters” due to extremely bitter and characteristic taste. The bitter principle Andrographolide was isolated in pure form by Gorter⁵. Whole plant of *Andrographis paniculata* has a wide range of pharmacological activity⁶. Due to presence of Andrographolide it is used in activities like anti-malarial activity⁷, anticancer activity⁸, anti-diabetic activity⁹, liver protection¹⁰ and many more diseases.

The genus *Andrographis* consists of 28 species of small annual herbs essentially distributed in Tropical Asia. Only a few species are of medicinal value out of which *Andrographis paniculata* (Burm. f.) Nees is the most popular. It is generally found in all kinds of vegetative lands i.e., in pine, evergreen, deciduous areas, along roads and villages. It is easily cultivated from seeds on all types of soil (Prajapati et al.¹¹ The plant is gregarious abundantly found in moist, shady waste grounds and sometimes in dry forests.

Extracts of different samples of *Andrographis paniculata* contain a mixture of bioactive and inactive chemical ingredients. Chemical composition of samples varies not only in different parts but also on growing region and the collection time. Therefore, efficient and effective methods of identification, extraction, standardizations, qualitative, quantitative and pharmacological testing's are essential. To evaluate

the therapeutic potential of the purified bioactive principles, method must be highly selective, in order to achieve the optimal outcomes. The main objective of this study was to purify one of these major components (Andrographolide) from *Andrographis paniculata*, to analyze its quality and to calculate yield on weight to weight bases and exact percentage of Andrographolide.

HPTLC and HPLC both are very effective methods and can be used for phytochemical profiling of whole plant, leaves and other parts of *Andrographis paniculata* and quantification of Andrographolide. In this study HPTLC was used. Preliminary tests and confirmatory tests were conducted to confirm the presence of Andrographolide in *Andrographis paniculata*. Andrographolide standard 99.8% (Product code A009, Lot No. T11B001) was procured from Natural Remedies, Bangalore and was used as standard biomarker to confirm the results and to find correct percentage of Andrographolide.

EXPERIMENTAL

Plant material

Two plants of *Andrographis paniculata* were obtained as a gift from Forest Research Institute (FRI), Dehradun, Uttarakhand, India in the month of June 2009. After one complete life cycle of the plants, fully matured seeds were collected and sown in nursery and the crop was grown as per cultivation practices developed by Pandey and Mandal¹². Whole plant material at different stages of life cycle of crop was collected.

Samples of whole plant material at different stages of life cycle of crop were –

Sample A-30 days

Sample B-60 days

Sample C-90 days

Sample D-110 days (after 110 days of plantation i.e., just before flowering)

Sample E-130 days (At maturity of the crop i.e., bearing flowers, fully matured seed capsules etc.)

All samples of whole plant material were dried under shade, powdered using blender and stored in air tight bottles.



Fig. 1: Different stages of life cycle of crop of *Andrographis paniculata* from 30 days to maturity of crop

Preliminary Test for *Andrographis paniculata* (Colour Test)

1 g of *Andrographis paniculata* powder with 20 mL of ethanol was boiled in a water bath for 5 minutes. 300 mg of activated charcoal was added, stirred and then filtered. The filtrate was used for the following two tests.

Test (1): The first test involved 2 drops of a 2% w/v solution of 3,5-dinitrobenzoic acid in ethanol and 2 drops of a 5.7% w/v solution of potassium hydroxide in ethanol being added to 0.5 mL of filtrate. A colour change to a purplish red indicated that the sample had active compounds (MPRI)¹³.

Test (2): The second test involved 3-5 drops of the 5.7% w/v solution of potassium hydroxide in ethanol being added to 0.5 mL of filtrate until a red colour appeared. The filtrate was set aside for 10 to 15 minutes. The red colour changed to yellow. These two tests indicate that the sample contained diterpene lactones as active constituents (MPRI)¹³.

Confirmatory test: To confirm that above obtained diterpene lactones obtained from *Andrographis paniculata* is Andrographolide the above filtrate was evaporated under reduced pressure until fully dried. About 5 mg of filtrate powder was dissolved in 5 mL of warm ethanol and 1 mg of standard Andrographolide was dissolved in 0.5 mL of warm ethanol separately. A pre coated plate of silica gel 60 F254 aluminum sheets (10 x 10 cm) was used and the mobile phase used was: - chloroform: methanol: ethyl acetate (8 : 1.5 : 1). 5 micro liters was used for each spot. TLC of the isolated samples was firstly detected by UV radiation (Electronic UV Trans illuminator, Quantum Scientific) and then confirmed by spraying with 2% w/v solution of 3,5-dinitrobenzoic acid in ethanol and an excess of 5.7% w/v of potassium hydroxide in ethanol. Results completely match with standard Andrographolide which clearly indicates that obtained diterpene lactones is Andrographolide.

Purification of andrographolide

After confirmation that *Andrographis paniculata* contained Andrographolide, a modification of the method reported by Rajani et al.¹⁴ was used to isolate Andrographolide from all samples of *Andrographis paniculata*. 200 g of whole dried crushed *Andrographis paniculata* was extracted, twice with a 1 : 1 mixture of dichloromethane and methanol by cold maceration (one with 1 Liter and then 0.2 Liter of the maceration mixture) (1 × 1 Liter and 1 × 0.2 Liter). Each extraction was conducted for 6 hours with constant stirring. The extracts were filtered and the filtrates were concentrated at a temperature below 70°C to produce a green mass. The green mass was washed with toluene several times until most of coloured matter was removed from the residue. The toluene was completely removed from the residue by evaporation. The residue was dissolved in 60-70°C hot ethanol and filtered while hot. The filtrate was cooled in a refrigerator for crystallization. This process was repeated several times until small yellow plates of Andrographolide of constant melting point, range of 226-229°C were obtained. Further isolation of Andrographolide was confirmed by melting point, UV absorption spectrum and HPTLC.

Melting point

Melting point was determined using a melting point apparatus (FSE, England). It was 229°C. This melting point matches with biomarker melting range from 224-230°C used in laboratories of government offices to test and control the quality of Andrographolide.

UV maximum absorption spectrum

UV spectrophotometry was used to determine the maximum absorption spectra within the range from 200 to 270 nm. Above purified Andrographolide was scanned in Shimadzu, UV-1700 Pharmaspec UV-vis-Spectrophotometer. The concentration of purified Andrographolide and standard Andrographolide

(purchased from Natural Remedies, Bangalore, India 99.8%) was 25 µg/mL in ethanol. The λ_{\max} was found to be 223 nm, which matches with standard value of Andrographolide.

High Performance thin layer chromatography (HPTLC)

Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system which distributed between two phases. Liquid chromatography (LC) is chromatographic procedure in which the mobile phase is a liquid. There are many types of chromatography but all are based on the principle that the mobile phase carries the compounds to be separated and a stationary phase binds these compounds through intermolecular forces.

Thin layer chromatography is employed to get TLC profile of various extracts and can be used in standardizing the raw drugs as well as the herbal formulations¹⁵. Ordinary TLC plates do not give better resolution particularly, when the herb contains many components similar to each other. Quantification of markers by conventional solvent extraction followed by calorimetric is laborious and expensive. Sethi¹⁶ offers better resolution and estimation of active principles with reasonable accuracy in a shorter time by HPTLC. Among the complex mixture of biologically active compound in *Andrographis paniculata*, Andrographolide can be used as an analytical marker compound to determine the quality of plant material of different sources. During crop improvement and drug analysis, a sensitive and accurate analytical method is required for the quantitation of important compound like Andrographolide, which is present in the plant. Although few methods such as gravimetric, colorimetric, spectrophotometric methods¹⁷ have been reported for the quantitative estimation of Andrographolide, many of these methods are time consuming, not very precise and required multiple step extraction and purification¹⁸. The present HPTLC method offers a sensitive, accurate and reliable method for routine detection and quantification of Andrographolide present in *Andrographis paniculata* and it is modified form of method suggested by Bhutani¹⁹. High-performance thin-layer chromatographic (HPTLC) methods have been developed for the simultaneous quantitative estimation of the biologically active diterpenoid Andrographolide in *Andrographis paniculata*. The present work focuses on development and validation of high performance thin layer chromatographic method for the determination of isolated major constituent in *Andrographis paniculata* extracts with an advantage of sensitive method with lower limit of detection (LOD), as well as precise even at lower concentration of isolated major compound in various samples of *Andrographis paniculata*. Methanol was found to be the most appropriate solvent for the exhaustive extraction of Andrographolide derivatives¹⁴. It was confirmed in the lab during experiments hence methanol was used as a solvent.

Equipment

A Camag HPTLC system equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner 3, Wincats and integration software 1.4 (Switzerland).

Stationary phase- Aluminum sheets (10 x 10 cm) pre coated silica gel 60 F254 (E. Merck).

Mobile phase- A mixture of 7 volumes of chloroform and 1 volume of methanol was used.

Test solution- 1 g of coarsely powdered samples under examination with 50 ml methanol each separately.

1-Reference solution- 0.5 g of tested *Andrographis paniculata* (used as working standard in Himalaya Herbal Healthcare, Dehradun, Uttarakhand, India.) with 50 mL methanol.

2- Reference solution- Standard Andrographolide solution.

Sample and standard preparation

Sample preparation

(1) 1 g of coarsely powdered drug (working standard from Himalaya Drug) was extracted with 10 mL absolute alcohol for 24 hours by cold extraction method. The extract was filtered by Whatmann filter paper 125 mm (containing ash less circles Cat No. -1441-125) and make up to 10 mL in a volumetric flask. Filtrate was concentrated to 2 mL and used for T.L.C.

(2) 1 g of coarsely powdered drug sample was extracted with 10 mL absolute alcohol for 24 hours by cold extraction method. The extract was filtered by Whatmann filter paper and make up to 10 mL in a volumetric flask. Same procedure was applied for all samples.

Standard preparation

5 mg of standard Andrographolide dissolved in 5 mL of absolute alcohol and made up to 5 mL in standard volumetric flask.

Procedure

The chromatographic estimation was performed using the following conditions, stationary phase was aluminum sheets (10 x 10 cm) pre coated silica gel 60 F254 (E. Merck) plates 0.2 mm thickness and the mobile phase used was :-Chloroform: Methanol : 7 : 1 v/v. Camag twin trough chamber was saturated with the developing solvent. The sample (5 μ L) was applied as bands on the plates using Camag linomate 5 sample applicator. The width of the applied band was 6.0 mm and the space between the bands was fixed as 13.0 mm. The speed of application was maintained at 150 μ L/sec. The chamber saturation time employed was 30 minutes and the developing distance was 8.5 cm. Scanning wavelength of 250 nm with a slit dimension of 6.0 x 0.45 mm and scanning speed of 20 mm/s were employed. When the solvent front reaches the other edge of the stationary phase, the plate was removed from the solvent reservoir. The developed plate was dried in air and examine in ultraviolet light at 250 nm using Camag TLC scanner 3 equipped with Wincat Version 1.4 software. The constituents of extracts were identified by simultaneously running standards with the unknown. The chromatographic profiles of the test solutions were similar to that of the reference solution and standard Andrographolide The fingerprint of HPTLC profile of all extracts and isolated compound Andrographolide were taken using computer by the help of software to increase the authenticity of results. R_f value of various samples was evaluated. The Peak table, Peak display and Peak densitogram were noted. The amount of Andrographolide in test samples was determined.

RESULTS AND DISCUSSION

In this study HPTLC plates were used to establish a TLC fingerprinting method. The chromatographic conditions, in particular the developing solvents (i.e., types of solvents and ratio), were carefully optimized before the crude drug and formulation samples were analyzed. Chromatogram scanned at 250 nm. The results observed under UV light showed a good separation for all compounds. A reference marker compound of Andrographolide was separated on the same plates for the authentication of each sample. Beside this the specificity of the method was ascertained by analyzing:

- Reference solution (1) *Andrographis paniculata* (working standard of Himalaya Herbal Healthcare, Dehradun, Uttarakhand, India.).
- Reference solution (2) Standard Andrographolide solution (99.8% Andrographolide procured from Natural Remedies, Bangalore).

- Isolated Andrographolide.
- Different samples of *Andrographis paniculata*.

The spots for Andrographolide in samples were confirmed by comparing the R_f and spectra of the spot with that of standard. The peak purity of Andrographolide was assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot. Wincats and integration software 1.4 (Switzerland) was used to calculate the amount of Andrographolide in test samples. It was determined according to the formula-

$$\% \text{ Content of Andrographolide} = \frac{\text{Standard area}}{\text{Sample area}} \times \frac{\text{Sample weight}}{\text{Sample dilution}} \times \frac{\text{Standard weight}}{\text{Standard dilution}} \times \% \text{ Purity of the standard}$$

Under the chromatographic conditions described in procedure the R_f for Andrographolide was found to be 0.31. For positive identification, the sample must exhibit bands with chromatographic characteristics, including colors and R_f values similar to those of reference marker compound. It was observed that Andrographolide appeared at R_f 0.31 in samples which is similar to that of Standard Andrographolide.

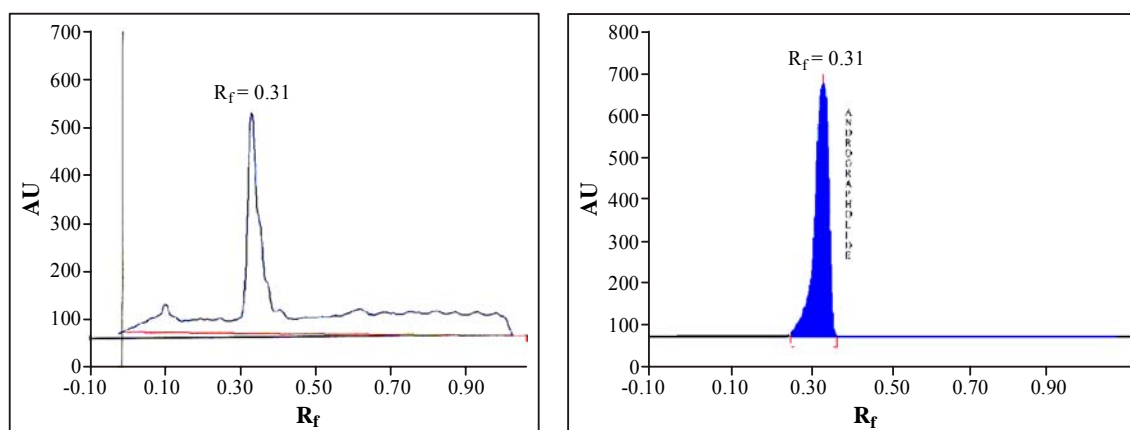


Fig. 2: Image of peak of standard biomarker of Andrographolide showing R_f

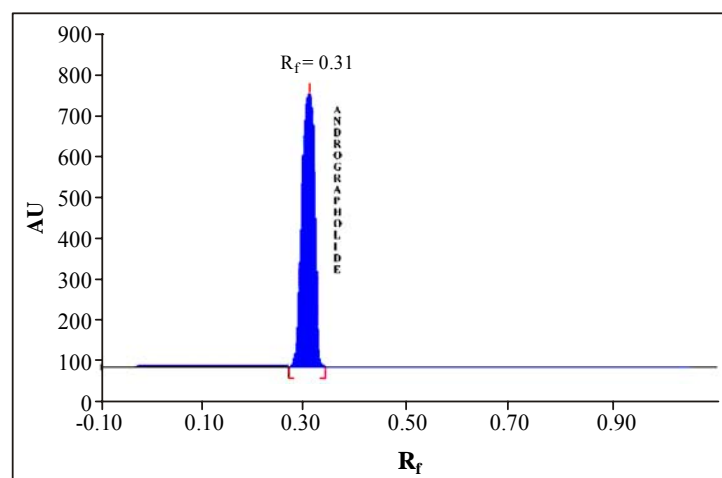


Fig 3: Image of peak of isolated Andrographolide showing R_f

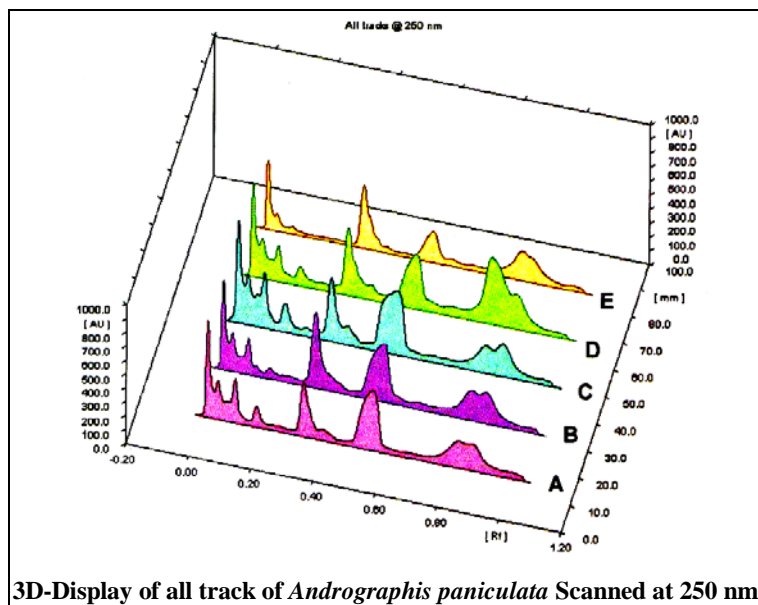


Fig. 4: Three dimensional image of the calibration spots for different samples of *Andrographis paniculata*.

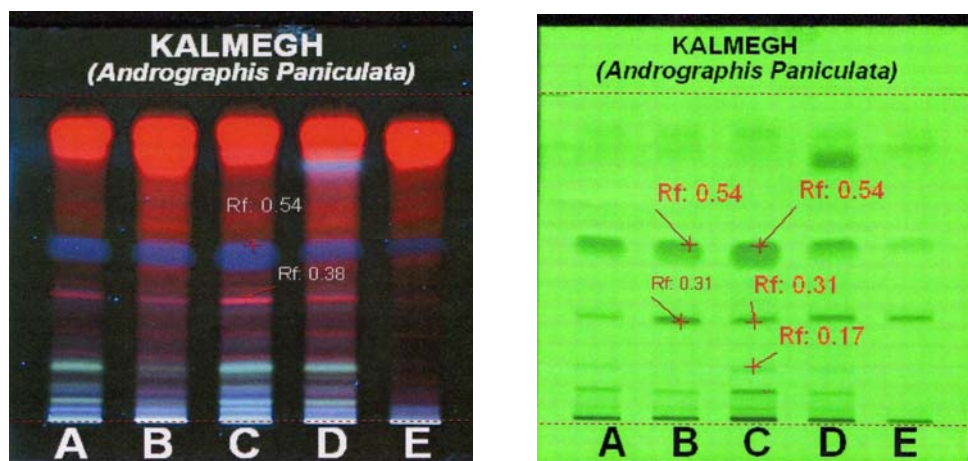


Fig. 5: Images of Fingerprinting of test samples of *Andrographis paniculata*

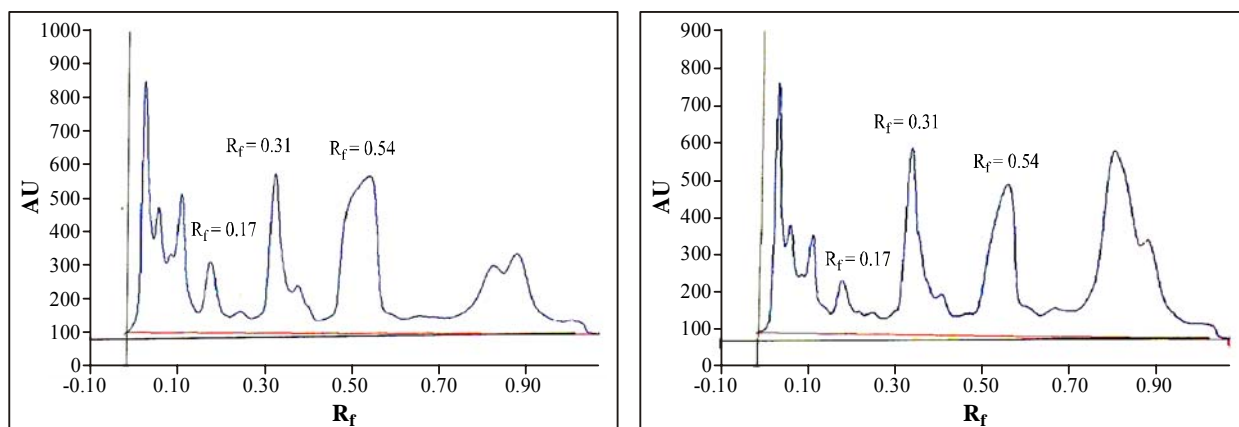
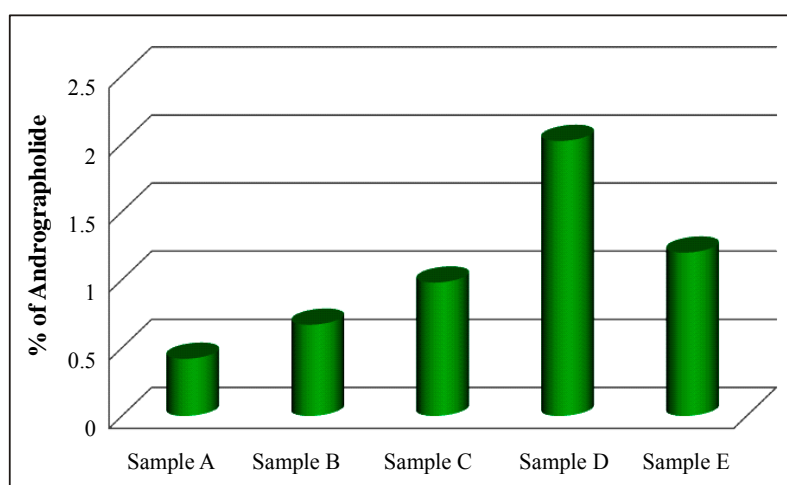


Fig. 6: Peaks of working standard and sample 4 of *Andrographis paniculata*.

Andrographolide content varied from 0.42% up to 2.02% in samples A to E. Result are given in Table 2.

Table2: Andrographolide content in different samples of *Andrographis paniculata*.

S. No.	Sample name	% of Andrographolide
1	A	0.42%
2	B	0.67%
3	C	0.98%
4	D	2.02%
5	E	1.20%



Sample A -30 days, Sample B-60 days, Sample C-90 days,
Sample D-110 days, Sample E-130 days

Fig. 7: Graph of Andrographolide content in different Samples of *Andrographis paniculata*

The maximum Andrographolide content (2.02 %) was found in the sample D harvested after 110 days of plantation i.e. just before flowering followed by sample E (1.2%). Results indicates that just before flowering is the correct time to collect plants of *Andrographis paniculata* to obtained highest percentage of Andrographolide content and to increase potency for medicinal uses.

CONCLUSION

The developed HPTLC method can be utilized for the quantitative determination of Andrographolide in *Andrographis paniculata* herb samples. The method developed is simple, sensitive and statistically validated. The above findings recommend the further investigation of *Andrographis paniculata* harvested after 110 days of plantation i.e. just before flowering to evaluate their chemical potential.

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